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(54) IMMUNOPHILIN LIGANDS AND METHODS FOR MODULATING IMMUNOPHILIN AND CALCIUM CHANNEL ACTIVITY

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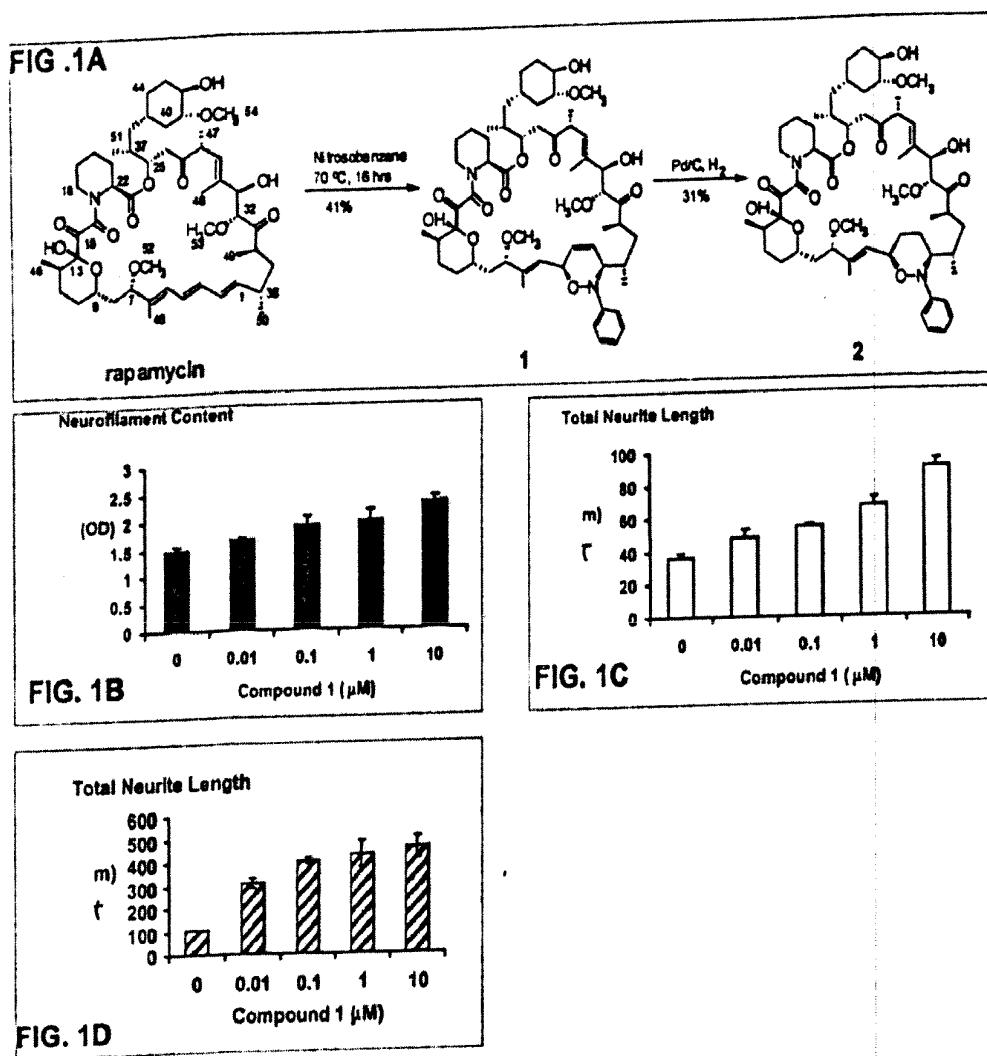
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C07D 498/22	(2006.01)
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A61P 25/06	(2006.01)
A61P 25/18	(2006.01)
A61P 25/22	(2006.01)
A61P 25/24	(2006.01)
A61P 25/14	(2006.01)
A61P 25/16	(2006.01)
A61P 25/08	(2006.01)
A61P 13/06	(2006.01)
A61P 9/06	(2006.01)
A61P 9/12	(2006.01)
A61P 9/10	(2006.01)
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(57) ABSTRACT

Immunophilin ligands and their uses as modulators of calcium channel activity are disclosed. Screening, therapeutic and prophylactic methods for conditions associated with calcium channel dysfunction, e.g., neurodegenerative and cardiovascular disorders, are also disclosed.



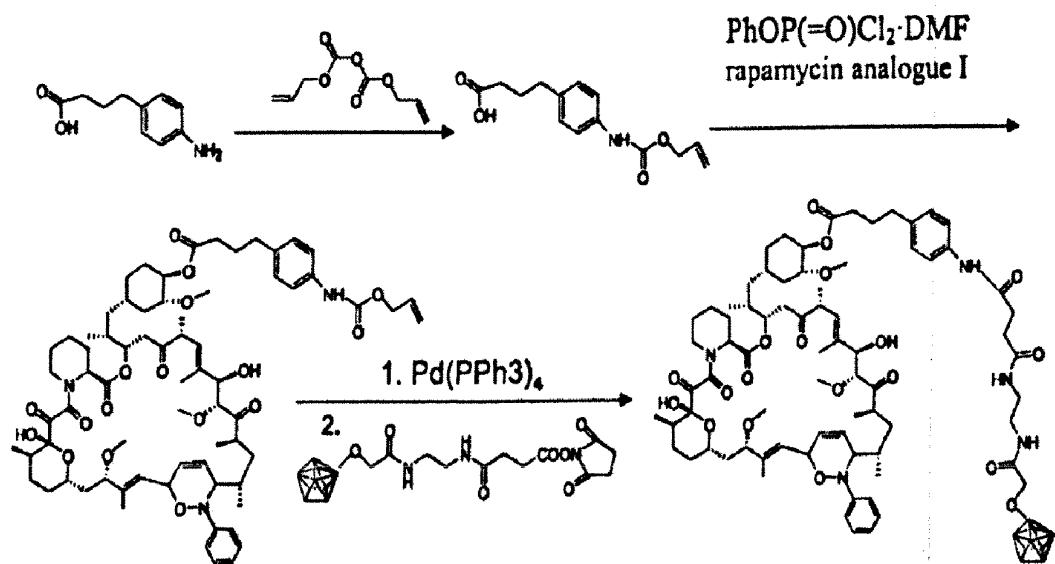


FIG. 2

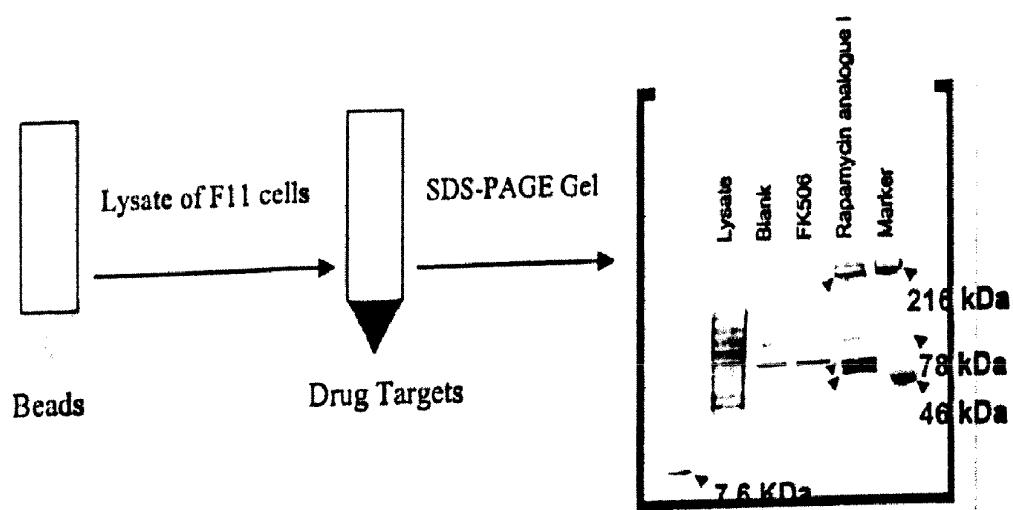


FIG. 3

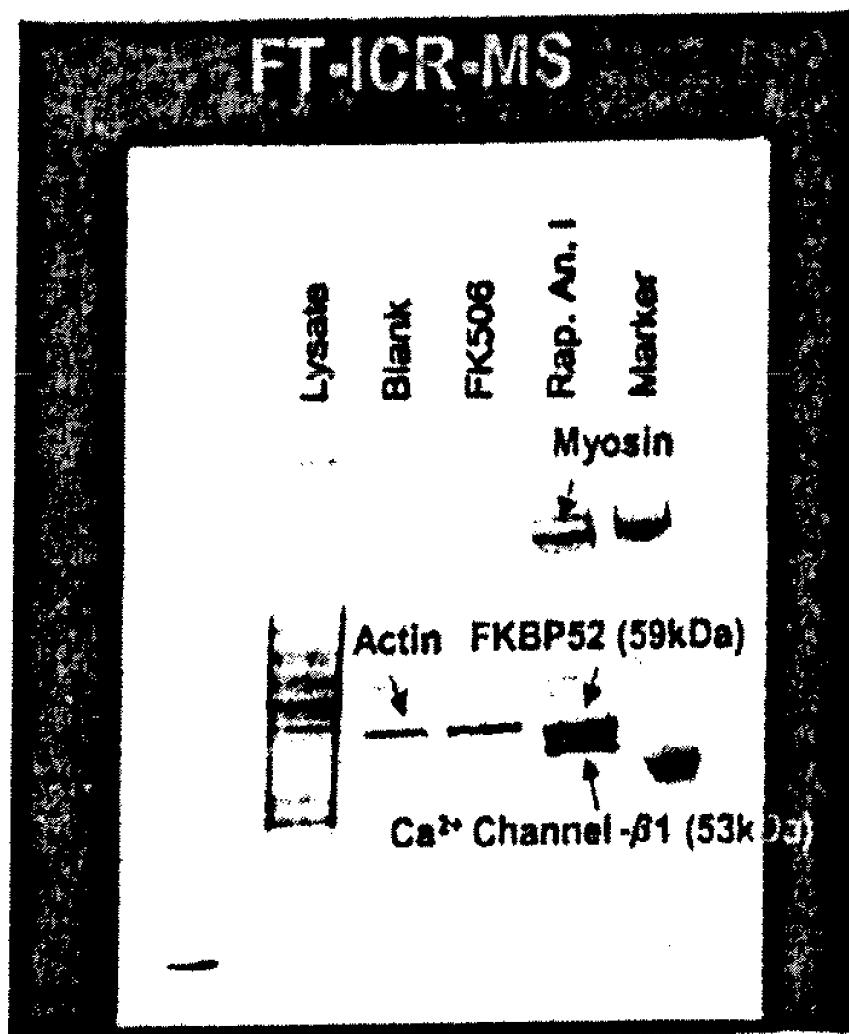
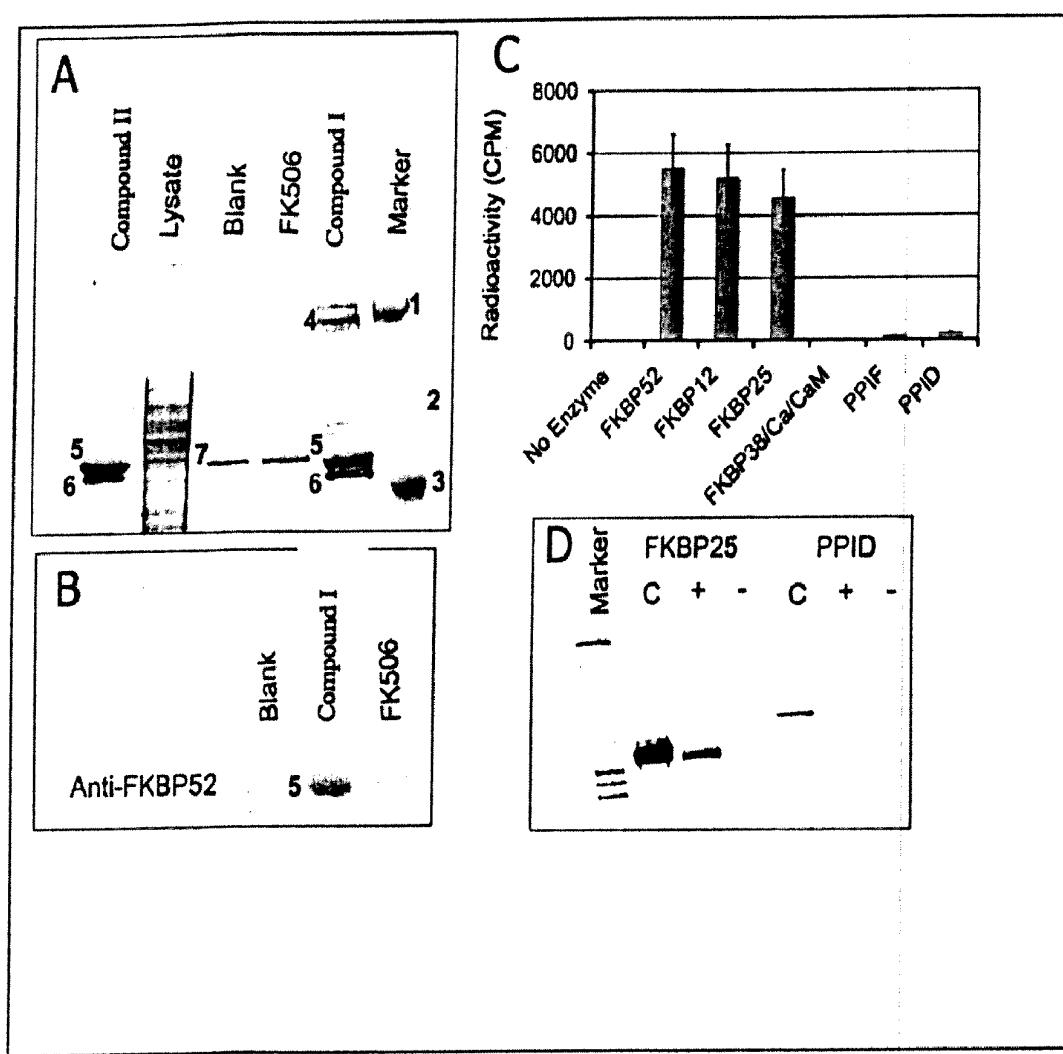
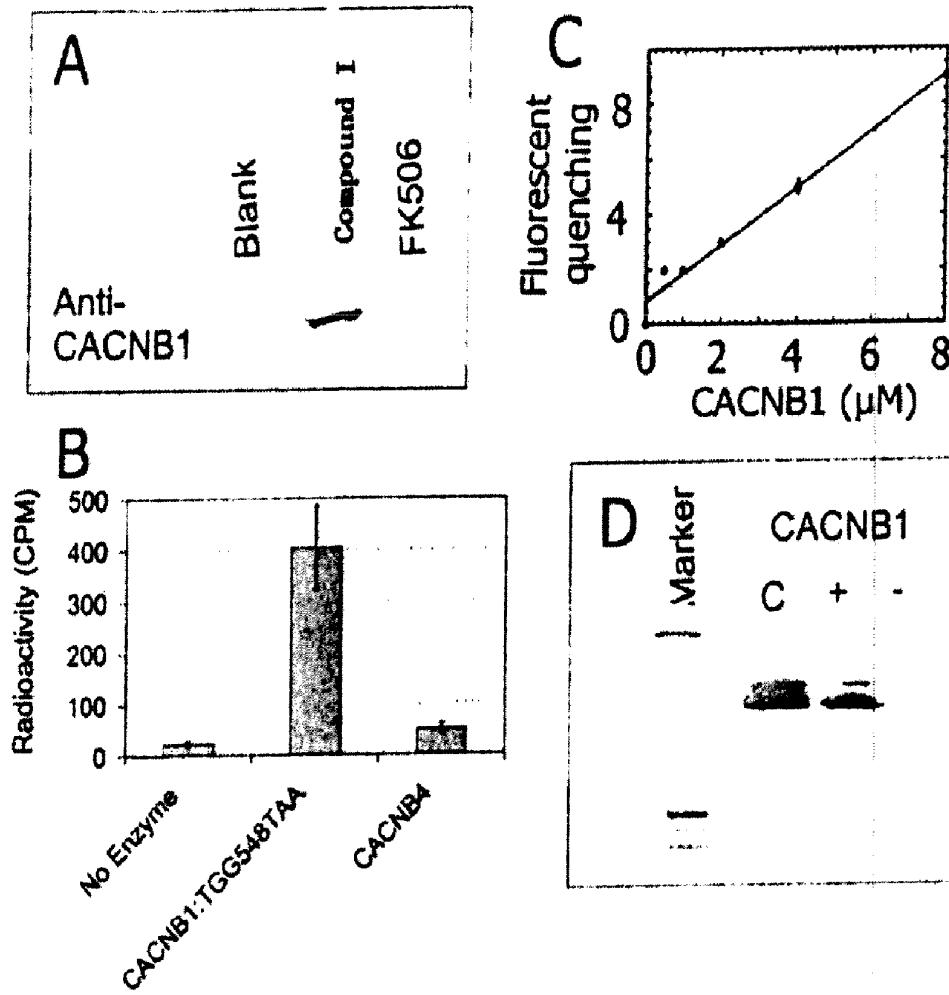


FIG. 4



FIGS. 5A-5D



FIGS. 6A-6D

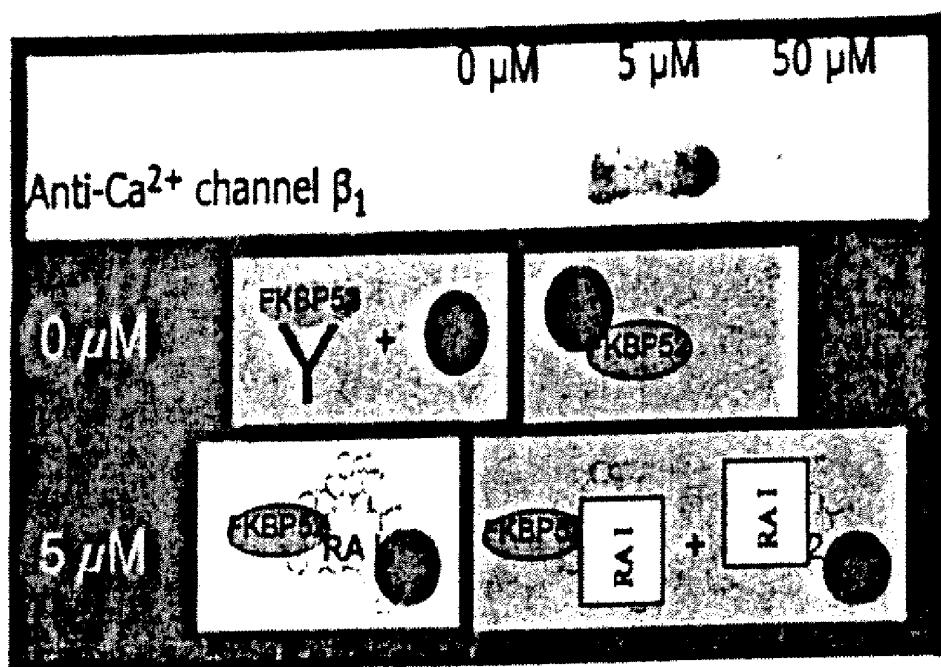
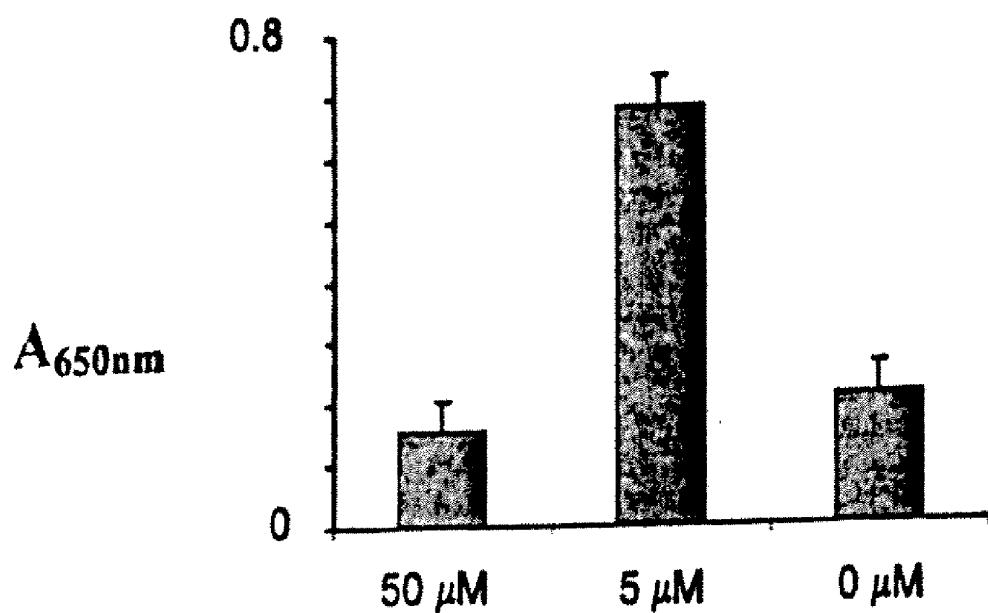
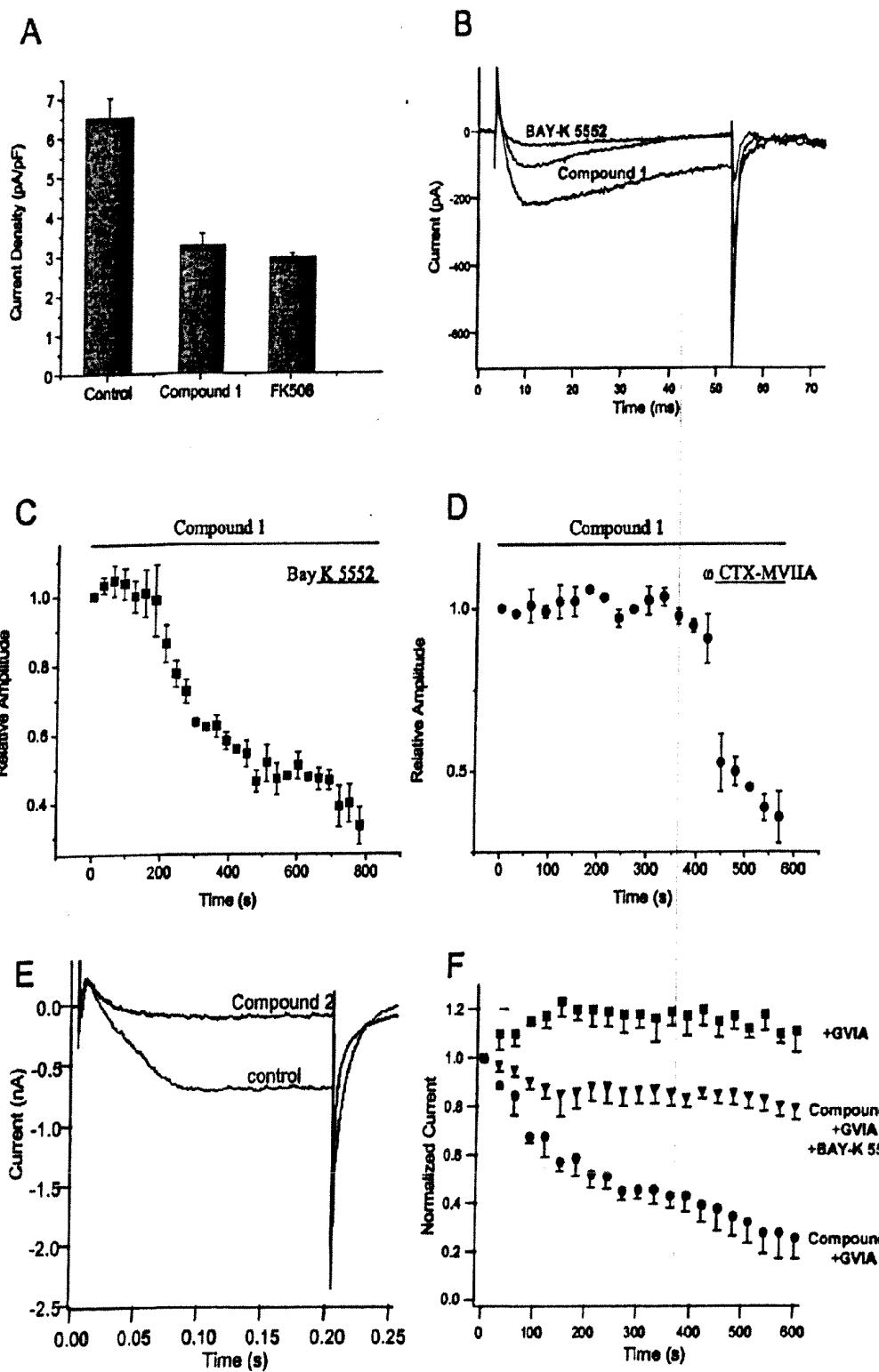


FIG. 7

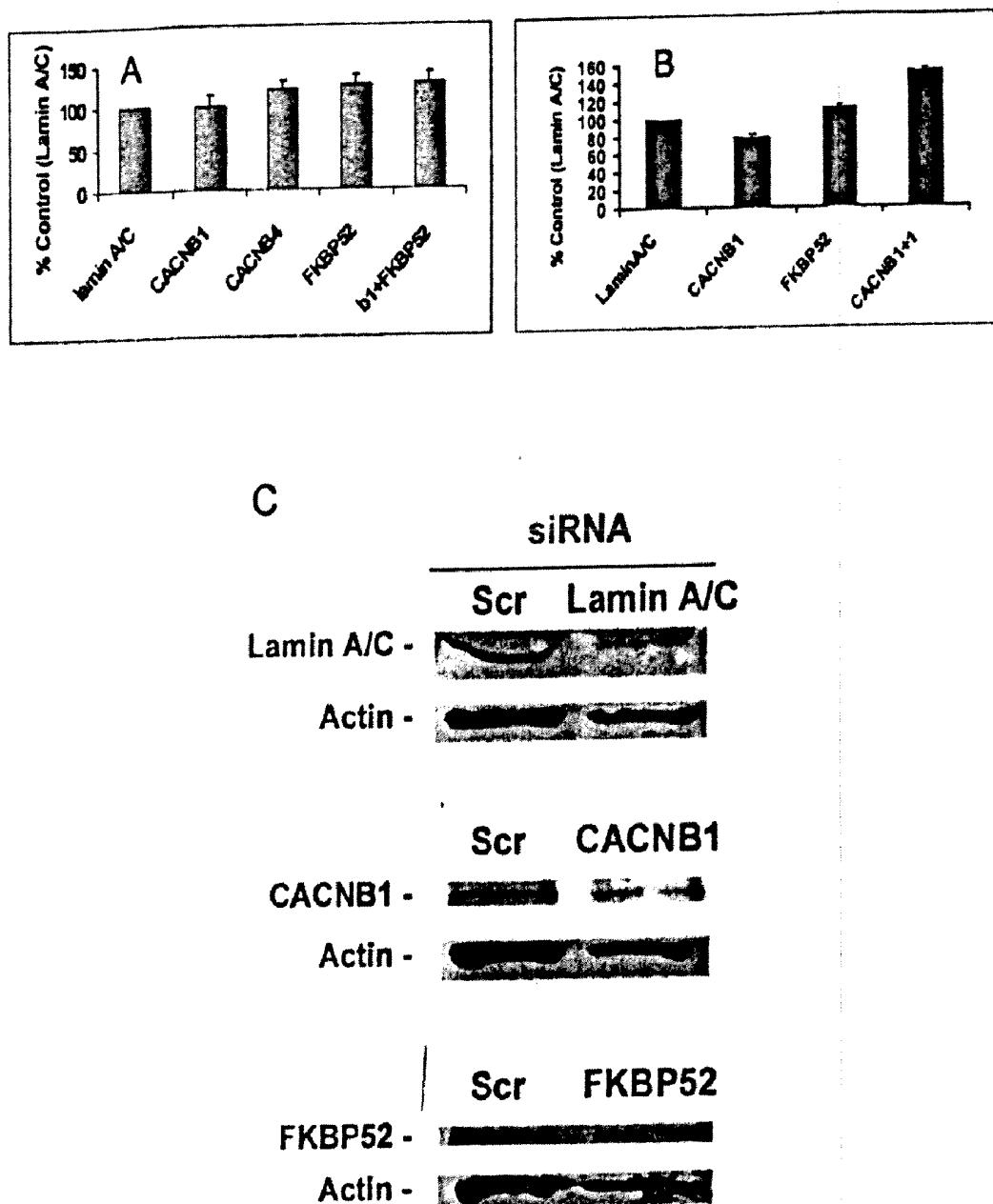


Rapamycin analogue I

FIG. 8



FIGS. 9A-9F



FIGS. 10A-10C

1 mvqktsmsrg pyppsqeipm evfdpspqk yskrkgrfkr sdgstssdtt snsfvrqgsa
61 esytsrpsds dvsleedrea lrkeaqral aqlekaktkp vafavrtvng ynpspgdevp
121 vqgvaitfep kdflhikeky nndwwigrlv kegcevgfip spvkldslrl lqeqklrqnr
181 lgssksgdns ssslgdvvtg trrptppasa kqkqkstehv ppydvvpsmr piilvgpslk
241 gyevtdmmqk alfdflkhrf dgrisitrvt adislakrsv lnnpshiiii ersntrssla
301 evqseierif elartlqlva ldadtinhpa qlsktslapl ivyikitspk vlqrlikarg
361 ksqsdkhlnvq iaaseklaqc ppemfdiild enqledaceh laeyleaywk athppsstpp
421 npllnrtmat aalaaspapv snlqgpylas gdqpleratg ehasmheypg elgqppglyp
481 sshppgragt lralsrqdtf dadtpgsrns aytelgdscv dmetdpsegp glgdpagggt
541 pparqgswed eeedyeeelt dnrnrgrnka rycaegggpv lgrnkneleg wgrgyvir
(SEQ ID NO:1)

FIG. 11A

1 gagggaaaggc aggaaggagg cagccgaagg ccgagctggg tggctggacc gggtgctggc
 61 tgcgcgcgc tgcttgcgc tcccacggc tetccatgc gctgagggag cccggctggg
 121 ccggccgcgc ggccggaggg gaggttcctc tccatgtcc agaagaccag catgtccgg
 181 ggcccttacc cacccttcca ggagatcccc atggaggtct tcgacccccc cccgcaggc
 241 aaatacagca agaggaagg gcgattcaa cggtcagatg ggagcacgtc ctggatacc
 301 acatccaaca gctttgtccg ccaggctca gcgaggtcct acaccagccg tccatcagad
 361 tctgtatgtat ctctggagga ggaccgggaa gccttaagga aggaagcaga ggcgcaggca
 421 tttagcgcage tggagaagge caagaccaag ccagtgcgtt tgctgtgcg gacaaatgtt
 481 ggctacaatc cgtctccagg ggatgggtg cctgtgcagg gagggccat caccttcgag
 541 cccaaagact tccatgcacat caaggagaaa tacataatg actgggtgat cggggggctg
 601 gtgaaggagg gctgtgaggt tggcttcatt cccagccccg tcaacttggc cagecttcgd
 661 ctgtgcagg aacagaagct ggcgcacaaac cgccttcggc ccagcaatc aggcgataac
 721 tccatgttcca gtctgggaga tggatggact ggacccccc geccccaccc cctgtccagt
 781 gccaaacaga agcagaaggc gacagagcat gtggccccc atgacgtggt gcttccatg
 841 aggccccatca ttctgggtgg accgtgcgtc aagggttcacg aggttacaga catgtatcg
 901 aaagctttat ttgtatgtt ttgtatgtt ggatctccat cactgtgtg
 961 acggcagata ttcccttgcg taagcttcgtca gtttcaaca accccagcaa acacatcate
 1021 attgagtcgtt ccaacacacg ctccagccgt gctggaggtgc agagttaat cgagcgtate
 1081 ttctggatgtgg cccggacccct tcaggatggc gctgtggatg ctgcacccat caatcccc
 1141 gcccggatgtt ccaagacccctc gctggccccc atcattttt acataaatg cacccttc
 1201 aaggacttcc aagggttcat caagtcccgaa ggaaatgttc agtccaaaca cctcaatgtc
 1261 caaatagccg ctcggaaaaa gctggcacag tgccccctg aaatgttgc catcatctg
 1321 gatgagaacc aattggagga tgcttcgtc catctggccg agtacttggg agcttattgg
 1381 aaggccacac accccggccag cagcacgcga cccaaatccgc tgctgaaccg caccatggc
 1441 accgcagccc tggctggccag cccgtccct gtcgtccaaacc tccaggacc ctacccctgt
 1501 tccggggacc agccacttggc acggggccacc ggggagcacg ccagcatgca cgagtaccca
 1561 ggggagctgg gccagcccccc aggcctttaa cccagcagcc acccaccagg cccggcaggc
 1621 acgttacggg cactgtcccg ccaagacact tttgtatggc acacccccc cagccgaaac
 1681 tctgtctaca cggagctggg agactcatgt gtggacatgg agactgaccc ctcaaggggg
 1741 ccagggtcttgg gagacccttgg agggggccgc acggccccccag cccgacaggg atccctgggg
 1801 gacgagggaa aagactatgg ggaagagctg accgacaaacc ggaaccgggg cccgaataag
 1861 gcccgtact ggcgtgggg tgggggttca gttttggggc gcaacaaaggaa tgatgtggag
 1921 ggctggggac gaggcgctca catttcgtca gaggcagggg ccacacggc ggagggaggg
 1981 ctctgagccc aggggggggg agggagcgag gggttccatc ctgacatgtt ttcgtccca
 2041 gggggcgttgc tccatgttcc ttcagatggc tttgtctaaa gttttgggtt tttttgggtt
 2101 taccatccccca gtcgggggg gggccatgg ccccaatggc ctttttttac ctgcctgttg
 2161 tggatggatg ggggataccct accttttctga agtgcgttccct ttcctccatc ttaaggggct
 2221 ctctccctcc acccttcctag agaaaagggtt cacttccttta acttttctta ctccggggcc
 2281 taatgtacgg ttcataatgg gatggatcttc cttttcccaaa gtcgttcgtac tggggaggg
 2341 ctggggcgttgc ttcctggaaa gggggggccac agatttttc ccatggggcc tctttcccc
 2401 agaccccaaga tccaaagggtcc ctcaccccttc ctcccaatgg cttggcagca
 2461 tcgtctggc ggtgaaagcc atagcatgttgc ccccaatgg ggatgttgc ttggggaggg
 2521 ttctgggtgg aagtcggcag gcatacagea ccccttacccttccgtggccca tggcaacgtc
 2581 caggggccag aacccttggg agtgagccgc cgagacgttgc ctccccccccc cccacccatca
 2641 tgcttcgtcc ttgttccatcc ccaggaatgg gttttggccctt caacatccct tgcctgtgc
 2701 cattatgttgc gggggccctt ctgtatgttgc gccccccatc cctgtggccat tgggggtgtgg
 2761 agcccatggg acaatctgtt ccgttccat ttaamccaaa aaactgttcc ttacccctca
 2821 ccctggggcc ccaggggggaga ggaccctgg gatgggttgc aggggttgc ttggggaccc
 2881 ggatcttcc tggggggctt ggatgttgc gttgtgttgc ttctgtgtt
 2941 ctgtttgttca cccatgttca ctccccccatc gagggggctt ggtacccccc ctccccctgg
 3001 gcatccctt gtcgttgc gatggatgttgc cccatggccat cccctccccc agatcccccc
 3061 ctggggcaga gagagccggag tggccaaaca aggactgggg cccggggcc tggccgcctc
 3121 agggatgggg acccttgc ttttttttgc ctttttttgc caatgtccca cccctccatc
 3181 ggggggggggg tgcgttcc acttactgtat tagaaagacac cactggccctt cttttcccc
 3241 tccctgttgc gtgttgcgttgc ccccaatgttgc ttttttttgc ttttttttgc
 3301 agaagtatgtt tccatgtataaaacccatgt ttttttttgc ttttttttgc ttttttttgc
 3361 gtttttttgc tccatgtataaaacccatgt ttttttttgc ttttttttgc ttttttttgc
 3421 gggggatgttgc aagggtgggtt ggttgcgttgc gttgtgttgc ttttttttgc
 3481 aagatgttcc ttgttgcgttgc ttttttttgc ttttttttgc ttttttttgc
 3541 cttccatgttgc tccatgtataaaacccatgt ttttttttgc ttttttttgc ttttttttgc
 3601 cccaaaggcaaa tggggaaatgttgc ttttttttgc ttttttttgc ttttttttgc
 3661 ttttttttgc ttttttttgc ttttttttgc ttttttttgc ttttttttgc ttttttttgc
 (SBQ ID NO:2)

1 mvqktsmsrg pyppsqeipm evfdpspqgk yskrkgrfkr sdgstssdtt snsfvrqgsa
61 esytsrpds dvsleedrea lrkeaqral aqleaktkp vafavrtng ynpspgdevp
121 vqgvaitfep kdflhikeky nndwwigrlv kegcevgfip spvkldslrl lqeqklrqnr
181 lgssksgdns ssslgdvvtg trrptppasg nemtnlafel dpleleeeea elgeqsgsak
241 tsvssvttpp phgkripffk ktehvppydv vpsmrpiilv gpslkgyevt dmmqkalfdf
301 lkhrrfdgris itrvtadisl akrsrvlnnps khiiiersnt rsslaevqse ierifelart
361 lqlvaldadrt inhpqqlskt slapiivyik itspkvlqr1 iksrgksqsk hlnvqiaase
421 klaqcqpemf diildenqle dacehlaeyl eaywkathpp stppnplln rtmataalaa
481 spapvsnlqv qvltslrrnl gfwgglessq rgsvvvpqeque ham (SEQ ID NO:3)

FIG. 11C

1 gagggaaaggc aggaaggagg cagccgaagg ccgagctggg tggctggacc gggtgctggc
61 tgcgccgcgc tgcttcggc tcccacggc tctccatgc gctgagggag cccggctggg
121 cggggccggc ggcgggaggg gaggctcctc tccatgtcc agaagaccag catgtccccgg
181 ggcccttacc caccctccca ggagatcccc atggaggtct tgcaccccaag cccgcaggc
241 aaatacagca agagaaaagg gcgattcaaa cggtagatg ggagcacgtc ctcggatacc
301 acatccaaca gcttgcggc ccagggctca gcgagtcct acaccagccg tccatcagac
361 tctgatgtat ctctggagga ggacccggaa gccttaagga aggaagcaga gcccaggca
421 tttagcgcage tcgagaaggc caagaccaag ccagtggcat ttgctgtgcg gacaatgtt
481 ggctacaatc cgtctccagg ggatgaggtg cctgtgcagg gagtgccat cacccatcgag
541 cccaaagact tccatcataatc caaggagaaa tacaataatg actgggtggat cgggcccgtg
601 gtgaaggagg gctgtgaggt tggcttcatt cccagccccg tcaaactggc cagccttcgc
661 ctgctgcagg aacagaagct gcgcacaaac cgcctcggt ccagcaaatc aggccataac
721 tccagttcca gtctgggaga tgtggtgact ggcacccggc gccccacacc ccctgccagt
781 ggtaatgaaa tgactaactt agcctttgaa ctagacccccc tagagttttaga ggaggaagag
841 gctgagctt gtgagcagag tggctctgcc aagacttagt ttagcagtgt caccaccccg
901 ccacccatg gcaaaacgcac ccccttcatt aagaagacag agcatgtgcc cccctatgac
961 gtgggcctt ccatgaggcc catcatcctg gtgggaccgt cgctcaaggg ctacgagtt
1021 acagacatga tgcagaaagc ttatgtac ttcttgaagc atcggtttga tggcaggatc
1081 tccatcactc gtgtgacggc agatatttc ctggctaagc gctcgttct caacaaccc
1141 agcaaaacaca tcatcattga ggcgtccaaac acacgttca gcctggctga ggtgcagagt
1201 gaaatcgagc gaatcttga gctggcccg acccttcaact tggctgtct ggatgtgac
1261 accatcaatc acccagccca gctgtccaaag acctcgctgg ccccatcat ttttacatc
1321 aagatcacct tcccaaggt acttcaaagg tcatcaagt cccgagggaa gtctcgttcc
1381 aaacacccca atgtccaaat agcggccctg gaaaagctgg cacagtggcc ccctgaaatg
1441 tttgacatca tccatggatga gaaccaattt gaggatgcct ggcagcatct ggccggagtac
1501 ttggaaagctt attggaaaggc cacacaccccg cccagcagca cggccacccca tccgctgtg
1561 aaccgcacca tggctaccgc agccctggct ggcaggccctg cccctgtctc caacccatcg
1621 gtacagggtgc tcacccctgtc caggagaaac ctggcttct gggggggct ggatcttc
1681 cagccccca gtgtgggtgc cccaggagcag gaacatgcca tggatgtggc gcccctggcc
1741 tttcccttc tgctctgggg tcggaaactgg agtgcaggaa acatggagga ggaaggaaag
1801 agtttatattt tgtaaaaaaa taagatgagc ggcaaaaaaa aaaaaaaaa (SEQ ID NO:4)

FIG. 11D

1 mvqktsmsrg pyppsqeipm evfdpspqk yskrkgrfkr sdgstssdtt snsfvrqgsa
61 esytsrpds dvsleedrea lrkeaqral aqleaktkp vafavrvnvg ynpspgdevp
121 vqgvaitfep kdflhikeky nndwwigrly kegcevqfip spvkldslrl lqeqlrqrn
181 lgssksgdns ssslgdvvvtg trrptppasa kqkqkstehv ppydvvpssmr piilvgpslk
241 gyevtdmmqk alfdf1khrl dgrisitrvt adislakrsv lnnpshilli ersntrssla
301 evqseierif elartlqlva ldadtinhpa qlsktslapi ivyikitspk vlqrliksrg
361 ksqskhlnvq iaaseklaqc ppremfdiild enqledaceh laeyleaywk athppsstpp
421 npllnrtmat aalaaspavy snlqvqvlts lrrnlgfwgg lessqrgsvv pqequeham
(SEQ ID NO:5)

FIG. 11E

1 gagggaaaggc aggaaggagg cagccgaagg ccgagctggg tggctggacc gggtgctggc
61 tgcccccgc tgcttcggc tcccacggcc tctccatgc gctgagggag cccggctggg
121 cccggccggc ggcgggaggg gaggctccct tccatggtcc agaagaccag catgtccccg
181 ggcccttacc cacccctcca ggagatcccc atggaggtct tcgaccccaag cccgcaggc
241 aaatacagca agagaaaagg gcgattcaaa cggtcagatg ggagcacgtc ctggatacc
301 acatccaaaca gcttgtccg ccagggctca gcggagtcct acaccagccg tccatcagac
361 tctgatgtat ctctggagga ggaccgggaa gcctaagga aggaagcaga ggcgcaggc
421 ttagcgcgc tcgagaaggc caagaccaag ccagtggcat ttgtgtgcg gacaaatgtt
481 ggctacaatc cgtctccagg ggatgaggtg cctgtgcagg gatggccat cacttcgag
541 cccaaagact tcctgcacat caaggagaaa tacaataatg actgggtggat cgggcggctg
601 gtgaaggagg gctgtgaggt tggcttcatt cccagcccg tcaacttggc cagccttcgc
661 ctgctgcagg aacagaagct ggcgcagaac cgcctcggtt ccagcaaatc aggcgataac
721 tccagttcca gtctggaga tgtggact ggcacccgcc gccccacacc ccctggcagt
781 gccaaacaga agcagaagtc gacagagcat gtggccctt atgacgtggt gccttccatg
841 aggcccatca tcctgggtgg accgtcgctc aagggtacg aggttacaga catgtgcag
901 aaagctttat ttgacttctt gaagcatcg tttgatggca ggatctccat cactcgtgt
961 acggcagata ttccctggc taagcgctca gttctcaaca accccagcaa acacatcatc
1021 attgagcgct ccaacacacg ctccagccctg gctgaggtgc agagttaaat cgagcgaatc
1081 ttcgagctgg cccgaccct tcagttggtc gctctggatg ctgacaccat caatcacca
1141 gcccagctgt ccaagacctc gctggccccc atcattgttt acatcaagat cacctctccc
1201 aaggtaatcc aaaggtcat caagtcccgaa ggaaagtctc agtccaaaca cctcaatgtc
1261 caaatagccg cctccggaaaa gctggcacag tgccccctg aaatgtttga catcatctg
1321 gatgagaacc aattggagga tgcctgcag catctggcg agtacttgg agcctattgg
1381 aaggccacac acccgccccag cagcacgcca cccaaatccgc tgcgtgaaccg caccatggct
1441 accgcagcccc tggctgccag ccctggccct gtcctcaacc tccaggtaca ggtgctcacc
1501 tgcgtcgaa gaaacctcggtt cttctggggc gggctggagt cctcacagcg gggcagtgt
1561 gtggcccgagg agcaggaaca tgccatgttag tgggcgcctt gcccgtcttc cctcctgctc
1621 tggggtcgga actggagtgc agggAACATG gaggaggaag ggaagagctt tattttgtaa
1681 aaaaataaga tgagcggcaa (SEQ ID NO: 6)

FIG. 11F

1 mvqksgmsrg pyppsqeipm evfdpspqk yskrkgrfkr sdgstssdtt snsfvrqgsa
61 esytsrpds dvsleedrea lrkeaqrlaqleaktp vafavrtvng ynpspgdevp
121 vqgvaitfep kdflihikeky nndwwigriv kegcevgfip spvkldslrl lqeqlrqnr
181 lsssksgdns ssslgdvvtg trrptppasg nemtnfafel dpleeeeeaa elgehggssak
241 tsvssvttpp phgkripffk ktehvppydv vpsmrpiilv gpslkgyevt dmmqkalfdf
301 lkhrgdgris itrvtadisl akrsvlnnps khiiersnt raslaevqse ierifelart
361 lqlvaldadlt inhpaqlskt slapiivyik itspkvlqrl iksrgksqsk hlnvqiaase
421 klaqcpcpemf diildengle dacehlaeyl eaywkathpp sstppnplln rtmataalaa
481 spapvsnlqv qvltslrrnl sfwgleasp rggdavaqpq eham (SEQ ID NO:7)

FIG. 11G

1 ttccggcggc ggcggcggcg acggcggcag cggccgcaga gagcacacgc cgagccggga
61 gggcaagcaa ggcggcgcgc gtgcagccgg aggtccagct gggagactgc acccggtgt
121 ggctgcgcga cgccgcgtg ctctggcgc ggacggcgc tccatgcgc tgagagcgcc
181 cggctggcgt gggaggcgg ccggaccggg ggatcccttc catggtccag aagagcggca
241 tgtccccggg cccttaccca ctttccaaag agatccctat ggaggtcttc gaccccgacc
301 cacagggcaa gtacagcaag agggaaaggcc ggttcaaaag gtcagacggg agtacgtct
361 cggatataaac atccaaacagc ttctccgcg agggtctcagc agatccctac acgagccgac
421 catcagactc tgatgtgtct ctggaggagg accggaaaggc cttaaaggaaag gaggcagagc
481 gccaggccctt agcccagctc gagaaagcca agaccaaacc agtggctttt gctgttcgga
541 caaatgttgg ctacaatccg tctccagggg atgaggtgcc tgacagggaa gtggccatca
601 ccttgagcc caaggacttc ctacacatca aggagaagta caataatgac tggtgattg
661 ggcggctggta agaggaaaggc tgcgagggtt gcttcatccc cagccggc aaactggaca
721 gccttcgtct gctgcaggaa cagaccctgc gccaagaaacc cctcagctcc agcaagttag
781 gtgacaactc cagttccagt ctgggagatg tggtaactgg caccggccgc cccacacccc
841 ctggcactgg taatgaaatg actaactttt cctttagct agacccctta gagtttagagg
901 aggaggaggc agagcttaggg gacgcacggcg gtcagccaa gactagctg agcagtgtca
961 ccacgcccggc accccacggc aacgcacatcc ctttcttaa gaagacagag cacgtcccc
1021 cctatgacgt ggtgccttcc atgaggccca tcatcctggt ggaccgtcg ctcaagggt
1081 atgaggtgac agacatgatg cagaaggctg tggtactt cctcaagcat cggtttgc
1141 gcaggatttc catcacccgg gtaacagctg acatccctt ggcacaaacgc tccgtctca
1201 acaaccccaag caaacacatc atcattgac gtcacaaacac gcttcacccgc ctggctgagg
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1321 acgctgacac catcaaccac ccagcccgac tctctaaaac gtcgctggc cccatcattg
1381 ttatcatcaa gatcacatct ccaaggtac tgcagaggct catcaaattcc cgagggaaagt
1441 ctcaatccaa acacccatc gtccaaatag cagcctcgga gaaatggca cagttccccc
1501 ccgaaatgtt tgacataatc ctggacgaga accaattggaa agatccctgc gacgtccgg
1561 ctgagttactt ggaaggctac tggaaaggccca cacatccggc tagcagcacg ccacccatc
1621 cgctgctgaa ccgcacccatg gtcacccgc ctctggctgc cagccctggc cccgtctcca
1681 acctccaggt acaggtgctc acctcgctca ggagaaatct cagttctgg ggccggctgg
1741 aggccctacc gcggggaggc gacgcgggtgg cccagctca ggacgcacggc atgtacccga
1801 tgccctctg gtcttccttc ccaccctggaa gtcacccggaa catgaggaag gaagggaaaga
1861 gctttatccc gtaaaaaacg tggtgacggc ca (SEQ ID NO:8)

FIG. 11H

1 mvqksgmsrg pyppsqeipm evfdpspqgk yskrkgrfkr sdgatssdtt snsfvrqgsa
61 esytsrpsds dvsleedrea lrkeaqral aqleakatk p vafavrtvg ynpspgdevp
121 vqgvaitfep kdflhikeky nndwwigrlv kegcevgfip spvkldslrl lqeqlrqr
181 lsssksgdns ssslgdvvtg trrptppasa kqkqkstehv ppydvvpsmr piilvgpslk
241 gyevtdmmqk alfdf1khrf dgrisitrvt adislakrs v lnnpshii ersntrssla
301 evqseierif elartlqlva ldadtinhpa qlsktslapi ivyikitspk vlqrlikr
361 ksqskskhlnvq iaaseklaqc ppemfdiild engledaceh laeyleaywk athppsstpp
421 npllnrtmat aalaaspav snlqgpylas gdqpldratg ehasvheypg elggppg1yp
481 snhplgragt lralsrqdtf dadtpgsrns aytepgdscv dmetdpsegp gpgdpagggt
541 pparqgswed eedyeeemtd nnrngrnk ar ycaegggpvl grnknelegw qqqvyr
(SEQ ID NO:9)

FIG. 11I

1 atggccaga agagcggcat gtccccgggc ctttaccac cttcccaaga gatccctatg
61 gaggtcttcg accccagccc acagggcaag tacagcaaga gaaaggcg gttcaaaagg
121 tcagacggga gtacgtcctc ggataacaaca tccaaacagct tcgtccgcca gggctcagca
181 gagtcctaca cgagccgacc attagactct gatgtgtctc tggaggagga ccgcgaagcc
241 ttaaggaagg aggccagagcg ccaggccta gcccagctcg agaaagccaa gccaaacca
301 gtggcttttg ctgttcggac aaatgttggc tacaatccgt ctccagggga tgaggtgcct
361 gtacagggag tggccatcac cttttagccc aaggacttcc tacacatcaa ggagaagtac
421 aataatgact ggtggattgg gcccgtggg aaggaaggct gcgaggttgg cttcatcccc
481 agcccggtca aactggacag cttcgtctg ctgcaggAAC agaccctcgcc ccagaaccgc
541 ctcagctcca gcaagtccagg tgacaactcc agttccagtc tggagatgt ggtactggc
601 accccggcccc ccacacccccc tgccagtgcc aaacagaagc agaaatcgac agagcacgt
661 cccccctatg acgtggtgcc ttccatgagg cccatcatcc tgggggacc gtcgtcaag
721 ggctatgagg tgacagacat gatgcagaaa gctgttttgc acttccatcaa gcatcggtt
781 gatggcagga ttccatcac cccggtaaca gctgacatcc ccctggccaa acgtccgtc
841 ctcaacaacc ccagcaaaca catcatcatt gagcgttcca acacgcgttc cagcctggct
901 gaggtacaga gtgaaattga gaggatctc gagctggccc ggaccttgqa gctggtcgccc
961 ttggacgctg acaccatcaa ccacccagcc cagctctcta aaacgtcgct ggccccatc
1021 attgtttaca tcaagatcac atctccaaag gtactgcaga ggctcatcaa atcccgaggg
1081 aagtctcaat ccaaacaccc caatgtccaa atagcagctt cggagaagct ggcacagtgt
1141 ccccccgaaa tggttgcacat aatccctggac gagaaccaat tggaaatgc ctgcgagcac
1201 ctggctgactt acttggaaage ctactggaaag gccacacatc cgcttagcag caccgcaccc
1261 aatccgctgc tgaaccgcac catggctacc gcagctctgg ctgcccggcc tgcccccgtc
1321 tccaaacctcc agggacccta ctttgcctcc ggggaccagg cgctggaccg ggccactgg
1381 gaacatgcca gtgtgcacga gtaccccggg gaattggcc agccccagg ctttacccc
1441 agcaaccacc cacttggccg ggcaggcacc ctgcgggcgc tatcccgccaa agacaccctt
1501 gatgtgtaca cccccggcag ccgaaattct gcctacacgg agccgggaga ctcgtgttg
1561 gacatggaga cagacccctc agagggccca gggcctggag accctgcagg gggaggcaca
1621 ccaccagccc ggcaggcacc ctggaaagac gaggaaact atgaggagga gatgaccgac
1681 aacaggaacc ggggcccggaa taaggcccgc tactgtgcgg aggtgttgg gccgggttctg
1741 gggcgcaata agaatgagct ggaggcgtgg ggacaaggcg tctacactcg ctga
(SEQ ID NO:10)

FIG. 11J

1 mttdegaknn eesptatvae qgeditskkd rgvlkivkrv gngeetpmig dkvyvhykgk
61 lsngkkfdss hdrnepfvfs lgkgqvikaw digvatmkg eichllckpe yaygsagslp
121 kipsnatlff eielldfkge dlfedggiir rtkrkgegys npnegatvei hlegrcggrm
181 fdcrdvaftv gegedhdipi gidkalekmq reeqcilylg prygfgeagk pkfgiepnae
241 liyevtlksf ekakeswemd tkekleqaai vkekgtvfyfk ggkymqaviq ygkivswlem
301 eyglsekesk asesflaaf lnlamcylkl reytkavecc dkalgltsan ekglyrrgea
361 qllmnefesa kgdfekvlev npqnkaarlq ismcqkkake hnerdrriya nmfkkfaeqd
421 akeeankamg kktsegvtne kgtdsqamee ekpeghv (SEQ ID NO:11)

FIG. 12A

1 gggccggctc gcgggcgtg ccagtctcg ggccgggtgt cccggcgcgc ggccggctgc
 61 tggggggct gaagggttag cgagacacgg gcaaggcggg gactgacgga gtcggcggc
 121 cccccggcgc acaggttctc tacttaaaag acaatgacta ctgatgaagg tgccaagaac
 181 aatgaagaaa gccccacage cactgtgt gacggggag agggatattac ctccaaaaaa
 241 gagaggggag tattaaagat tgc当地aa gatggggatgt gtgaggaaac gcccgtgatt
 301 ggagacaaag ttatgttcc ttacaaagga aaattgtcaa atggaaaagaa gtttgattcc
 361 agtcatgata gaaatgaacc atttgcttt agtcttggca aaggccaaatg catcaaggca
 421 tgggacattg ggggtggctac catgaagaaa ggagagatgt gcccatttact gtgcaaaacca
 481 gaatatgcat atggctcggt tggcagtctc ccttcaatgc aactctttt
 541 tttgagattt agtcttgc tttcaaaagga gaggatttat ttgaagatgg aggcatata
 601 cggagaaacca aacggaaagg agaggatgt tcaatccaa acgaaggagc aacagttagaa
 661 atccacctgg aaggccgtg tggggaaagg atggttgcact gcaaggatgtt ggcatttact
 721 gtggggaaag gagaagacca cgacattcca attggaaattt gcaaggatgtt ggagaaaatg
 781 cagcgggaaag aacaatgtat ttatatctt ggaccaagat atggttttgg agaggcagg
 841 aaggccaaat ttggcattga acttaatgtt gacgttataat atgaagttac acttaaagage
 901 ttcgaaaaagg ccaaaagaaatc ctggggatgt gatccaaag aaaaatttgg gcaaggctg
 961 attgttcaaaag agaaggaaac cgtataacttc aaggggggca aatacatgca ggcgggtgatt
 1021 cagttatggaa agatgtgtc ctgggttagag atggaaatgtt gtttacgaa aaggaaatcg
 1081 aaggcttctg aatcattttctt ccttgc ttttgc tggccatgtg ctatctgaaag
 1141 cttagagaat acaccaaaatc tggtaatgc tggcataagg cccttggact ggacagtgc
 1201 aatgagaagaaatc gttttatag gagggtggaa gcccacgtgc tcatgaaatc gtttgc
 1261 gccaagggtt acttttggaa atgtgtttt gtaaaaaaaa agaataaggc tgcaagactg
 1321 cagatcttca tggccagaa aaggccaaatc gggccaaatc agcgggaccc caggatata
 1381 gccaacatgt tcaaaatgtt tggcagacgg gatggccaaatc aaaaaggccaaatc
 1441 ggcaaaagaaatc cttagaaagg ggttactaat gaaaaaggaa cagacatgtc agcaatggaa
 1501 gaagagaaac ctggggccaa cgtatgtacgc caccggccaaatc agggaaatgtt cccatgt
 1561 tggcccttc ctcataatggc ttccccccaa ctcaggacag aacatgtttt aatgtaaatg
 1621 ttgttataatgtt ctatgttattt ctggaaagca atggccaaatc cagtagcttcc
 1681 ccccccctgtt gttttttttt ggttactgtt gggggatgtt cccggggactt ccagggtggaa
 1741 caaacagaaatc tgactgtgtt ttggggggatgtt gggccatgtt gtttgc
 1801 agtttctatc aacccatgtt ttttttttttccatgttccatgttccatgttccatgtt
 1861 gggctgttag ttttttttttccatgttccatgttccatgttccatgttccatgttccatgtt
 1921 gcttcatgtt ttttttttttccatgttccatgttccatgttccatgttccatgttccatgtt
 1981 gtttttttttccatgttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 2041 cacatgttccatgttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 2101 gaaatccatgttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 2161 agccttccatgttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 2221 ttcatttttttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 2281 ttcatttttttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 2341 tttttttttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 2401 agcattgttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 2461 ttgtgttttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 2521 gcaatgttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 2581 aatccatgttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 2641 ttttttttttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 2701 ttcacccatgttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 2761 ctggcgtggatgttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 2821 taaatccatgttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 2881 gtttccatgttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 2941 agaaacacttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 3001 ttcacccatgttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 3061 tatgtatgttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 3121 tcaatgttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 3181 agttttttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 3241 tttatgttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 3301 tttatgttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 3361 caatgttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 3421 gtttccatgttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 3481 gggaaacttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 3541 actacagggttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 3601 taatgttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 3661 tatgtatgttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 3721 atttagatgttccatgttccatgttccatgttccatgttccatgttccatgttccatgtt
 3781 a (SEQ ID NO:12)

FIG. 12B

1 mttdegtssn genpaatmte qgedittkkd rgvlkivkrv gtsdeapmfg dkvyvhykgm
61 lsdgkkfdss hdrkpfafs lgqqqvikaw digvstmkkg eichllckpe yaygsagh1q
121 kipsnatlif eielldfkge dlfedsgvir rikrkgegys npnegatvkv hlegccggrt
181 fdcrdvffvv gegehdipi gidkalvkmq reeqcilylg prygfgeagk pkfidpnae
241 lmyevtlksf ekakeswemd tkekltqaai vkektvyfk ggkytqaviq yrkivswlem
301 eyglsekesk asesflaaf lnlamcylkl reynkavecc dkalgldean ekglyrrgea
361 qllmndfesa kgdfekvlav npqnraarlq ismcqrkake hnerdrruya nmfkkfaerd
421 akeeaskags kkavegaagk qhesqameeg kakghv (SEQ ID NO:13)

FIG. 12C

1 cccggcggcgg tgccggcgc gcggctgagc gaccgagcgt gcgacggag cgccggcgt
61 ctggggcgggc tgaggcggc gcggggcggc ggagagacgc ggagcgaggg acgcggcggc
121 ggcggacgcg ggcacaggc ttctacttac aaaggacaat gactactgtat gagggccacca
181 gtaacaatgg agagaaccca gcagccacca tgactgagca gggtaagat atcaactacga
241 agaaaagacag aggagtatta aagattgtca aaagagtggg gactagtgc gaggccccaa
301 tgggggtga caaagttat gtccactaca aaggatgtt gtcagatgga aagaagtttq
361 atccagtca tgacagaaag aagccatttgc cctttagcct tggccaaggc caggttatca
421 aagcctggga cattggggtg tctactatga agaaaaggcga gatctgcctt ttattatgt
481 aaccagaata tgcttatggc tcggctggcc acctccaaaa aattccatca aatgcacac
541 tcttttttga gattgagcgc ctgttattca aagggtgagga ttatgttga gattcaggcg
601 ttatccgtat aatccaaacgg aaaggcgagg gatactcaaa cccaaacgaa ggagcaacgg
661 taaaagtcca ccttggaaaggc tgctgtggg gaaggacatt tgattggcga gatgtgggt
721 tcgttgttgg ggaaggagaa gaccacgaca ttccgatrgg gatcgacaaa gcccgggtga
781 agatgcagag agaagaacag tgtatctat atttggacc acgtatggt ttggagaag
841 ccgggaagcc taagttggc attgacccca atgctgagct tatgtacgag gtcaccccta
901 agagcttcga gaaggccaaa gaatcttggg agatggacac caaagaaaag ctgacgcagg
961 ctgccatcgt gaaagagaag ggaactgtgt acttcaaggg aggaagatc acgcaggccg
1021 tgattcgtat caggaagata gtgtctggc tgagatgga atacggctg tcagagaagg
1081 agtccaaacgc ctcagagtcg ttcccttcgc cagcccttcg gaaacctggc atgtgttacc
1141 tgaagctccg agagtacaac aaagccgtgg agtgcgtcga caaggccctt ggactggaca
1201 gtgcctaattga gaaaggcttg tacagaaggg gcgaggccca gtcgtcatg aatgacttgc
1261 agtcggccaa gggcgacttc gagaagggtgt tggcgtcaat tcetcagaac agggccgtc
1321 gcctgcagat ctccatgtgc cagaggaaagg cggaggagca caacgaggcgg gaccgcagg
1381 tgcacggccaa catgttcaag aagttcgac agcggggacgc aaaggaggg gccagcaaag
1441 ctgggagccaa gaaggctgtt gaaaggagccg ctggcaaaaca acacgagat caggdcatgg
1501 aagaaggaaaa ggccaaaggc catgtatgc gtcgcgcac ggagggaaga gatgtctaat
1561 gaactcggcc ctcctcgtg ggctcgccctc caactcagga ctgaaacagt tttatgttaa
1621 ggtttgttac agtctctgtt attcttggaaag caaatggcat accagtagt tcccaatgt
1681 ccacctgctg ctgcgggggg gttgggggtgg gggacatgca agaaacacgc agagaaggcc
1741 gctgggtgtga agagaccagg ccagcagcgc agtccagccc atttcagtt gtcacccctt
1801 agtgtccagc acagcatccc tgcgttacca gggcccagct gctgtgggtt ctacatccgc
1861 actagggtca cactgcagaa accgttgata aaacaaaactc agtgcacatc gctttccat
1921 tgggggcatt ggcagggggcg ggtgtatgaga ttgttttgc actgactgac tggcctgct
1981 agaacacaag cccacagcca ggggtccctt ggtccacage tgggtctcag gccccttacc
2041 tgccttccaa gtccttcgc agactcttgc gttgggtttt ctgttccatgc cagcatgtcc
2101 cacagactct tttgttccctt caacgcggcgt cattatgtac agttttctt ctgttccatgc
2161 gtgggtgttga gatgggttag aagttagttt atcttccctt cttttttttt cttttttttt
2221 cgatgttagc gtcagccat ccctggccag cacagtcgtc gtcctcatg ctccccccccc
2281 cacccttgcgt gtgcagaaccc taaggcttgc tccctggccca gactcgatgt gacggacatc
2341 catgtccacc caggatttgc gaaggaaaggc acttcacact cctctatcat gaaaaaaaaatag
2401 ttttcaatttt ttactttttt gcttgggttt taaaaggcaaa actcttaggag gctttttttt
2461 ggcaggtaact cttaaaggcga ggtagatacc tcatcttgcg cccgagccctc tttttttttt
2521 tggggactgt aagggttgcg ggcttccggc gtcctgtcat gggccaaag tctccaaaac
2581 ttggggatttgc aatttagggca aatcttaat agttttgggg tggattttgc tttttttttt
2641 tgatgttgc acgtggctgg ctttccacca gcttaaggaa ccccatggg gacacacccca
2701 ccacccaaatg acccatgcac ctctggccgg acagtcact tccagcccttca aggacactg
2761 tgctcaagat gtgtggctgt agtaaagacag ttcatgtca ggcattaaatg caaggccctt
2821 ctteacaccc tggggacacgc gcttcttgcg ctccagtttct gctctgtctg aagcaggagg
2881 gatgggttgc gaaaggatctt gggatgtgc tggaccctc gcatctcaca ctggccactc
2941 tgccttccatc catccatgtt tgagggccctt ggtcttccatca ggaagtgcag agcaggagg
3001 gactgtgtgt gtaatgtctt agtgggttgc gttctttca tttttttttt tttttttttt
3061 aggccgggctt ggaatttttgc cttaaacatgt taggtttttt tttttttttt tttttttttt
3121 ctgttccca agtgcgtatt gtggcccaag gagactggta cttttttttt tttttttttt
3181 ggttccaaaggc cagaaatggc aggtggccag ccccttggc tttttttttt tttttttttt
3241 agtaatctca gacgatttac acggaaatca gaagatgtct tttttttttt tttttttttt
3301 tagagtgtat gggaaaggcga tgatgttgc ttccatgtt tttttttttt tttttttttt
3361 gctggggatc atgggtacac acacccttgg tccctgtacc tttttttttt tttttttttt
3421 caaaacaaaaaa aatcatttag atgcatttttgc tttttttttt tttttttttt tttttttttt
3481 aaaaaaaaaaaaaaa (SEQ ID NO: 14)

FIG. 12D

IMMUNOPHILIN LIGANDS AND METHODS FOR MODULATING IMMUNOPHILIN AND CALCIUM CHANNEL ACTIVITY

BACKGROUND

[0001] Entry of calcium into mammalian cells through voltage-gated calcium channels mediates a wide variety of cellular and physiological responses, including excitation-contraction coupling, hormone secretion and gene expression (Miller et al. (1987) *Science* 235:46-52; Augustine et al. (1987) *Annu. Rev. Neurosci.* 10:633-93). Calcium channels directly affect membrane potential and contribute to diverse electrical properties in neurons. Calcium entry further influences neuronal function by regulating calcium-dependent ion channels and modulating the activity of calcium-dependent enzymes, such as protein kinase C and calmodulin-dependent protein kinase II. An increase in calcium concentration at the presynaptic nerve terminal typically triggers neurotransmitter release and increases in calcium channel activity. Such calcium increases have been implicated in a number of human disorders, including, but are not limited to, neurological and cardiac disorders (e.g., congenital migraine, cerebellar ataxia, angina, epilepsy, hypertension, ischemia, and some arrhythmias).

[0002] In view of the widespread role of voltage-gated calcium channels in physiological and pathological functions, the need still exists for identifying novel modulators of calcium channel activity and understanding their mechanism of action.

SUMMARY

[0003] Methods and compositions for modulating immunophilin and calcium channel activity are disclosed. In one embodiment, immunophilin ligands modified at the mTOR binding region of rapamycin have been shown to decrease the activity of FKBP52 and voltage-gated L-type calcium channels, in particular, the $\beta 1$ subunits of the L-type calcium channels. Such decreased activity has been shown to be associated with a concomitant increase in neurite outgrowth and neuronal survival. Without being bound by theory, it is believed that the decrease in FKBP52 and channel activity occurs, at least in part, via the formation of a complex that includes an immunophilin ligand, one or both of an immunophilin (e.g., FKBP52) and/or the $\beta 1$ subunit of the L-type calcium channel. Accordingly, the present invention provides methods for modulating, e.g., inhibiting, decreasing and/or reducing, the activity of the immunophilin and/or the β subunit of the L-type calcium channel using immunophilin ligands, e.g., immunophilin ligands modified at the mTOR binding region. In other aspects, methods for treating or preventing conditions associated calcium channel dysfunction, e.g., neurodegenerative and cardiovascular disorders, using immunophilin ligands are also disclosed. Methods and reagents of identifying compounds that modulate an activity of the immunophilin and/or the calcium channel subunit are additionally encompassed by the invention.

[0004] In one aspect, the invention provides a purified complex that includes an immunophilin ligand (e.g., a rapamycin or a meridamycin analogue (e.g., a known or an unknown analogue)), and one or both of (i) an immunophilin or a functional variant thereof, and/or (ii) a calcium channel subunit or a functional variant thereof. Accordingly, exemplary complexes of the invention may include an immunophilin

ligand and an immunophilin or functional fragment thereof; an immunophilin ligand and a calcium channel subunit or a functional variant thereof; and an immunophilin ligand, an immunophilin or a functional variant thereof, and a calcium channel subunit or a functional variant thereof. It shall be understood that the complexes of the invention may include additional polypeptides or fragments thereof.

[0005] In one embodiment, the rapamycin analogue is modified at the mTOR binding region of rapamycin, e.g., has a heteroatom substituent at positions 1 and 4 of the rapamycin backbone (see FIG. 1A). In other embodiments, the rapamycin analogue has a cyclic structure at positions 1, 2, 3 and/or 4 of the rapamycin backbone. In other embodiments, the rapamycin analogue has a chemical formula as described herein (e.g., formulae I, Ia and/or Ib). In other embodiments, the rapamycin analogue has the structure of the compounds referred to herein as "rapamycin I" and "rapamycin II" (FIG. 1A) (also referred to herein as "Compound I" and "Compound II," respectively). In other embodiments, the immunophilin ligand binds to an immunophilin, e.g., FKBP-52, with a selectivity, relative to other immunophilins (e.g., FKBP12), that is at least 100, 200, 300, 400, 500, 600, 700, 800 or higher than that of rapamycin.

[0006] In embodiments, the immunophilin is an FK506 binding protein, e.g., FKBP52 (e.g., a mammalian FKBP52), or a functional variant thereof. In other embodiments, the calcium channel subunit is a subunit of the voltage gated L-type calcium channel, e.g., a $\beta 1$ subunit (e.g., a mammalian $\beta 1$ subunit), or a functional variant thereof. A functional variant of a polypeptide described herein includes a fragment, mutated form, fusion protein, labeled form (e.g., radiolabeled) that retains one or more activities of the unmodified form, e.g., retains the ability to bind to an immunophilin ligand and/or form a complex as described herein. The terms "immunophilin" and "calcium channel," or the like, include "functional variants thereof," although the phrase "functional variants thereof" may or may not be repeated throughout for ease of reading.

[0007] In another aspect, the invention provides a method, or an assay, for identifying a test compound (e.g., a rapamycin or a meridamycin analogue as described herein) that interacts with (e.g., binds to) and/or modulates (e.g., decreases or increases) an activity of (i) an immunophilin, e.g., an immunophilin as described herein (e.g., FKBP52), (or a functional variant thereof), and/or (ii) a calcium channel subunit (e.g., a calcium channel subunit as described herein (e.g., $\beta 1$ subunit)), (or a functional variant thereof). The method, or the assay, includes: contacting the immunophilin, and/or the calcium channel subunit, with a test compound under conditions that allow an interaction and/or modulation of activity to occur; detecting a change in the interaction and/or activity of the immunophilin and/or the calcium channel subunit in the presence of the test compound relative to a reference, e.g., a reference sample (e.g., a control sample not exposed to the test compound, or a control sample exposed to rapamycin). A change (e.g., an increase or a decrease) in the level of interaction and/or activity of the immunophilin and/or the calcium channel subunit, in the presence of the test compound, relative to the reference, indicates that said test compound interacts with and/or affects (e.g., increases or decreases) the activity of the immunophilin and/or a calcium channel subunit.

[0008] In embodiments, the interaction between the test compound and one or both of the immunophilin and/or the

calcium channel subunit is detected by the formation of a complex (e.g., a complex between one or more of the following: the test compound and the immunophilin; the test compound and the calcium channel subunit; or, the test compound, the immunophilin and the calcium channel subunit). A change in the formation and/or stability of the complex in the presence of the test compound, relative to the reference indicates that said test compound interacts with one or both of the immunophilin and/or a calcium channel subunit.

[0009] In yet another aspect, the invention provides a method, or an assay, for identifying a neurotrophic and/or neuroprotective compound. The method, or the assay, includes: contacting (i) an immunophilin (e.g., an immunophilin as described herein (e.g., FKBP52)) (or a functional variant thereof), and/or a (ii) calcium channel subunit (e.g., a calcium channel subunit as described herein (e.g., β 1 sub-unit)) (or a functional variant thereof), with a test compound under conditions that allow the interaction and/or modulation of activity to occur; detecting a change in the interaction and/or activity of the immunophilin and/or the calcium channel subunit in the presence of the test compound relative to a reference, e.g., a reference sample (e.g., a control sample not exposed to the test compound, or a control sample exposed to rapamycin). An increase in the level of interaction, and/or a decrease in the activity of the immunophilin and/or the calcium channel subunit, in the presence of the test compound, relative to the reference, is indicative of a potential neurotrophic and/or neuroprotective compound. In embodiments, the increase in the interaction between the test compound and the immunophilin and/or the calcium channel subunit is detected by an increase in the formation and/or stability of a complex between two or more of the aforesaid components. In other embodiments, the decrease in activity is determined by detecting a decrease in calcium channel activity, e.g., as described in more detail herein. A decrease in immunophilin activity can be detected by, e.g., measuring glucocorticoid receptor activation.

[0010] Additional embodiments of the aforesaid screening methods and assays may include one or more of the following features:

[0011] In embodiments, the immunophilin and/or the calcium channel subunit are present in a sample. The sample can be a cell lysate or a reconstituted system (e.g., cell membrane or soluble components). Alternatively, the sample can include cells in culture, e.g., purified cultured or recombinant cells, or in vivo in an animal subject. A change in the interaction and/or activity between the test compound or neurotrophic compound and the immunophilin and/or the calcium channel subunit can be determined by detecting one or more of: a change in the binding or physical formation of the complex itself, e.g., by biochemical detection, affinity based detection (e.g., Western blot, affinity columns), immunoprecipitation, fluorescence resonance energy transfer (FRET)-based assays, spectrophotometric means (e.g., circular dichroism, absorbance, and other measurements of solution properties); a change, e.g., an increase or a decrease, in signal transduction, e.g., calcium-dependent phosphorylation and/or transcriptional activity (e.g., a transcriptional profile as described herein); a change, e.g., increase or decrease, in calcium channel activity (e.g., electrophysiological activity, calcium kinetics), and/or a change, e.g., increase or decrease, in neuronal survival, differentiation and/or neurite outgrowth. In one embodiment, the test compound or the neurotrophic compound is identified and re-tested in the same or a different

assay. For example, a test compound or a neurotrophic compound is identified in an in vitro or cell-free system, and re-tested in an animal model or a cell-based assay. Any order or combination of assays can be used. For example, a high throughput assay can be used in combination with an animal model or tissue culture.

[0012] In other embodiments, the method, or assay, includes providing a step based on proximity-dependent signal generation, e.g., a two-hybrid assay that includes a first fusion protein (e.g., a fusion protein comprising an immunophilin portion), and a second fusion protein (e.g., a fusion protein comprising a β subunit portion), contacting the two-hybrid assay with a test compound, under conditions wherein said two hybrid assay detects a change in the formation and/or stability of the complex, e.g., the formation of the complex initiates transcription activation of a reporter gene.

[0013] In other embodiments, the method, or assay, further includes the step of contacting the immunophilin and/or the calcium channel subunit with a known immunophilin ligand (e.g., a rapamycin analogue modified at the mTOR binding region of rapamycin as described herein); detecting the interaction and/or activity of the known immunophilin ligand with the immunophilin and/or the calcium channel subunit in the absence or presence of a test compound. A change in binding (e.g., complex formation) and/or activity of the immunophilin and/or the calcium channel subunit, in the presence or absence of the test compound, is indicative that the test compound interacts with and/or binds to the immunophilin and/or the calcium channel subunit.

[0014] In other embodiments, the method, or assay, further includes the step(s) of comparing binding of the test compound to the complex compared to the binding of the known immunophilin ligand to the complex. The method, or assay, can additionally, optionally, include detecting the interaction (e.g., binding) of the test compound to a complex of the immunophilin and/or the calcium channel subunit, relative to the individual components.

[0015] In some embodiments, the method further includes the step of evaluating a change, e.g., increase or decrease, in neuronal activity, e.g., one or more of neuronal survival, differentiation and/or neurite outgrowth. An increase in one or more of neuronal survival, differentiation and/or neurite outgrowth is indicative of a neurotrophic and/or neuroprotective compound. The evaluation step can be performed in cells in culture or in an animal model as described herein.

[0016] Candidate test or neurotrophic compounds increase the formation of the complex described herein and/or inhibit calcium channel or immunophilin activity. In one embodiment, the test compound binds with higher affinity to the complex relative to its binding to the individual components of the complex. The test or neurotrophic compound can be a natural product or a chemically synthesized compound. For example, the test compound can be a polyketide obtained from a naturally-occurring or modified (e.g., recombinantly modified) prokaryotic (e.g., Actinomycete such as *Streptomyces*, e.g. *S. hygroscopicus*) or eukaryotic (e.g., a fungal or mammalian) cell. In embodiments, the test compound is a rapamycin or a meridamycin, or an analogue thereof (e.g., a rapamycin or meridamycin compound described herein, or an analogue thereof).

[0017] Compounds disclosed herein and/or identified by the methods or assays described herein are also within the scope of the invention. Compositions, e.g., pharmaceutical compositions, that include the compounds of the invention

and a pharmaceutically-acceptable carrier are disclosed. In one embodiment, the compositions include the compounds of the invention in combination with one or more agents, e.g., therapeutic agents. In one embodiment, the second agent is a calcium channel antagonist, e.g., an antagonist of an L-type calcium channel. Examples of antagonists of L-type calcium channels include dihydropyridines, phenylalkylamines and benzothiazepines diphenylbutylpiperidine class of antischizophrenic neuroleptic drugs. In certain embodiments, the amount of the immunophilin ligand and/or calcium channel antagonist administered present in the composition is lower than the amount of the drug present in compositions administered individually.

[0018] In another aspect, the invention provides a host cell comprising one or more nucleic acids encoding one or more of the polypeptide constituents of the complex disclosed herein. In one embodiment, the host cell contains a first nucleic acid that includes a nucleotide sequence encoding an immunophilin, e.g., an FKBP52 (e.g., a mammalian FKBP52) (or a functional variant thereof); and/or a second nucleic acid that includes a nucleotide sequence encoding a subunit of the voltage gated L-type calcium channel, e.g., a $\beta 1$ subunit (e.g., a mammalian ($\beta 1$ subunit), (or a functional variant thereof). In some embodiments, recombinant immunophilin and the calcium channel subunit and/or control regulatory sequences thereof are exogenously added.

[0019] In yet another aspect, the invention provides an antibody, or antigen-binding fragment thereof, that binds to the complexes disclosed herein. In certain embodiments, the antibody or fragment thereof increases the formation of a complex disclosed herein.

[0020] In other embodiments, the antibody or fragment thereof decreases or inhibits the formation of a complex disclosed herein. In one embodiment, the antibody or fragment thereof selectively binds to the complex, but does not significantly bind to the individual components of the complex. The complex can include the immunophilin ligand or test compound and the immunophilin and/or the calcium channel, as described herein.

[0021] In another aspect, the invention provides a method of making an antibody or antigen binding fragment thereof. The method includes using the complex described herein as an antigen (e.g., an immunogen in an animal model or phage display selection), and selecting antibodies or binding fragments thereof on the basis of binding to the complex. The method may, optionally, include the step of confirming binding of the antibody or fragment thereof to the complex and comparing binding of the antibody to the individual components of the complex, or a complex that contains the three components of the complex. Antibodies or fragments thereof that selectively bind to the complex over the individual components or a complex thereof are preferred.

[0022] In another aspect, the invention provides a method of modulating (e.g., decreasing) the activity of an immunophilin (or a functional variant thereof), and/or a calcium channel subunit (or a functional variant thereof). The method includes: contacting one or both of (i) an immunophilin, e.g., an FKBP52, as described herein; and/or (ii) a subunit of a calcium channel, e.g., a $\beta 1$ subunit, as described herein, with an immunophilin ligand (e.g., a rapamycin or meridamycin analogue as described herein), under conditions that allow an interaction (e.g., binding) to occur. In embodiments, the activity modulated (e.g., increased) is the formation and/or stability of a complex that includes the immunophilin ligand,

and one or both of the immunophilin, and/or the calcium channel subunit. In one embodiment, the contacting step can be effected in vitro, e.g., in a cell lysate or in a reconstituted system. Alternatively, the subject method can be performed on cells in culture, e.g., in vitro or ex vivo. For example, cells (e.g., purified or recombinant cells) can be cultured in vitro and the contacting step can be effected by adding the immunophilin ligand, e.g., the rapamycin or meridamycin analogue, to the culture medium. Typically, the cell is a mammalian cell, e.g., a human cell. In some embodiments, the cell is a neuronal or a cardiovascular cell.

[0023] In another aspect, the invention provides a method of modulating, e.g., inhibiting, calcium channel activity (e.g., voltage-gated calcium channel activity) and/or immunophilin activity, in a cell. The method includes: contacting a cell that expresses (i) an immunophilin, e.g., an FKBP52 (e.g., a mammalian FKBP52) (or a functional variant thereof); and/or (ii) a subunit of the voltage gated L-type calcium channel, e.g., a $\beta 1$ subunit (e.g., a mammalian ($\beta 1$ subunit), (or a functional variant thereof), with an immunophilin ligand, e.g., a rapamycin or meridamycin analogue as described herein, under conditions that allow an interaction between (e.g., formation of a complex that includes) the ligand, and one or both of the immunophilin and/or the subunit to occur, thereby inhibiting the calcium channel and/or immunophilin activity. Typically, the cell is a mammalian cell, e.g., a human cell. In some embodiments, the cell is a neuronal or a cardiovascular cell. The method can be performed in cells in cultured medium as described herein.

[0024] In yet another aspect, the invention provides a method of increasing neuronal function, e.g., neurite outgrowth and/or survival. The method includes: contacting a neuronal cell with an immunophilin ligand in an amount sufficient to promote neuronal function. In embodiments, the immunophilin ligand is present at a concentration that elicits one or more of the following: (i) downregulates expression and/or activity at least one component of the calcium signaling pathways (e.g., calcium- influx channels, N-methyl D-aspartate subtype of glutamate (NMDA) receptors, plasminogen activator (PLAU), SHT3R channels); (ii) decreases immunophilin (e.g., FKBP52) activity and/or expression; (iii) reduces or inhibits the activity and/or expression of a calcium channel (e.g., an L-type calcium channel); (iv) activates steroid receptor signaling (e.g., glucocorticoid receptor signaling); (v) induces formation of a complex that includes the immunophilin ligand, the immunophilin (e.g., FKBP52) and/or a subunit of the voltage gated L-type calcium channel, e.g., a $\beta 1$ subunit; and/or (vi) protects neurons from calcium-induced cell death.

[0025] In yet another aspect, the invention features a method of treating or preventing, in a subject, a disorder associated with calcium channel dysfunction(e.g., a disorder associated with L-type calcium channel function). In embodiments, the disorder is not associated with a ryanodine receptor channelopathy. The method includes administering to a subject an immunophilin ligand in an amount sufficient to treat or prevent the disorder. In embodiments, the immunophilin ligand is present at a concentration that elicits one or more of the following: (i) downregulates expression or activity at least one component of the calcium signaling pathways (e.g., calcium- influx channels, NMDA receptors, plasminogen activator (PLAU), SHT3R channels); (ii) decreases immunophilin (e.g., FKBP52) activity and/or expression; (iii) reduces or inhibits the activity and/or expression of a calcium

channel (e.g., an L-type calcium channel); (iv) activates steroid receptor signaling (e.g., glucocorticoid receptor signaling); (v) induces formation of a complex that includes the immunophilin ligand, the immunophilin (e.g., FKBP52) and/or a subunit of the voltage gated L-type calcium channel, e.g., a $\beta 1$ subunit; and/or (vi) protects neurons from calcium-induced cell death.

[0026] Additional embodiments of the aforesaid methods of modulating activity and treating or preventing disorders may include one or more of the following features.

[0027] In one embodiment, the immunophilin ligand is a rapamycin analogue which is modified at the mTOR binding region, e.g., has a heteroatom substituent at positions 1 and 4 of the rapamycin backbone (see FIG. 1A). In other embodiments, the rapamycin analogue has a cyclic structure at positions 1, 2, 3 and/or 4 of the rapamycin backbone. In other embodiments, the rapamycin analogue has a chemical formula as described herein (e.g., formulae I, Ia and/or Ib). In other embodiments, the rapamycin analogue has the structure of the compounds referred to herein as "rapamycin I" and "rapamycin II" (FIG. 1A). In other embodiments, the immunophilin ligands binds to an immunophilin, e.g., FKBP-52, with a selectivity, relative to another immunophilin (e.g., FKBP-12), that is at least 100, 200, 300, 400, 500, 600, 700, 800 or higher than that of rapamycin.

[0028] In other embodiments, the method can be performed on cells (e.g., neuronal cells) present in a subject, e.g., as part of an *in vivo* (e.g., therapeutic or prophylactic) protocol, or in an animal subject (e.g., an *in vivo* animal model). For *in vivo* methods, the immunophilin ligand, e.g., the rapamycin or meridamycin analogue, alone or in combination with another agent, can be administered to a subject, e.g., a mammal, suffering from a disorder, e.g., a neurodegenerative or a cardiovascular disorder, in an amount sufficient to form and/or stabilize the complex.

[0029] In some embodiments, a therapeutic amount or dosage can be determined, e.g., prior to administration to the subject, by testing *in vitro* the amount of immunophilin ligand required to elicit one or more of the following: (i) induce complex formation; (ii) downregulate expression or activity at least one component of the calcium signaling pathways; (iii) reduce or inhibit the activity of a calcium channel (e.g., an L-type calcium channel); and/or (iv) activate steroid receptor signaling (e.g., glucocorticoid receptor signaling). The *in vivo* method can, optionally, include the step(s) of identifying (e.g., evaluating, diagnosing, screening, and/or selecting) a subject at risk of having, or having, one or more symptoms associated with a disorder associated with calcium channel dysfunction (e.g., a disorder associated with L-type calcium channel function). In embodiments, the disorder is not associated with a ryanodine receptor channelopathy.

[0030] The subject can be a mammal, e.g., a human, suffering from, for example, a neurodegenerative or a cardiovascular disorder. In embodiments, the subject is a mammal having one or more symptoms associated with a disorder associated with calcium channel dysfunction (e.g., a disorder associated with L-type calcium channel function). In embodiments, the disorder is not associated with a ryanodine receptor channelopathy. For example, the subject is a mammal (e.g., a human patient) suffering from a disorder chosen from one or more of: stroke, Parkinson's disease, epilepsy, angina, cardiac arrhythmia and ischemia. In other embodiments, the subject is a mammal suffering from one or more of: migraine, neuropathic pain, acute pain, mood disorders, schizophrenia,

depression, anxiety, cerebellar ataxia, tardive dyskinesia, hypertension and/or urinary incontinence.

[0031] The immunophilin ligand, e.g., the rapamycin or meridamycin analogue, can be administered to the subject alone, or in combination with one or more agents, e.g., therapeutic agents. In one embodiment, the second agent is a calcium channel antagonist, e.g., an antagonist of an L-type calcium channel. Examples of antagonists of L-type calcium channels include dihydropyridines, phenylalkylamines and benzothiazepines diphenylbutylpiperidine class of antischizophrenic neuroleptic drugs. In certain embodiments, the amount of the immunophilin ligand and/or calcium channel antagonist administered in combination is lower than the amount of the drug administered individually. The agents can be administered simultaneously or sequentially.

[0032] In yet another aspect, the invention provides a method of stimulating one or more of neurite outgrowth, survival, and/or differentiation of a neuronal cell (e.g., a dopaminergic, cholinergic, cortical, and spinal cord neuronal cell). The method includes contacting the cell with an antagonist of an immunophilin (e.g., FKBP52) and/or a calcium channel β subunit, e.g., a $\beta 1$ subunit of the voltage gated L-type calcium channel. The antagonist can also be an inhibitor of activity and/or expression of the immunophilin (e.g., FKBP52) or calcium channel β subunit. In one embodiment, the inhibitor is an intracellular antagonist of a calcium channel, e.g., an antagonist of a calcium channel β subunit. In another embodiment, the antagonist is an immunophilin ligand, e.g., a rapamycin or meridamycin analogue as described herein. Typically, the immunophilin ligand is administered in an amount sufficient to form and/or stabilize a complex that includes the ligand, an immunophilin (or a functional variant thereof), and/or a calcium channel subunit (or a functional variant thereof). In other embodiment, the antagonist is an inhibitor of transcription of the immunophilin (e.g., FKBP52) and/or calcium channel β subunit, e.g., RNAi. The contacting step can be effected *in vitro*, e.g., in culture, or *in vivo*, e.g., by administration to a subject, as described herein.

[0033] As used herein, the articles "a" and "an" refer to one or to more than one (e.g., to at least one) of the grammatical object of the article.

[0034] The term "or" is used herein to mean, and is used interchangeably with, the term "and/or", unless context clearly indicates otherwise.

[0035] The terms "proteins" and "polypeptides" are used interchangeably herein.

[0036] Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF DRAWINGS

[0037] FIG. 1A provides a diagram of chemical synthesis and structures of rapamycin analogues I and II (referred to interchangeably in the Figure (and throughout) as "1" and "2," or "Compound 1" and "Compound 2," respectively). The rapamycin structure using the numbering system referenced herein is also provided.

[0038] FIG. 1B provides a bar graph depicting promotion of neuronal survival in cortical neurons in response to rapamycin analogue I (referred to in the Figure as "Compound 1").

[0039] FIG. 1C provides a graph depicting neurite outgrowth in cortical neurons in response to rapamycin analogue I (referred to in the Figure as "Compound 1").

[0040] FIG. 1D provides a graph depicting neurite outgrowth in F-11 cells in response to rapamycin analogue I (referred to in the Figure as "Compound 1").

[0041] FIG. 2 provides a diagram showing preparation of affinity matrices of several rapamycin analogues I, II, FK506 and rapamycin.

[0042] FIG. 3 provides an SDS-PAGE gel photograph of the mobility of the proteins isolated by affinity precipitation from lysates of F11 cells (fusion between mouse embryonic neuroblastoma and rat dorsal root ganglion (DRG) neurons).

[0043] FIG. 4 provides Fourier transform ion cyclotron resonance mass spectrometric (FT-ICR-MS) analysis of tryptic digested bands from the SDS-PAGE gel. "Rap. An. I" represents rapamycin analogue I.

[0044] FIGS. 5A-5D depict the characterization of immunophilin binding of rapamycin analogues I and II.

[0045] FIG. 5A provides an SDS-PAGE gel analysis of proteins that bound to the various affinity matrixes. The bands found in the marker lane are (1) 220 kDa, (2) 78 kDa, (3) 45.7 kDa, in the rapamycin analogue I pull-down fraction are (4) Myosin, (5) FKBP52, (6) CACNB 1, FKBP25 and FKBP 12, in the blank bead control is (7) actin, and in the rapamycin analogue II pull-down fraction are (5) FKBP52, and (6) CACNB 1. "Compound 1" represents rapamycin analogue I and "Compound II" represents rapamycin analogue II.

[0046] FIG. 5B provides a Western blot analysis using anti-FKBP52 and anti-Ca²⁺ channel β₁-subunit antibodies to detect the presence of the corresponding antigens on affinity beads coating with rapamycin analogue I and FK506.

[0047] FIG. 5C are bar graphs depicting the results of size exclusion chromatography to measure the fraction of [¹⁴C]-1 that binds to the purified recombinant immunophilins and cyclophilins.

[0048] FIG. 5D is a blot depicting the results of affinity chromatography to test the binding of FKBP25 and PPID proteins to Compound 2. Lanes were labeled as follows: "C" represent a protein standard; "+" represents a protein incubated with Compound 2-containing beads; "-" represents a protein incubated with blank beads.

[0049] FIGS. 6A-6D depict the characterization of the binding of Compounds I and II to the L-type calcium channel beta subunits.

[0050] FIG. 6A depicts Western analysis of fractions for the presence of CACNB 1 using the corresponding antibody.

[0051] FIG. 6B are bar graphs depicting the results from size exclusion chromatography to measure the fraction of [¹⁴C]-1 that binds to the purified recombinant CACBN1 and CACBN4.

[0052] FIG. 6C depicts the results of fluorescent analysis to measure the fluorescent quenching induced upon binding of Compound 2 (1 μM) to CACNB1 (0-8 μM).

[0053] FIG. 6D depicts the results of affinity chromatography to test the binding of CACNB1 to Compound 2. Lanes were labeled as follows: "C" represent a protein standard; "+" represents a protein incubated with Compound 2-containing beads; "-" represents a protein incubated with blank beads.

[0054] FIG. 7 provides an immunoblot of the co-immunoprecipitate of the lysate of F11 cells exposed to various concentrations of the rapamycin analogue I (0 μM, 5 μM or 50 μM) precipitated using an anti-FKBP52 antibody. The immunoprecipitated fractions were immunoblotted with an anti-

Ca²⁺ channel β₁-subunit antibody. The lower panels provide diagrams summarizing the protein interactions. "RA I" represents rapamycin analogue I.

[0055] FIG. 8 provides a bar graph depicting the effect of various concentrations of rapamycin analogue I (50 μM, 5 μM, or 0 μM) on neurite outgrowth of F11 cells using neurofilament ELISA.

[0056] FIGS. 9A-9F depict the biological effect of Compounds 1 and 2 on calcium currents.

[0057] FIG. 9A is a bar graph of the mean Ca²⁺ current density from whole-cell recording in F-11 cells treated with 5 μM of Compound 1, FK-506 or vehicle in the bath for 2 hrs. Recordings were performed from 7 cells in each condition.

[0058] FIG. 9B depicts representative Ca²⁺ currents with internally applied Compound 1 (10 μM in pipette) at time 0 sec (bottom trace), 800 sec (middle trace) and in the presence of the L-type Ca²⁺ channel blocker Bay-K 5552 (top trace) externally.

[0059] FIG. 9C depicts a graph of the time course of the experiment illustrated in FIG. 9B. Whole cell, and subsequent diffusion of Compound 1 into the cell, begins at time 0. Once current stabilizes after 400 sec, 10 μM BayK-5552 is applied in the bath. (n=3)

[0060] FIG. 9D depicts similar conditions as in FIG. 9C, except that after 300 sec 100 nM ωCTX MVIIA is applied via the bath. (n=2)

[0061] FIG. 9E depicts the Ca²⁺ current trace from hippocampal neuron immediately upon break-in to whole-cell (control) and after 10 minutes of recording with 10 μM Compound 2 internally and ωCTX GVIA externally.

[0062] FIG. 9F depicts the mean responses (+/-SEM) normalized to the initial current from hippocampal neurons. Compound 2 (10 μM) applied internally via the recording pipette, beginning at time 0, where indicated (● and ▼). External solution contains 1 μM TTX+100 nM ωCTX GVIA+10 μM Bay-K 5552 (▼, n=4) or 1 μM TTX+100 nM ωCTX GVIA (●, n=5). Control without compound (■) contained 100 nM ωCTX GVIA externally (n=3).

[0063] FIG. 10A provides a graph demonstrating the effect of siRNA-driven reduction of FKBP52 and CACNB1 on neurite outgrowth.

[0064] FIG. 10B provides a graph demonstrating the effect of siRNA-driven reduction of FKBP52 and CACNB1 on neuronal survival.

[0065] FIG. 10C shows Western blots confirming that siRNA treatment reduced lamin A/C, CACNB1 or FKBP52 protein expression in cortical neurons after 24 hours.

[0066] FIGS. 11A-11B provide the amino acid sequence and nucleotide sequence of human Ca²⁺ channel β₁ subunit isoform 1 (SEQ ID NOS: 1-2, respectively).

[0067] FIGS. 11C-11D provide the amino acid and nucleotide sequence of human Ca²⁺ channel β₁ subunit isoform 2 (SEQ ID NOS: 3-4).

[0068] FIGS. 11E-11F provide the amino acid and nucleotide sequence of human Ca²⁺ channel β₁ subunit isoform 3 (SEQ ID NOS: 5-6).

[0069] FIGS. 11G-11H provide the amino acid and nucleotide sequence of a mouse (*Mus musculus*) Ca²⁺ channel β₁ subunit isoform A (SEQ ID NOS: 7-8).

[0070] FIG. 11I-11J provide the amino acid sequence of a mouse (*Mus musculus*) Ca²⁺ channel β₁ subunit isoform B (SEQ ID NOS: 9-10).

[0071] FIGS. 12A-12B provide the amino acid and nucleotide sequence of human FKBP52 (SEQ ID NOS: 11-12).

[0072] FIGS. 12C-12D provide the amino acid sequence of mouse (*Mus musculus*) FKBP52 (SEQ ID NOs:13-14).

DETAILED DESCRIPTION

[0073] The present invention is based, at least in part, on the discovery that immunophilin ligands, e.g., a rapamycin analogues modified at the mTOR binding region, interact with, e.g., bind to, the immunophilin FKBP52 and/or the voltage gated L-type calcium channel β 1 subunit. Inhibition of FKBP52 and/or CACNB1 by these compounds stimulates neurite outgrowth and/or neuronal survival. Thus, interaction (and complex formation) between these components is believed to inhibit the activity of the β 1 subunit and stimulate neurite outgrowth, implicating voltage gated L-type calcium channels in some of the neurotrophic and/or neuroprotective activities exhibited by immunophilin ligands, such as the rapamycin or meridamycin analogues described herein.

[0074] Applicants have additionally shown in the appended Examples that at least one of the immunophilin ligands disclosed herein (rapamycin analogue II) showed a significant increase in binding selectivity for FKBP52, relative to FKBP12 binding, of at least 600 fold higher compared to rapamycin. Without being bound by theory, it is believed that inhibition of FKBP52 activity mediates neurite outgrowth, presumably by activating steroid, e.g., glucocorticoid receptors. Furthermore, treatment of cortical neurons with the immunophilin ligands disclosed herein caused an overall downregulation of calcium signaling pathways and partial inhibition of L-type calcium channels. A significant effect on neurite outgrowth of neuronal cells was also detected by selectively reducing the expression of the β 1 subunit and FKBP52 in culture.

[0075] The data disclosed herein demonstrate that modification of rapamycin at the mTOR binding region can provide significantly non-immunosuppressive compounds with unusual selectivity for FKBP52 and potent neurotrophic activities. FKBP52 appears to mediate immunophilin ligand-mediated neurite outgrowth, presumably by the activation of steroid receptors (including glucocorticoid receptors), as demonstrated by neurite outgrowth observed in FKBP52 siRNA treated cortical neurons. Further, the ability of these rapamycin analogues to partially inhibit L-type Ca^{2+} channels and reduce transcription of various Ca^{2+} signaling proteins indicates that these analogues can protect neurons from Ca^{2+} induced neuronal cell death, which is consistent with their effect on neuronal survival.

[0076] Calcium channels are present in various tissues, including neuronal and cardiovascular tissues, and have important roles in a number of vital processes in animals, including neurotransmitter release, muscle contraction, pacemaker activity, and secretion of hormones and other substances. Entry of calcium into neuronal cells through voltage-gated calcium channels mediates a wide variety of cellular and physiological responses, including, but not limited to, modulating the activity of calcium-dependent enzymes such as protein kinase C and calmodulin-dependent protein kinase II; controlling membrane potential and contributing to electrical properties such as excitability and repetitive firing patterns; and increasing neurotransmitter release. These processes, are involved in human disorders, such as neurological and cardiovascular disorders. Therefore, methods of inhibiting the function of voltage-dependent calcium channels by forming immunophilin-calcium channel complexes are use-

ful for treating, preventing and/or alleviating symptoms of calcium channel disorders, as described in more detail herein.

[0077] In order that the present invention may be more readily understood, certain terms are described in more detail herein and throughout the detailed description.

[0078] Calcium channels are membrane-spanning, multi-subunit proteins that allow controlled entry of Ca^{2+} ions into cells from the extracellular fluid. The most common type of calcium channel is voltage dependent. "Excitable" cells in animals, such as neurons of the central nervous system (CNS), peripheral nerve cells, and muscle cells (including those of skeletal muscles, cardiac muscles, and venous and arterial smooth muscles) have voltage-dependent calcium channels. Voltage-gated calcium channels allow for influx of Ca^{2+} ions into a cell, and typically require a depolarization to a certain level of the potential difference between the inside of the cell bearing the channel and the extracellular environment bathing the cell. Voltage-gated calcium channels have been classified by their electrophysiological and pharmacological properties into L-, N-, P/Q-, R- and T-types (reviewed in Catterall, 2000; Huguenard 1996; Dolphin, A. C. (2003) *Pharmacological Reviews* 55:607-627). The L-, N- and P/Q-type channels activate at positive potentials (high voltage-gated). T-type (or low voltage-gated) channels describe a broad class of molecules that transiently activate at negative potentials and are highly sensitive to changes in resting potential.

[0079] High voltage-gated calcium channels are composed of four distinct polypeptides: α_1 , $\alpha_2\delta$, β and γ (reviewed by Stea et al., 1994; Catterall, 2000). The β subunit (also referred to herein as "CACB1") is a soluble intracellular protein encoded by at least four known separate genes, each of which is processed into multiple splice variants. In embodiments, the β subunit has one or more of the following features: (i) an amino acid sequence of a naturally occurring mammalian (e.g., human or rodent) subunit or a fragment thereof, e.g., the amino acid sequence as shown in FIGS. 11A-11J (SEQ ID NOs:1-10) or a fragment thereof; (ii) an amino acid sequence substantially homologous to the amino acid sequence shown in FIGS. 11A-11J (SEQ ID NOs:1-10) or a fragment thereof; (iii) an amino acid sequence that is encoded by a naturally occurring mammalian (e.g., human or rodent) β 1 subunit nucleotide sequence or a fragment thereof, e.g., an amino acid sequence encoded by the nucleotide sequence as shown in FIGS. 11A-11J (SEQ ID NOs:1-10) or a fragment thereof; (iv) an amino acid sequence encoded by a nucleotide sequence which is substantially homologous to the nucleotide sequence shown in FIGS. 11A-11J (SEQ ID NOs:1-10) or a fragment thereof; (v) an amino acid sequence encoded by a nucleotide sequence degenerate to a naturally occurring β 1 subunit nucleotide sequence or a fragment thereof, e.g., the nucleotide sequence shown in FIGS. 11A-11J (SEQ ID NOs:1-10) or a fragment thereof; or (vi) a nucleotide sequence that hybridizes to one of the foregoing nucleotide sequences under stringent conditions, e.g., highly stringent conditions. In some embodiments, the β subunit or functional variant (e.g., fragment) thereof exhibits one or more activities of the naturally-occurring sequence, including but not limited to, (i) forms a complex as described herein; (ii) interacts with, e.g., binds to, the α -subunit; (iii) facilitates the localization or trafficking of the voltage-gated calcium channel, e.g., the α_1 subunit, to the cellular plasma membrane; (iv) modulates gating of the channel (e.g., alters activation and inactivation kinetics, causes a leftward shift in the I-V curve and, at a

single channel level, induces an increase in the channel opening probability); or (v) controls transcriptional activity of one or more of the genes described herein (e.g., calcium- influx channels, NMDA receptors, plasminogen activator (PLAU), SHT3R channels).

[0080] In other embodiments, the β subunit has a sequence substantially identical to that disclosed in Powers et al. (1992) *J. Biol. Chem.* 267(32):22967-22972; Collin et al. (1993) *Circ. Res.* 72(6):1337-1344; Hogan, K. et al. (1999) *Neurosci. Lett.* 277 (2), 111-114; Foell et al. (2004) *Physiol. Genomics* 17 (2), 183-200 (human 131 and 32 subunits); Toba et al. (2005) *Eur. J. Neurosci.* 22 (1), 79-92 (murine beta 1 subunit isoform); Serikov et al. (2002) *Biochem. Biophys. Res. Commun.* 293 (5), 1405-1411; Pragnell et al. (1991) *FEBS Lett.* 291 (2), 253-258; Cahill et al. (2000) *J. Neurosci.* 20 (5), 1685-1693 (2000) (bovine beta 1, 2 and 3 subunits); Rosenfeld et al. (1993) *Ann. Neurol.* 33 (1), 113-120; Taviaux et al. (1997) *Hum. Genet.* 100 (2), 151-154 (human genes for beta 2 and beta 4 subunits); Colecraft et al. (2002) *J. Physiol. (Lond.)* 541 (Pt 2), 435-452 (human beta 2a, 2c, 2d and 2e subunits); Opatowsky et al. (2003) *J. Biol. Chem.* 278 (52), 52323-52332 (rat beta 2 subunit); Yamada et al. (2001), *J. Biol. Chem.* 276 (50), 47163-47170 (2001) (rat beta 2 subunit); Strausberg et al. (2002) *PNAS U.S.A.* 99 (26), 16899-16903 (human beta 3 subunit, murine beta 4 subunit); Murakami et al. (1996) *Eur. J. Biochem.* 236 (1), 138-143 (1996) (murine calcium channel beta 3 subunit); Yamada et al. (1995) *Genomics* 27 (2), 312-319 (human calcium channel alpha 1 subunit (CACNL1A2) and beta subunit (CACNLB3) genes); Chen et al. (2004) *Nature* 429 (6992), 675-680 (human beta 4 subunit); Helton et al. (2002) *J. Neurosci.* 22 (5), 1573-1582 (2002) (beta 4 subunit); Badou et al. (2005) *Science* 307 (5706), 117-121 (2005) (calcium channel beta4 subunit); the contents of all of which are hereby incorporated by reference. Other β subunit sequences are disclosed in Genbank Accession Numbers: NP_666235, Q9Y698, Q02641, Q9MZL3 and P54288_2.

[0081] Immunophilins are soluble cytosolic proteins that form complexes with immunophilin ligands, which in turn serve as ligands for other cellular targets involved in signal transduction. Classes of immunophilins include cyclophilins and FK506-binding proteins (e.g., FKBP), such as FKBP-12 and FBBP-52. Cyclosporin A is a macrolide immunophilin ligand that binds to cyclophilins. Other macrolide immunophilin ligands, such as meridamycin, FK506, FK520, and rapamycin, are understood to bind to FKBP. Binding of FK506, FK520 and rapamycin to FKBP typically occurs through structurally similar segments of the polyketide molecules, referred to as "FKBP-binding domain."

[0082] Gene sequences corresponding to more than two-dozen FKBP have been found in the human genome (Dornan et al., *Curr. Top. Med. Chem.* 3, 1392-1409 (2003)). They are expressed 10-50 fold higher in central nervous system (CNS) and peripheral nervous system (PNS) tissue than in immune tissue (Lyons et al., *J. Neurosci.* 15, 2985-2994 (1995)), and their expression is increased following the onset of neurological disease (Kihira et al., *Neuropathology* 22, 269-274 (2002)). Interestingly, FKBP12, FKBP12.6 and FKBP52 were reported as channel-gating-FKBP proteins, modulating ryanodine receptor (RYR) (Huang et al., *Proc. Natl. Acad. Sci. USA.* 103, 3456-3461 (2006)), inositol 1,4,5-trisphosphate receptor (IP₃R) (Cameron et al., *Proc. Natl. Acad. Sci. USA.* 92, 1784-1788 (1995)) and transient receptor potential channels (TRPC) (Sinkins et al., *J. Biol. Chem.* 279, 34521-

34529 (2004)). FKBP52 and FKBP51 associate with three types of steroid receptor complexes that mediate the down-stream responses to estrogen, androgen and glucocorticoid hormones (Steiner et al., *Proc. Natl. Acad. Sci. USA.* 94, 2019-2024 (1997)). The nuclear FKBP25 regulates gene expression through associating with histone deacetylase, casein kinase II, nucleolin and transcription factor YY1 (Yao and Yang, *Curr. Cancer Drug Targets* 5, 595-610 (2005)). FKBP38 is constitutively inactive and located at the mitochondria and endoplasmic reticulum. Interestingly, high levels of Ca²⁺ and calmodulin (CaM) are required for FKBP38 to bind Bcl-2 (Edlich et al., *EMBO J.* 24, 2688-2699 (2005)). Immunophilin ligands cause various down-stream biological activities by disruption of the natural FKBP-containing complexes (Gold *Drug Metab. Rev.* 31, 649-663 (1999); Edlich et al., *J. Biol. Chem.* 281, 14961-14970 (2006)) and by formation of novel complexes, such as FKBP12-FK506-calmodulin or FKBP12-rapamycin-mammalian target of rapamycin (mTOR) (Kissinger et al., *Nature* 378, 641-644 (1995); Choi et al., *Science* 273, 239-42 (1996)).

[0083] FKBP52 is a member of the FK506-binding class of immunophilins. Binding of FK506 to the glucocorticoid receptor (GR)-associated FKBP52 caused increased nuclear translocation of GR in response to dexamethasone and potentiation of GR-mediated gene expression (Sanchez and Ning (1996) *Methods: A Companion to Meth. Enzymol.* 9:188-200). Immunophilins such as FKBP52 and CyP40 and non-immunophilin proteins such as PP5, p60, and Mas70p, have one or more tetratricopeptide repeat (TPR) domains (Ratajczak et al. (1993) *J. Biol. Chem.* 268:13187-13192) that bind to the TPR-binding domain of hsp90. The number of TPR domains in a protein appears to correlate with its hsp90-binding affinity. Regions bordering the TPR domain also participate in binding, e.g., residues 232-271 of FKBP52 (Ratajczak and Carrello (1996) *supra*).

[0084] In some embodiments, the immunophilin has one or more of the following features: (i) an amino acid sequence of a naturally occurring mammalian (e.g., human or rodent) FKBP52 or a fragment thereof, e.g., the amino acid sequence as shown in FIGS. 12A-12D (SEQ ID NOs:11-14) or a fragment thereof; (ii) an amino acid sequence substantially homologous to the amino acid sequence shown in FIGS. 12A-12D (SEQ ID NOs:11-14) or a fragment thereof; (iii) an amino acid sequence that is encoded by a naturally occurring mammalian (e.g., human or rodent) FKBP52 nucleotide sequence or a fragment thereof, e.g., an amino acid sequence that is encoded by the nucleotide sequence as shown in FIGS. 12A-12D (SEQ ID NOs:11-14) or a fragment thereof; (iv) an amino acid sequence encoded by a nucleotide sequence which is substantially homologous to the nucleotide sequence shown in FIGS. 12A-12D (SEQ ID NOs:11-14) or a fragment thereof; (v) an amino acid sequence encoded by a nucleotide sequence degenerate to a naturally occurring FKBP52 nucleotide sequence or a fragment thereof, e.g., the nucleotide sequence shown in FIGS. 12A-12D (SEQ ID NOs:11-14) or a fragment thereof; or (vi) a nucleotide sequence that hybridizes to one of the foregoing nucleotide sequences under stringent conditions, e.g., highly stringent conditions. In some embodiments, the FKBP52 or functional variant (e.g., fragment) thereof exhibits one or more activities of the naturally-occurring sequence, including but not limited to, forms a complex as described herein; binds to FK506; increases nuclear translocation of a glucocorticoid receptor in response

to dexamethasone; potentiates glucocorticoid receptor -mediated gene expression; and/or binds to a heat shock protein, e.g., hsp90.

[0085] Exemplary amino acid and nucleotide sequences for FKBP52 are disclosed in Sanchez et al. (1990) *Biochemistry* 29 (21), 5145-5152; and Peattie et al. (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89 (22), 10974-10978, the contents of both of which are hereby incorporated by reference.

[0086] In one embodiment, β subunit or immunophilin polypeptides of this invention include, but are not limited to, fragments of native polypeptides from any animal species (including humans, rodents), and variants (e.g., functional variants) thereof (human and non-human) polypeptides and their fragments, provided that they have a biological activity in common with a respective native polypeptide. "Fragments" comprise, in one embodiment, regions within the sequence of a mature native polypeptide. Any form of the β subunit or immunophilin, e.g., FKBP52, of less than full length can be used in the methods and compositions of the present invention, provided that it is still functional, e.g., retains at least one activity of the naturally-occurring sequence (e.g., retains the ability to form a complex as described herein). β subunits of less than full length can be produced by expressing a corresponding fragment of the polynucleotide encoding the full-length β subunit protein in a host cell. These corresponding polynucleotide fragments are also part of the present invention. Modified polynucleotides as described above may be made by standard molecular biology techniques, including construction of appropriate desired deletion mutants, site-directed mutagenesis methods or by the polymerase chain reaction using appropriate oligonucleotide primers.

[0087] A "variant" of a polypeptide, or fragment thereof, such as, for example, a variant of a β 1 subunit or FKBP52 includes chimeric proteins, labeled proteins (e.g., radiolabeled proteins), fusion proteins, mutant proteins, proteins having similar (e.g., substantially similar) sequences (e.g., proteins having amino acid substitutions (e.g., conserved amino acid substitutions), deletions, insertions), protein fragments, mimetics, so long as the variant has at least a portion of an amino acid sequence of a native protein, or at least a portion of an amino acid sequence of substantial sequence identity to the native protein. A "functional variant" includes a variant that retains at least one function of the native protein, e.g., retains the ability to interact an immunophilin ligand with and/or form a complex as described herein.

[0088] A "chimeric protein" or "fusion protein" is a fusion of a first amino acid sequence encoding a polypeptide with a second amino acid sequence, wherein the first and second amino acid sequences do not occur naturally as part of a single polypeptide chain.

[0089] As used herein, the term "substantially similar" (or "substantially" or "sufficiently" "homologous" or "identical") is used herein to refer to a first amino acid or nucleotide sequence that contains a sufficient number of identical or equivalent (e.g., with a similar side chain, e.g., conserved amino acid substitutions) amino acid residues or nucleotides to a second amino acid or nucleotide sequence such that the first and second amino acid or nucleotide sequences have similar activities. Sequences similar or homologous (e.g., at least about 85% sequence identity) to the sequences disclosed herein are also part of this application. In some embodiments, the sequence identity can be about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or higher. Alternatively,

substantial identity exists when the nucleic acid segments hybridizes under selective hybridization conditions (e.g., highly stringent hybridization conditions), to the complement of the strand. The nucleic acids may be present in whole cells, in a cell lysate, or in a partially purified or substantially pure form.

[0090] Calculations of "homology" or "sequence identity" between two sequences (the terms are used interchangeably herein) are performed as follows. The sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). Typically, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, 90%, 100% of the length of the reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

[0091] The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In one embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch ((1970) *J. Mol. Biol.* 48:444-453) algorithm which has been incorporated into the commercially available GAP program in the GCG software package, using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another embodiment, the percent identity between two nucleotide sequences is determined using the commercially available GAP program in the GCG software package, using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 30 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. Parameters typically used to determine percent homology are a Blossum 62 scoring matrix with a gap penalty of 12, a gap extend penalty of 4, and a frameshift gap penalty of 5. The percent identity between two amino acid or nucleotide sequences can also be determined using the s algorithm of E. Meyers and W. Miller ((1989) *CABIOS* 4:11-17) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

[0092] As used herein, the term "hybridizes under stringent conditions" describes conditions for hybridization and washing. Stringent conditions are known to those skilled in the art and can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. Aqueous and non-aqueous methods are described in that reference and either can be used. An example of stringent hybridization conditions are hybridization in 6 \times sodium chloride/sodium citrate (SSC) at about 45°C., followed by one or more washes in 0.2 \times SSC, 0.1% SDS at 50° C. Another example of stringent hybridization conditions are hybridization in

6×SSC at about 45° C., followed by one or more washes in 0.2×SSC, 0.1% SDS at 55° C. A further example of stringent hybridization conditions are hybridization in 6×SSC at about 45° C., followed by one or more washes in 0.2×SSC, 0.1% SDS at 60° C. Typically, stringent hybridization conditions are hybridization in 6×SSC at about 45° C., followed by one or 20 more washes in 0.2×SSC, 0.1% SDS at 65° C. More typically, the highly stringent conditions used are 0.5M sodium phosphate, 7% SDS at 65° C., followed by one or more washes at 0.2×SSC, 1% SDS at 65° C.

[10093] It is understood that the variants of the polypeptide disclosed herein may have additional conservative or non-essential amino acid substitutions, which do not have a substantial effect on antigen binding or other immunoglobulin functions. A “conservative amino acid substitution” is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, praline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., s tyrosine, phenylalanine, tryptophan, histidine).

[10094] A “non-essential” amino acid residue is a residue that can be altered from the wild-type sequence of a hybrid antibody, without abolishing or more preferably, without substantially altering a biological activity, whereas an “essential” amino acid residue results in such a change.

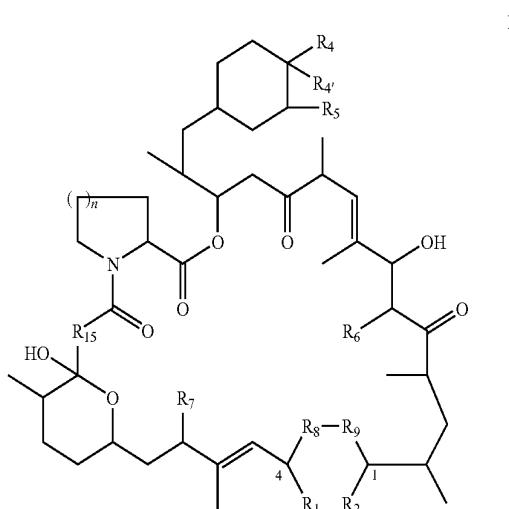
Immunophilin Ligands

[10095] Immunophilin ligands bind to immunophilins to activate other cellular targets, primarily in the immune and nervous system. Several immunophilins are immunosuppressive, e.g., cyclosporin A, FK506 and rapamycin, whereas other less immunosuppressive immunophilins show neurotrophic activities. For example, meridamycin is substantially non-immunosuppressive and shows significant neuroprotective activity in vitro (US 2005/0272133 by He, M. et al. published on Dec. 8, 2005, and US 2005/0197356 by Grizzani, E. et al. published on Sep. 8, 2005). Preferably, immunophilin ligands identified by, or used in, the methods of the invention are substantially non-immunosuppressive, but retain a desirable activity, e.g., a neurotrophic activity. Preferred immunophilin ligands increase the formation of a complex as described herein and/or reduce FKBP and/or calcium channel activity.

[10096] In some embodiments, the immunophilin ligands are modified at the mTOR binding domain. The mTOR binding domain of rapamycin is believed to localize at the macrocycle core at about positions 1-7 and 27-36 of FIG. 1A. For example, the immunophilin ligands can have a heteroatom substituent at positions 1 and 4 of the rapamycin backbone (FIG. 1A). In other embodiments, the rapamycin analogues have a cyclic structure at positions 1, 2, 3 and/or 4 (FIG. 1A). Such rapamycin analogues are disclosed in commonly assigned co-pending published application U.S. 2006/0135549 entitled “Rapamycin Analogues and the Uses Thereof in the Treatment of Neurological, Proliferative, and Inflammatory Disorders,” published on Jun. 22, 2006 from

U.S. Ser. No. 11/300,839, the entire content of which is hereby incorporated by reference.

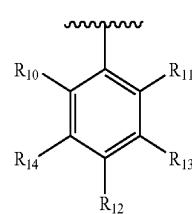
[0097] In one embodiment, the rapamycin analogues have the formula I:



[0098] R₁ and R₂ in the above-noted formula are different, independent groups and are selected from among OR₃ and N(R₃)(R_{3''}) or R₁ and R₂ are different, are connected through a single bond, and are selected from O and NR₃. R₃, R_{3'}, and R_{3''} are independently selected from among H, C₁ to C₆ alkyl, C₁ to C₆ substituted alkyl, C₃ to C₈ cycloalkyl, substituted C₃ to C₈ cycloalkyl, aryl, substituted aryl, heteroaryl, and substituted heteroaryl. R₄ and R_{4'} are (a) independently selected from among H, OH, O(C₁ to C₆ alkyl), O(substituted C₁ to C₆ alkyl), O(acyl), O(aryl), O(substituted aryl), and halogen; or (b) taken together to form a double bond to O. R₅, R₆, and R₇ are independently selected from among H, OH, and OCH₃. R₈ and R₉ are connected through a (i) single bond and are CH₂ or (ii) double bond and are CH. R₁₅ is selected from among C=O, CHO, and CH₂ and n is 1 or 2; or pharmaceutically acceptable, salts, prodrugs, or metabolites thereof.

[0099] In further embodiments, R₁ and R₂ are connected through a single bond and are selected from O and NR₃. In still a further embodiment, R₁ is O and R₂ is NR₃.

[0100] In one embodiment, R₃, or R_{3''} is an aryl or substituted aryl group, or a substituted benzene ring. In another embodiment, substituted benzene groups at R₃, or R_{3''} include rings of the following structure:

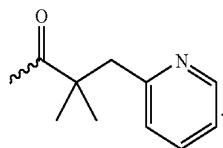


[0101] R₁₀, R₁₁, R₁₂, R₁₃, and R₁₄ are independently selected from among H, C₁ to C₆ alkyl, substituted C₁ to C₆ alkyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, halogen, acyl, OH, O(alkyl), O(substituted alkyl), O(aryl), O(substituted aryl), O(acyl), NH₂, NH(alkyl), NH(substituted alkyl), NH(aryl), NH(substituted aryl), and NH(acyl).

[0102] In further embodiments, R₃, R_{3'} or R_{3''} are phenyl optionally substituted by 1 or 2 substituents selected from C₁ to C₆ alkyl and halogen. In still further embodiments, R₃, R_{3'} or R_{3''} are phenyl optionally substituted with 1 or 2 methyl or chloro substituents, e.g. phenyl and 3-methyl, 4-chlorophenyl.

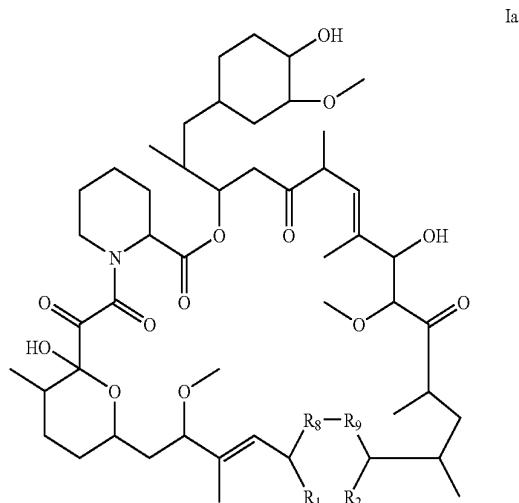
[0103] In one embodiment, R₄ or R_{4'} are OH or O(acyl), e.g., where the acyl is

[0104] —C(O)— optionally substituted alkyl, in particular where alkyl can be straight or branched and optionally substituted e.g. by heterocyclic such as aromatic heterocyclic such as pyridyl. An example is:



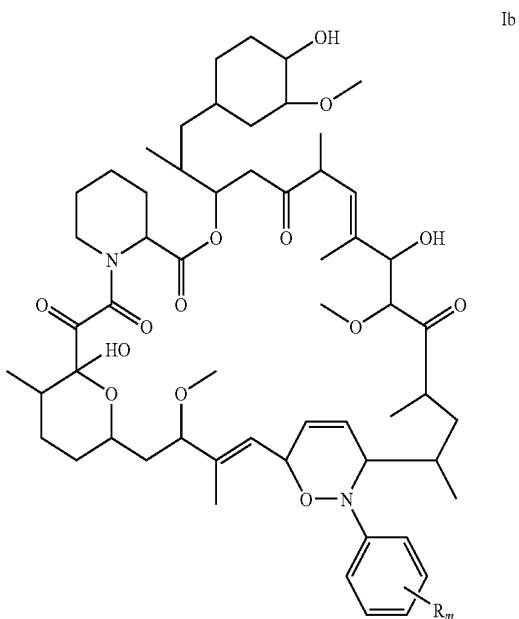
[0105] In other embodiments, rapamycin analogues of formula I include those where R₅, R₆ and R₇ are OCH₃, those where the nitrogen containing ring at positions 17-22 of the rapamycin backbone is a piperidine ring, or where R₁₅ is a carbonyl.

[0106] In one embodiment, the rapamycin analogues have the formula Ia:



[0107] where R₁, R₂, R₃, and R₉ are defined as noted above.

[0108] In another embodiment, the rapamycin analogues have the following formula Ib:



[0109] In formula Ib, R is independently selected from among H, C₁ to C₆ alkyl, substituted C₁ to C₆ alkyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, halogen, acyl, OH, O(alkyl), O(substituted alkyl), O(aryl), O(substituted aryl), O(acyl), NH₂, NH(alkyl), NH(substituted alkyl), NH(aryl), NH(substituted aryl), and NH(acyl) and m is 1 to 5.

[0110] Specific rapamycin analogues are illustrated herein and include 9,27-dihydroxy-3-[2-[4-hydroxy-3-methoxycyclohexyl]-1-methylethyl]-10,21-dimethoxy-6,8,12,14,20,26-hexamethyl-37-phenyl-4,9,10,12,13,14,15,18,21,22,23,24,25,26,27,32,33,34,34a-nonadecahydro-3H-23,27-epoxy-18,15-(epoxyimino)pyrido[2,1-c][1,4]

oxazacycloheptenatriacontine-1,5,11,28,29(6H, 31H)-pentone; 9,27-dihydroxy-3-[2-[4-hydroxy-3-methoxycyclohexyl]-1-methylethyl]-10,21-dimethoxy-6,8,12,14,20,26-hexamethyl-37-phenyl-4,9,10,12,13,14,15,16,17,18,21,22,23,24,

25,26,27,32,33,34,34a-henicosahydro-3H-23,27-epoxy-18,15-(epoxyimino)pyrido[2,1-c][1,4]

oxazacycloheptenatriacontine-1,5,11,28,29(6H, 31H)-pentone;

37-(4-chloro-3-methylphenyl)-9,27-dihydroxy-3-[2-[4-hy-

droxy-3-methoxycyclohexyl]-1-methylethyl]-10,21-

dimethoxy-6,8,12,14,20,26-hexamethyl-4,9,10,12,13,14,15,

18,21,22,23,24,25,26,27,32,33,34,34a-nonadecahydro-3H-

23,27-epoxy-18,15-(epoxyimino)pyrido[2,1-c][1,4]

oxazacycloheptenatriacontine-1,5,11,28,29(6H, 31H)-pentone;

37-(2,6-dichlorophenyl)-9,27-dihydroxy-3-[2-[4-hydroxy-

3-methoxycyclohexyl]-1-methylethyl]-10,21-dimethoxy-6,

8,12,14,20,26-hexamethyl-4,9,10,12,13,14,15,18,21,22,23,

24,25,26,27,32,33,34,34a-nonadecahydro-3H-23,27-epoxy-

18,15-(epoxyimino)pyrido[2,1-c][1,4]

oxazacycloheptenatriacontine-1,5,11,28,29(6H, 31H)-pentone;

9,27-dihydroxy-3-[2-[4-hydroxy-3-methoxycyclohexyl]-1-methylethyl]-10,21-dimethoxy-6,8,12,14,20,26-hexamethyl-37-phenyl-4,9,10,12,13,14,15,18,21,22,23,24,25,26,

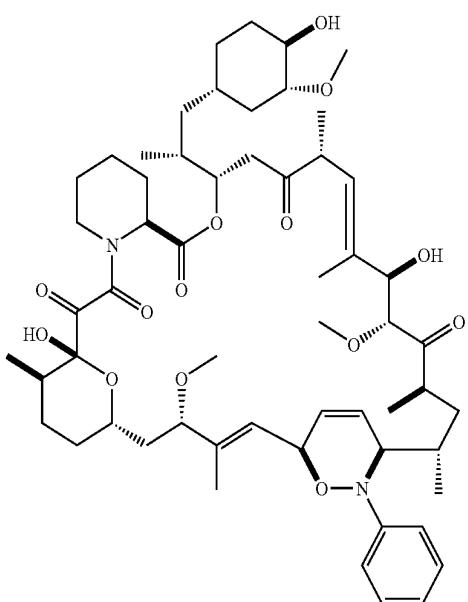
27,32,33,34,34a-nonadecahydro-3H-23,27-epoxy-18,15-

(epoxyimino)pyrido[2,1-c][1,4]

oxazacycloheptenatriacontine-1,5,11,28,29(6H, 31H)-pentone with -2,2-dimethyl-3-

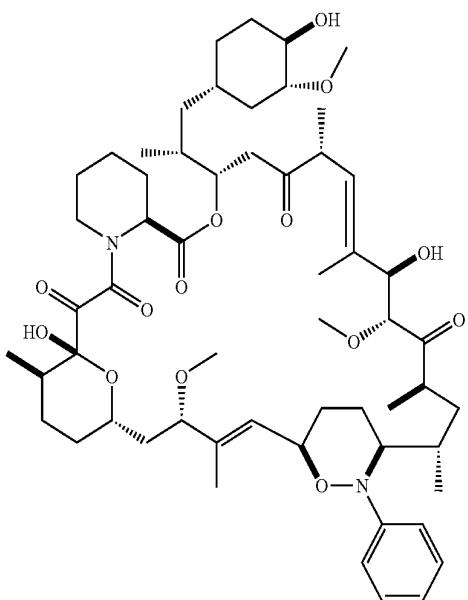
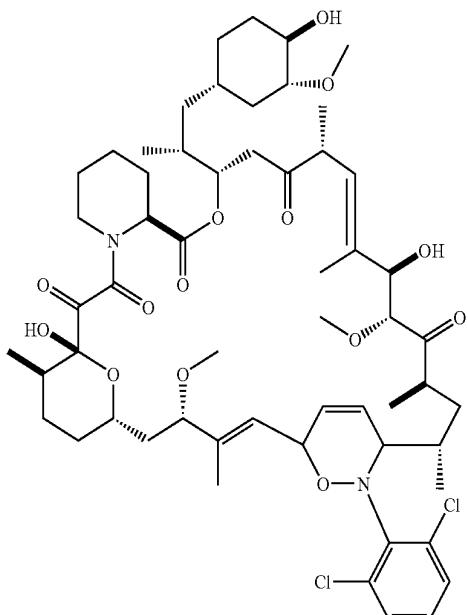
(pyridin-2-yl)-propionic acid; 37-(2,6-dichlorophenyl)-9,27-dihydroxy-3-{2-[4-hydroxy-3-methoxycyclohexyl]-1-methylethyl}-10,21-dimethoxy-6,8,12,14,20,26-hexamethyl-4,9,10,12,13,14,15,18,21,22,23,24,25,26,27,32,33,34,34a-nonadecahydro-3H-23,27-epoxy-18,15-(epoxyimino)pyrido[2,1-c][1,4]oxazacyclohepten-1,5,11,28,29(6H, 31H)-pentone; or pharmaceutically acceptable, salts, prodrugs, or metabolites thereof. The invention is not limited to these illustrative compounds.

[0111] In another embodiment, the specific compounds include the following:

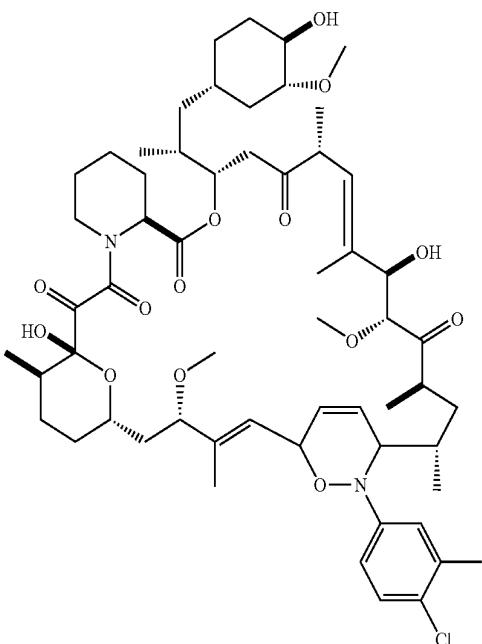


Rapamycin I

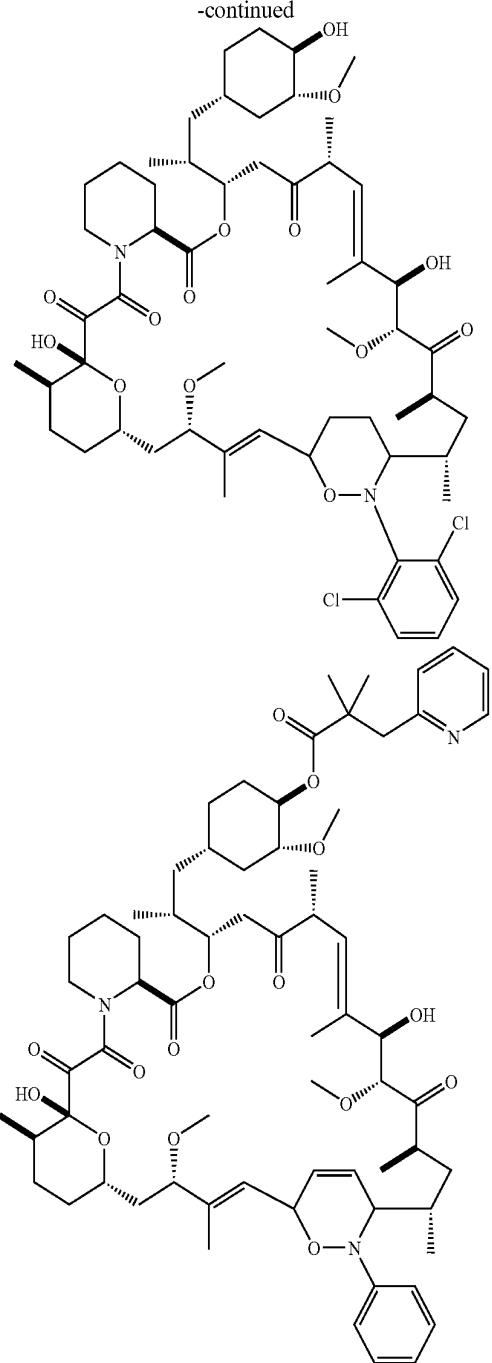
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Rapamycin II



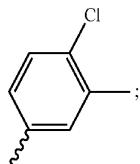
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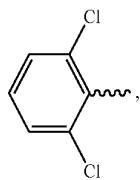
[0112] Rapamycin analogues I and II, referred to throughout the application, are represented by the first and second chemical structures, respectively, shown from the top left.

[0113] Rapamycin analogues also include compounds where R₁ and R₂ are connected through a single bond; R₁ is O; R₂ is NR₃; R₃ is phenyl; R₄ is OH; R₅-R₇ are OCH₃; and R₈ and R₉ are HC=CH; a compound where R₁ is OR₃; R₂ is N(R₃)(R_{3..}); R₃ is H; R_{3..} is H; R_{3..} is phenyl; R₄ is OH; R₅-R₇ are OCH₃; and R₈ and R₉ are H₂C—CH₂; a compound where R₁ and R₂ are connected through a single bond; R₁ is O; R₂ is NR₃; R₃ is phenyl; R₄ is OH; R₅-R₇ are OCH₃; and R₈ and R₉ are H₂C—CH₂; a compound where R₁ and R₂ are connected

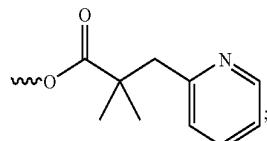
through a single bond; R₁ is O; R₂ is NR₃; R₄ is OH; R₅-R₇ are OCH₃; R₈ and R₉ are HC=CH; and R₃ is



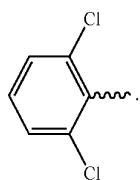
[0114] a compound where R₁ and R₂ are connected through a single bond; R₁ is O; R₂ is NR₃; R₄ is OH; R₅-R₇ are OCH₃; R₈ and R₉ are HC=CH; and R₃ is



[0115] a compound where R₁ and R₂ are connected through a single bond; R₁ is O; R₂ is NR₃; R₃ is phenyl; R₅-R₇ are OCH₃; R₈ and R₉ are HC=CH; and R₄ is



[0116] and a compound where R₁ and R₂ are connected through a single bond; R₁ is O; R₂ is NR₃; R₄ is OH; R₅-R₇ are OCH₃; R₈ and R₉ are H₂C—CH₂; and R₃ is



[0117] The compounds can contain one or more asymmetric carbon atoms and some of the compounds can contain one or more asymmetric (chiral) centers and can thus give rise to optical isomers and diastereomers. While shown without respect to stereochemistry, when the compounds can contain one or more chiral centers, preferably at least one of the chiral centers is of S-stereochemistry. Thus, the compound includes such optical isomers and diastereomers; as well as the racemic and resolved, enantiomerically pure stereoisomers; as well as other mixtures of the R and S stereoisomers, and pharmaceutically acceptable salts, hydrates, metabolites, and prodrugs thereof.

[0118] The term “alkyl” is used herein to refer to both straight- and branched-chain saturated aliphatic hydrocarbon groups having 1 to 10 carbon atoms, and desirably about 1 to

8 carbon atoms. The term “alkenyl” is used herein to refer to both straight- and branched-chain alkyl groups having one or more carbon-carbon double bonds and containing about 2 to 10 carbon atoms. In one embodiment, the term alkenyl refers to an alkyl group having 1 or 2 carbon-carbon double bonds and having 2 to about 6 carbon atoms. The term “alkynyl” group is used herein to refer to both straight- and branched-chain alkyl groups having one or more carbon-carbon triple bond and having 2 to 8 carbon atoms. In another embodiment, the term alkynyl refers to an alkyl group having 1 or 2 carbon-carbon triple bonds and having 2 to 6 carbon atoms.

[0119] The term “cycloalkyl” is used herein to refer to an alkyl group as previously described that is cyclic in structure and has about 4 to 10 carbon atoms, or about 5 to 8 carbon atoms.

[0120] The terms “substituted alkyl”, “substituted alkenyl”, and “substituted alkynyl” refer to alkyl, alkenyl, and alkynyl groups, respectively, having one or more substituents including, without limitation, halogen, CN, OH, NO₂, amino, aryl, heterocyclic, alkoxy, aryloxy, alkylcarbonyl, alkylcarboxy, and arylthio, which groups can be optionally substituted e.g. by 1 to 4 substituents including halogen, CN, OH, NO₂, amino, alkyl, cycloalkyl, alkenyl, alkynyl, alkoxy, aryloxy, alkylcarbonyl, alkylcarboxy, aminoalkyl, and arylthio. These substituents can be attached to any carbon of an alkyl, alkenyl, or alkynyl group provided that the attachment constitutes a stable chemical moiety.

[0121] The term “aryl” as used herein refers to an aromatic system, e.g., of 6-20 carbon atoms, which can include a single ring or multiple aromatic rings fused or linked together (e.g. two or three) where at least one part of the fused or linked rings forms the conjugated aromatic system. The aryl groups can include, but are not limited to, phenyl, naphthyl, biphenyl, anthryl, tetrahydronaphthyl, phenanthryl, indene, benzophenyl, fluorenyl, and carbazolyl.

[0122] The term “substituted aryl” refers to an aryl group which is substituted with one or more substituents including halogen, CN, OH, NO₂, amino, alkyl, cycloalkyl, alkenyl, alkynyl, alkoxy, aryloxy, alkylcarbonyl, alkylcarboxy, aminoalkyl, and arylthio, which groups can be optionally substituted. In one embodiment, a substituted aryl group is substituted with 1 to 4 substituents including halogen, CN, OH, NO₂, amino, alkyl, cycloalkyl, alkenyl, alkynyl, alkoxy, aryloxy, alkylcarbonyl, alkylcarboxy, aminoalkyl, and arylthio.

[0123] The term “heterocyclic” as used herein refers to a stable 4- to 7-membered monocyclic or multicyclic heterocyclic ring which is saturated, partially unsaturated, or wholly unsaturated, including aromatic such as pyridyl. The heterocyclic ring has carbon atoms and one or more heteroatoms including nitrogen, oxygen, and sulfur atoms. In one embodiment, the heterocyclic ring has 1 to 4 heteroatoms in the backbone of the ring. When the heterocyclic ring contains nitrogen or sulfur atoms in the backbone of the ring, the nitrogen or sulfur atoms can be oxidized. The term “heterocyclic” also refers to multicyclic rings, e.g., of 9 to 20 ring members in which a heterocyclic ring is fused to an aryl ring. The heterocyclic ring can be attached to the aryl ring through a heteroatom or carbon atom, provided the resultant heterocyclic ring structure is chemically stable. A variety of heterocyclic groups are known in the art and include, without limitation, oxygen-containing rings, nitrogen-containing rings, sulfur-containing rings, mixed heteroatom-containing rings,

fused heteroatom containing rings, and combinations thereof. Oxygen-containing rings include, but are not limited to, furyl, tetrahydrofuryl, pyranyl, pyronyl, and dioxinyl rings. Nitrogen-containing rings include, without limitation, pyrrolyl, pyrazolyl, imidazolyl, triazolyl, pyridyl, piperidinyl, 2-oxopiperidinyl, pyridazinyl, pyrimidinyl, pyrazinyl, piperazinyl, azepinyl, triazinyl, pyrrolidinyl, and azepinyl rings. Sulfur-containing rings include, without limitation, thieryl and dithietyl rings. Mixed heteroatom containing rings include, but are not limited to, oxathiolyl, oxazolyl, thiazolyl, oxadiazolyl, oxatriazolyl, dioxazolyl, oxathiazolyl, oxathiolyl, oxazinyl, oxathiazinyl, morpholinyl, thiamorpholinyl, thiamorpholinyl sulfoxide, oxepinyl, thiepinyl, and diazepinyl rings. Fused heteroatom-containing rings include, but are not limited to, benzofuryl, thionaphthene, indolyl, benazolyl, purindinyl, pyranopyrrolyl, isoindazolyl, indoxazinyl, benzoxazolyl, anthranilyl, benzopyranyl, quinolinyl, isoquinolinyl, benzodiazonyl, naphthylridinyl, benzothienyl, pyridopyridinyl, benzoxazinyl, xanthenyl, acridinyl, and purinyl rings.

[0124] The term “substituted heterocyclic” as used herein refers to a heterocyclic group having one or more substituents including halogen, CN, OH, NO₂, amino, alkyl, cycloalkyl, alkenyl, alkynyl, alkoxy, aryloxy, alkylcarbonyl, alkylcarboxy, aminoalkyl, and arylthio, which groups can be optionally substituted. In one embodiment, a substituted heterocyclic group is substituted with 1 to 4 substituents.

[0125] The term “acyl” refers to a —C(O)— group, which is substituted at the carbon atom. The acyl group can be substituted or a terminal acyl group such as an HC(O)— group. The substituents can include any substituents noted above for alkyl groups, viz. one or more substituents including, without limitation, halogen, CN, OH, NO₂, amino, aryl, heterocyclic, alkoxy, aryloxy, alkylcarbonyl, alkylcarboxy, and arylthio, which groups can be optionally substituted. Examples include —C(O)-alkoxy (e.g. —OMe or —OEt) or —C(O)-alkyl where alkyl can be straight or branched and optionally substituted e.g., by heterocyclic (such as pyridyl).

[0126] The term “alkoxy” as used herein refers to the O(alkyl) group, where the point of attachment is through the oxygen-atom and the alkyl group is optionally substituted.

[0127] The term “aryloxy” as used herein refers to the O(aryl) group, where the point of attachment is through the oxygen-atom and the aryl group is optionally substituted.

[0128] The term “alkyloxy” as used herein refers to the alkylOH group, where the point of attachment is through the alkyl group.

[0129] The term “arylthio” as used herein refers to the S(aryl) group, where the point of attachment is through the sulfur-atom and the aryl group can be optionally substituted.

[0130] The term “alkylcarbonyl” as used herein refers to the C(O)(alkyl) group, where the point of attachment is through the carbon-atom of the carbonyl moiety and the alkyl group is optionally substituted.

[0131] The term “alkylcarboxy” as used herein refers to the C(O)O(alkyl) group, where the point of attachment is through the carbon-atom of the carboxy moiety and the alkyl group is optionally substituted.

[0132] The term "aminoalkyl" as used herein refers to both secondary and tertiary amines where the point of attachment is through the nitrogen-atom and the alkyl groups are optionally substituted. The alkyl groups can be the same or different.

[0133] The term "halogen" as used herein refers to Cl, Br, F, or I groups.

[0134] The rapamycin analogues can be prepared from a rapamycin starting material. Preferably, the rapamycin starting material includes, without limitation, rapamycin, norrapamycin, deoxorapamycin, desmethylrapamycins, or desmethoxyrapamycin, or pharmaceutically acceptable salts, prodrugs, or metabolites thereof. However, one of skill in the art would readily be able to select a suitable rapamycin starting material that can be utilized to prepare the novel rapamycin analogues of the present invention.

[0135] The term "desmethylrapamycin" refers to the class of rapamycin compounds which lack one or more methyl groups. Examples of desmethylrapamycins that can be used according to the present invention include 29-desmethylrapamycin (U.S. Pat. No. 6,358,969), 7-O-desmethyl-rapamycin (U.S. Pat. No. 6,399,626), 17-desmethylrapamycin (U.S. Pat. No. 6,670,168), and 32-O-desmethylrapamycin, among others.

[0136] The term "desmethoxyrapamycin" refers to the class of rapamycin compounds which lack one or more methoxy groups and includes, without limitation, 32-desmethoxyrapamycin.

[0137] The rapamycin analogues can be prepared by combining a rapamycin starting material and a dienophile. The term "dienophile" refers to a molecule that reacts with a 1,3-diene to give a [4+2] cycloaddition product. Preferably, the dienophile utilized in the present invention is an optionally substituted nitrosobenzene. A variety of nitrosobenzenes can be utilized in the present invention and include nitrosobenzene, 2,6-dichloronitrosobenzene, and 1-chloro-2-methyl-4-nitrosobenzene, among others. One of skill in the art would readily be able to select the amount of nitrosobenzene that would be effective in preparing the rapamycin analogues of the present invention. Preferably, an excess of the nitrosobenzene is utilized, and more preferably in a 5:1 ratio of nitrosobenzene to rapamycin starting material. However, even a 1:1, 2:1, or 3:1 ratio of nitrosobenzene to rapamycin can be utilized as determined by one of skill in the art.

[0138] The nitrosobenzene and rapamycin starting material is combined in a solvent. The solvent preferably dissolves the nitrosobenzene and/or rapamycin on contact, or dissolves the nitrosobenzene and rapamycin as the reaction proceeds. Solvents that can be utilized in the present invention include, without limitation, dimethylformamide, dioxane such as p-dioxane, chloroform, alcohols such as methanol and ethanol, ethyl acetate, water, acetonitrile, tetrahydrofuran, dichloromethane, and toluene, or combinations thereof. However, one of skill in the art would readily be able to select a suitable solvent based upon the solubility of the rapamycin starting material and nitrosobenzene, as well as the reactivity of the solvent with the same. The amount of solvent utilized depends upon the scale of the reaction and specifically the amount of rapamycin starting material and nitrosobenzene

present in the reaction mixture. One of skill in the art would readily be able to determine the amount of solvent required.

[0139] Typically, the solution containing the nitrosobenzene, rapamycin starting material, and solvent is maintained at elevated temperatures, and preferably a temperature that does not promote decomposition of the rapamycin and nitrosobenzene. In one embodiment, the solution is maintained a temperature of about 30 to about 70° C., and preferably about 50° C. The components are heated for a period of time sufficient to permit reaction between the rapamycin and nitrosobenzene. One of skill in the art using known techniques would readily be able to monitor the progress of the reaction during heating and thereby determine the amount of time required to perform the reaction. In one preferred embodiment, the rapamycin and nitrosobenzene are combined with p-dioxane and maintained at a temperature of about 50° C.

[0140] Isolation and purification of the rapamycin analogue is well within one of skill in the art and include chromatography including, without limitation, and recrystallization, high performance liquid chromatography (HPLC) such as reverse phase HPLC, and normal phase HPLC, and size-exclusion chromatography.

[0141] Once the rapamycin analogue is obtained, it can be reduced to form a more saturated rapamycin analogue. One of skill in the art would readily be able to select a suitable reducing agent for use in the present invention. Preferably, reduction of the rapamycin analogue can be effected using a hydrogenation agent. One of skill in the art would readily be able to select a suitable hydrogenation agent for use in the present invention. Typically, transition metal catalysts or transition metals on a support, preferably a carbon support, among others, in the presence hydrogen gas, are utilized to carry out the reduction. In a preferred embodiment, the reduction is performed using palladium metal on carbon in the presence of hydrogen gas.

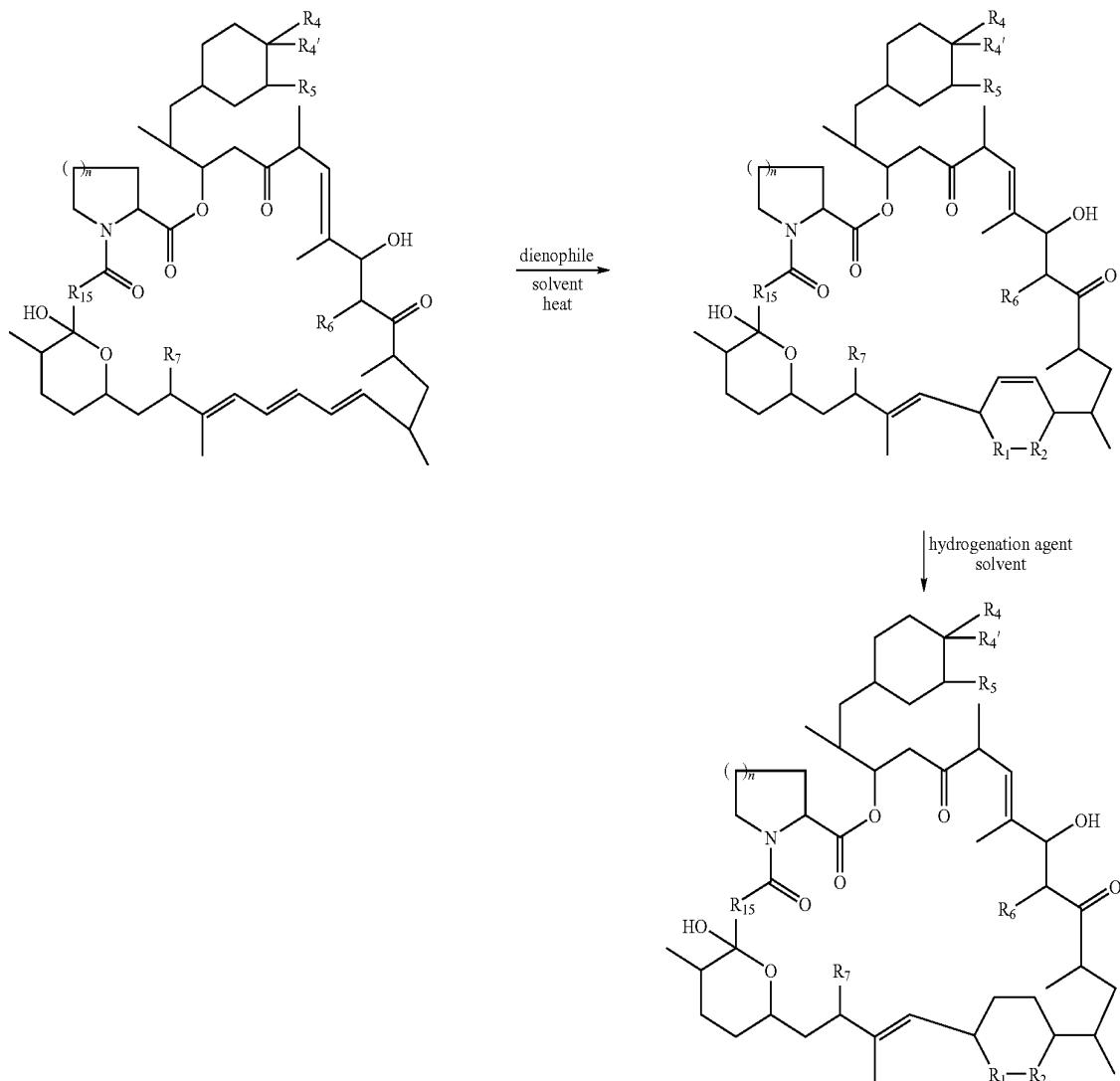
[0142] Reduction of the rapamycin analogue is typically carried out in a solvent. A variety of solvents can be utilized in the reduction and include, without limitation, alcohols such as methanol. However, one of skill in the art would readily be able to select a suitable solvent for use in the present invention and depending on the hydrogenation catalyst and rapamycin analogue being reduced. The amount of solvent depends on the scale of the reaction, and specifically the amount of rapamycin analogue being reduced.

[0143] The amount of hydrogenation agent utilized in the present invention can readily be determined by one of skill in the art. However, one of skill in the art would be able to determine and adjust the amount of hydrogenation agent necessary to perform the reduction and to form the more saturated rapamycin analogues of the present invention. Further, a variety of apparatuses can be utilized to perform the hydrogenation of the present invention and include Parr apparatuses, among others. The selection of the particular apparatus for the hydrogenation is well within one of skill in the art.

[0144] A preferred method of preparing the rapamycin analogues of the present invention is summarized in Scheme 1 below:

[0147] Additional synthetic routes and characterization of the rapamycin analogues are provided in Examples 1-3 of commonly assigned co-pending published application US

Scheme 1



[0145] where R_1 , R_2 , R_4 , $R_{4'}$, R_5 , R_6 , R_7 , R_{15} , and n are defined above.

[0146] The rapamycin analogues can be utilized in the form of pharmaceutically acceptable salts, prodrugs, or metabolites thereof derived from pharmaceutically or physiologically acceptable acids or bases. These salts include, but are not limited to, the following salts with mineral or inorganic acids such as hydrochloric acid, sulfuric acid, nitric acid, phosphoric acid and organic acids such as acetic acid, oxalic acid, succinic acid, and maleic acid. Other salts include salts with alkali metals or alkaline earth metals, such as sodium, potassium, calcium or magnesium in the form of esters, carbamates and other conventional "pro-drug" forms, which, when administered in such form, convert to the active moiety in vivo.

2006/0135549, entitled "Rapamycin Analogues and the Uses Thereof in the Treatment of Neurological, Proliferative, and Inflammatory Disorders," published on Jun. 22, 2006, referenced hereinabove.

[0148] Other examples of rapamycin analogues that can be used in the methods of the invention are disclosed in commonly owned published application U.S. 2006/0135550 entitled "Rapamycin Derivatives and the Uses Thereof in the Treatment of Neurological Disorders," published on Jun. 22, 2006, from U.S. Ser. No. 11/300,941, the entire content of which is hereby incorporated by reference.

[0149] In other embodiments, the immunophilin ligand is a meridamycin analogue. Examples of meridamycin analogues that can be used in the methods of the invention include those disclosed in, e.g., U.S. 2005/0197379, U.S. 2005/0272133,

U.S. 2005/0197356, WO 2005/084673, WO 2005/085257, as well as the following commonly owned provisional applications: U.S. Ser. No. 60/664,483 entitled “Meridamycin Derivatives and Uses Thereof,” filed Mar. 23, 2005 (publicly available through USPTO PAIR; and U.S. Ser. No. 60/779,940 entitled “Meridamycin Analogues for the Treatment of Neurodegenerative Disorders,” filed Mar. 7, 2006. (The entire contents of all of which are hereby incorporated by reference.) Some of the neurotrophic effects of the immunophilin ligands disclosed may be mediated by the formation of complexes described herein. In one embodiment, the meridamycin analogue has the chemical formula of compound I in U.S. 2005/0197379.

[0150] Several of the aforesaid rapamycin and meridamycin analogues have been demonstrated to have potent neurotrophic (e.g., neuroprotective, neuroregenerative and/or stimulating neurite outgrowth) activities in cultured cortical, dopaminergic and spinal cord neurons.

Immunophilin Complexes

[0151] In one aspect, the invention relates to the discovery of, immunophilin complexes. In some embodiments, the complexes includes an immunophilin ligand (e.g., a rapamycin or a meridamycin analogue as described herein), an immunophilin (e.g., FKBP52) or a functional variant thereof, and a calcium channel subunit (e.g., a β 1 subunit of the voltage gated L-type calcium channel) or a functional variant thereof.

[0152] As used herein, the terms “binding” and “complex formation” refer to a direct or indirect association between two or more molecules, e.g., polypeptides, macrolides, among others. Direct associations may include, for example, covalent, electrostatic, hydrophobic, ionic and/or hydrogen-bond interactions under physiological conditions. Indirect associations include, for example, two or more molecules that are part of a complex but do not have a direct interaction. In one embodiment, the association between the molecules is sufficient to maintain a stable complex under physiological conditions.

[0153] A complex of the invention may be obtained in isolated, recombinant, or purified form. The term “purified” or “isolated” as qualifiers of “protein” or “complex” refers to a preparation of a protein or proteins which are substantially free of other proteins normally associated with the protein(s) in a cell or cell lysate. For example, the phrase “substantially free” encompasses preparations comprising less than 40%, 30%, 20% (by dry weight) contaminating protein, and typically comprises less than 5% contaminating protein. By “purified” or “isolated,” it is meant, when referring to component protein preparations used to generate a reconstituted protein mixture, that the indicated molecule is present in the substantial absence of other biological macromolecules, such as other proteins (particularly other proteins which may substantially mask, diminish, confuse or alter the characteristics of the component proteins either as purified preparations or in their function in the subject reconstituted mixture). The term “purified” or “isolated” as used herein preferably means at least 80% by dry weight, typically in the range of 85% by weight, more typically 95-99% or higher by weight, of biological macromolecules of the same type present (but water, buffers, and other small molecules, especially molecules having a molecular weight of less than 5000, can be present). In one embodiment, the complex or protein is substantially free

of purification materials, e.g., matrices or other materials. In other embodiments, the complex or protein is associated with the purification materials.

[0154] The term “recombinant” “protein” or “complex” refers to a protein(s) that form a complex, which are produced by recombinant DNA techniques. Generally, the DNA(s) encoding the expressed protein(s) is inserted into a suitable expression vector which is in turn used to transform a host cell (also referred to herein as a “recombinant cell”) to produce the heterologous protein. Moreover, the phrase “derived from,” with respect to a recombinant gene encoding the recombinant protein is meant to include within the meaning of “recombinant protein” those proteins having an amino acid sequence of a native protein, or an amino acid sequence similar thereto which is generated by mutations including substitutions, insertions, and deletions of a naturally occurring protein.

[0155] In an embodiment, the invention provides a complex prepared, for example, by extraction from a cell, e.g., an immunophilin-treated cell, that comprises the components of the complex (e.g., a naturally occurring or a recombinant cell). Extraction from a cell may be accomplished by any of the methods known in the art. For example, a complex may be extracted from the cell by a series of traditional protein purification steps, such as centrifugation, gel filtration, ion exchange chromatography, affinity chromatography and/or affinity purification. It will generally be preferable to select purification steps and conditions that do not dissociate the complex. As described in the appended Examples, a lysis buffer (e.g., 6 ml; 50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na₃VO₄, 1% Nonidet P40 (NP40), 0.1% mercaptoethanol and 2% protease inhibitor cocktails) can be used. For example, affinity matrices linking an immunophilin ligand, e.g., a rapamycin analog, to a resin can be prepared as described by Fretz et al. (1991) *J. Am. Chem. Soc.* 113:1409. In one embodiment, affinity matrices can be prepared by using Affigel10 resin through amino-phenyl-butyric acid (FIG. 1). Briefly, the amino group of amino-phenyl-butyric acid can be protected by treating with a protecting group such as diallyldicarbonate. The acid group of the resulting complex can be activated with PhOP(O)Cl₂ DMF complex in CH₂Cl₂. After the reaction is quenched, the ester product can be purified by, e.g., HPLC, and characterized by, e.g., MS and NMR. After removing the allyloxycarbonyl group, the amino group of the product can be linked to Affigel-10 matrix. The resulting Affigel-immunophilin ligand affinity matrix can be washed and stored. After extraction, aliquots of cell lysates can be mixed with affinity beads, such as Affigel10-immunophilin ligand. Beads can be analyzed on, e.g., 4-20% SDS-PAGE gel. The protein bands can be digested and further analyzed by, e.g., FT-ICR-MS analysis.

[0156] In other embodiments, the complex can be prepared by purifying recombinant polypeptides expressed in cells, such as *E. coli*, and reconstituting the complex in vitro. In certain embodiments, one or more of the constituent polypeptides of a complex is expressed from an endogenous gene of a cell. In certain embodiments, complexes are recombinant complexes wherein one or more of the constituent polypeptides are expressed from a recombinant nucleic acid. In certain embodiments, the invention also includes labeled protein complexes, wherein at least one polypeptide of the complex is labeled. For example, the label is a detectable label can be chosen from, e.g., one or more of radioisotopes, fluorescent compounds, enzymes, and enzyme co-factors. In another

embodiment, the label facilitates purification, isolation, or detection of the polypeptide. The label may be a polyhistidine, FLAG, Glu-Glu, glutathione S transferase (GST), thioredoxin, protein A, protein G, and an immunoglobulin heavy chain constant region. In one embodiment, the labeled protein is FKBP52. In another embodiment, the labeled protein is a calcium channel subunit. The labeled complex or a component thereof can be purified by an appropriate affinity purification (e.g., as described above, or by contacting the complex with a nickel or copper resin in the case of a hexahistidine tag, contacting with a glutathione resin in the case of a GST tag).

[0157] In certain embodiment, a complex of the invention is in water-soluble form (a “soluble complex”). For example, a soluble complex may include soluble cytoplasmic portions of an immunophilin and/or a calcium channel subunit. In other embodiments, the complex may be less soluble in water or in membrane-associated form. For example, a complex comprising a protein having a transmembrane domain will generally be water insoluble. Insoluble complexes may be prepared, for example, as lipid micelles, detergent micelles or mixed micelles comprising lipids, detergents and/or other components. Insoluble complexes may also be prepared as membrane fractions from a cell. A membrane fraction may be a crude membrane fraction, wherein the membrane portion is simply separated from the soluble portion of a cell by, for example, centrifugation or filtration. A membrane fraction may be further purified by, for example, affinity purification directed to an affinity tag present in one or more of the proteins of a complex. Where a complex is present in a lipid bilayer, the lipid bilayer may, for example, be a vesicle (optionally inverted, i.e., with the normally extracellular face facing inwards towards the interior of the vesicle) or a planar bilayer.

[0158] Crystallized forms of the complex are also within the scope of the invention.

[0159] In one embodiment, the complex is cross-linked. Crosslinked complexes can be prepared using crosslinking reagents which are multifunctional or bifunctional agents. Such agents include the diamine group of compounds, such as, for example, hexamethylenediamine, diaminooctane, ethylenediamine, 4-(4-N-Maleimidophenyl)butyric acid hydrazide.HCl(MPBH), 4-(N-Maleimidomethyl)cyclohexane-1-carboxy-hydrazide.HCl ($M_2 C_2 H$), and 3-(2-Pyridylidithio)propionylhydrazide(PDPH) and other amine alkenes. Examples of such crosslinking agents are glutaraldehyde, succinaldehyde, octanedialdehyde and glyoxal. Additional multifunctional crosslinking agents include halo-triazines, e.g., cyanuric chloride; halo-pyrimidines, e.g., 2,4,6-trichloro/bromo-pyrimidine; anhydrides or halides of aliphatic or aromatic mono- or di-carboxylic acids, e.g., maleic anhydride, (meth)acryloyl chloride, chloroacetyl chloride; N-methylol compounds, e.g., N-methylol-chloro acetamide; di-isocyanates or di-isothiocyanates, e.g., phenylene-1,4-di-isocyanate and aziridines. Other crosslinking agents include epoxides, such as, for example, di-epoxides, tri-epoxides and tetra-epoxides. For a representative listing of other available crosslinking reagents see, for example, the Pierce Catalog and Handbook, Pierce Chemical Company, Rockford, Ill. (1997) and also S. S. Wong, Chemistry of Protein Conjugation and Cross-Linking, CRC Press, Boca Raton, Fla. (1991).

[0160] Alternatively, reversible crosslinkers can be used. Examples of reversible crosslinkers are described in T. W.

Green, Protective Groups in Organic Synthesis, John Wiley & Sons (Eds.) (1981). Any variety of strategies used for reversible protecting groups can be incorporated into a crosslinker suitable for at least one crosslinking in producing carbohydrate crosslinked glycoprotein crystals capable of reversible, controlled solubilization. Various approaches are listed, in Waldmann's review of this subject, in Angewandte Chmie Intl. Ed. Engl., 35, p. 2056 (1996). Other types of reversible crosslinkers are disulfide bond-containing crosslinkers.

[0161] The invention further provides methods for modulating (e.g., increasing) the formation and/or stability of a complex described herein. The method includes: contacting an immunophilin, e.g., an FKBP52 (e.g., a human FKBP52) or a functional variant thereof; and a subunit of the voltage gated L-type calcium channel, e.g., a $\beta 1$ subunit (e.g., a human $\beta 1$ subunit), or a functional variant thereof, with an immunophilin ligand, e.g., a rapamycin or meridamycin analogue as described herein, under conditions that allow the formation of the complex to occur. The contacting step can occur *in vitro*, e.g., in a cell lysate or in a reconstituted system. Alternatively, the method can be performed on cells (e.g., neuronal or cardiovascular cells) present in a subject, e.g., a human or an animal subject (e.g., an *in vivo* animal model).

[0162] The subject method can also be used on cells in culture. For example, cells (e.g., purified or recombinant cells) can be cultured *in vitro* and the contacting step can be effected by adding the immunophilin ligand, e.g., the rapamycin or meridamycin analogue, to the culture medium. Typically, the cell is a mammalian cell, e.g., a human cell. In some embodiments, the cell is a neuronal or a cardiovascular cell. In some embodiments, the cell is a recombinant cell, e.g., a host cell. Such methods include (i) introducing into the cell one or more polynucleotides encoding the immunophilin and/or the calcium channel subunit; (ii) contacting said cell with an immunophilin ligand, e.g., a rapamycin or meridamycin analog as described herein; (iii) thereby forming a complex.

Host Cells

[0163] In another aspect, the invention features host cells comprising one or more nucleic acids encoding one or more of the polypeptide constituents of the complex disclosed herein. In one embodiment, the host cells contain a first nucleic acid that includes a nucleotide sequence encoding an immunophilin, e.g., an FKBP52 (e.g., a mammalian FKBP52 as described herein) or a functional variant thereof; and/or a second nucleic acid that includes a nucleotide sequence encoding a subunit of the voltage gated L-type calcium channel, e.g., a $\beta 1$ subunit (e.g., a mammalian $\beta 1$ subunit as described herein), or a functional variant thereof. In one embodiment, the first nucleic acid comprises a nucleotide sequence encoding the amino acid sequence shown as FIG. 13A-13B (SEQ ID NOs:6-7), or a sequence substantially identical thereto. In other embodiments, the second nucleic acid comprises a nucleotide sequence encoding the amino acid sequence shown as FIG. 12A-12E (SEQ ID NO:1-5), or a sequence substantially identical thereto.

[0164] “Host cells,” “recombinant cells,” and “recombinant host cells” are terms used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences,

such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

[0165] The term "recombinant nucleic acid" includes any nucleic acid that includes at least two sequences which are not present together in nature. A recombinant nucleic acid may be generated in vitro, for example by using the methods of molecular biology, or in vivo, for example by insertion of a nucleic acid at a novel chromosomal location by homologous or non-homologous recombination.

[0166] In some embodiments, host cells may be used, for example, for purifying, making or studying a protein or protein complex. Optionally, host cells may be used, for example, for testing compounds in assay protocols such as those described below.

[0167] In certain embodiments, recombinant expression of polypeptides of a complex of the invention may be performed separately, and complexes formed therefrom. In another embodiment, recombinant expression of such polypeptides of a complex of the invention may be performed in the same cell, and complexes formed therefrom.

[0168] Suitable host cells for recombinant expression include bacteria such as *E. coli*, *Clostridium* sp., *Pseudomonas* sp., yeast, plant cells, insect cells (such as) and mammalian cells such as fibroblasts, lymphocytes, U937 cells (or other promonocytic cell lines) and Chinese hamster ovary cells (CHO cells).

[0169] For the purpose of host cell expression, the recombinant nucleic acid may be operably linked to one or more regulatory sequences in an expression construct. Regulatory nucleotide sequences will generally be appropriate for the host cell used for expression. Numerous types of appropriate expression vectors and suitable regulatory sequences are known in the art for a variety of host cells. Typically, said one or more regulatory nucleotide sequences may include, but are not limited to, promoter sequences, leader or signal sequences, ribosomal binding sites, transcriptional start and termination sequences, translational start and termination sequences, and enhancer or activator sequences. Constitutive or inducible promoters as known in the art are contemplated by the invention. The promoters may be either naturally occurring promoters, or hybrid promoters that combine elements of more than one promoter. An expression construct may be present in a cell on an episome, such as a plasmid, or the expression construct may be inserted in a chromosome. In a preferred embodiment, the expression vector contains a selectable marker gene to allow the selection of transformed host cells. Selectable marker genes are well known in the art and will vary with the host cell used.

[0170] The expression vector may also include a fusion domain (typically provided by the expression vector) so that the recombinant polypeptide of the invention is expressed as a fusion polypeptide with said fusion domain. The main advantage of fusion domains are that they assist identification and/or purification of said fusion polypeptide and also enhance protein expression level and overall yield.

Antibodies

[0171] In yet another aspect, the invention features an antibody, or antigen-binding fragment thereof that binds to the complexes disclosed herein. In certain embodiments, the antibodies increase the formation and/or stability of a complex disclosed herein. In other embodiments, the antibodies, or antigen-binding fragments thereof, decrease or inhibit the

formation and/or stability of a complex disclosed herein. Exemplary antibody molecules include full immunoglobulin molecules, or portions thereof that contain, for example, the antigen binding site (including those portions of immunoglobulin molecules known in the art as F(ab), F(ab'), F(ab')₂, humanized chimeric antibody, and F(v)). Polyclonal or monoclonal antibodies can be produced by methods known in the art. (Kohler and Milstein (1975) *Nature* 256, 495-497; Campbell "Monoclonal Antibody Technology, the Production and Characterization of Rodent and Human Hybridomas" in Burdon et al (eds.) (1985) "Laboratory Techniques in Biochemistry and Molecular Biology", Vol. 13, Elsevier Science Publishers, Amsterdam); Harlow and Lane (eds) (1988) In "Antibodies A Laboratory Manual", Cold Spring Harbor Press, Cold Spring Harbor, N.Y; the contents of all of which are hereby incorporated by reference.

[0172] Purified complexes of the invention, or the polypeptide components thereof, can be used to immunize animals to obtain polyclonal and monoclonal antibodies which specifically react with the complex. Such antibodies may be obtained using the entire complex or full length polypeptide components as an immunogen, or by using fragments thereof. Smaller fragments of the polypeptides may also be used to immunize animals. The peptide immunogens additionally may contain a cysteine residue at the carboxyl terminus and are conjugated to a hapten such as keyhole limpet hemocyanin (KLH). Additional peptide immunogens may be generated by replacing tyrosine residues with sulfated tyrosine residues. Methods for synthesizing such peptides are known in the art, as described in, for example, Ausbel et al. (eds) (1987) *In Current Protocols In Molecular Biology*, John Wiley and Sons (New York, N.Y.).

[0173] Modified antibodies, or antigen-binding fragments thereof, can be generated by techniques known in the art as disclosed in, e.g., Wood et al., International Publication WO 91/00906, Kucherlapati et al., International Publication WO 91/10741; Lonberg et al., International Publication WO 92/03918; Kay et al., International Publication WO 92/03917; Lonberg et al. (1994) *Nature* 368:856-59; Green et al. (1994) *Nat. Genet.* 7:13-21; Morrison et al. (1994) *Proc. Natl. Acad. Sci. U.S.A.* 81:6851-55; Bruggeman et al. (1993) *Year Immunol.* 7:33-40; Tuaillon et al. (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:3720-24; Bruggeman et al. (1991) *Eur. J. Immunol.* 21:1323-1326; Larrick et al. (1991) *Biotechniques* 11:152-56; Robinson et al., International Patent Application PCT/US86/02269; Akira et al., European Patent Application 184,187; Taniguchi, European Patent Application 171,496; Morrison et al., European Patent Application 173,494; Neuberger et al. International Publication WO 86/01533; Cabilly et al. U.S. Pat. No. 4,816,567; Better et al. (1988) *Science* 240:1041-43; Liu et al. (1987) *Proc. Natl. Acad. Sci. U.S.A.* 84:3439-43; Liu et al. (1987) *J. Immunol.* 139:3521-26; Sun et al. (1987) *Proc. Natl. Acad. Sci. U.S.A.* 84:214-18; Nishimura et al. (1987) *Canc. Res.* 47:999-1005; Wood et al. (1985) *Nature* 314:446-49; Shaw et al. (1988) *J. Natl. Cancer Inst.* 80:1553-59; Morrison (1985) *Science* 229:1202-07; Oi et al. (1986) *BioTechniques* 4:214; and Queen et al. U.S. Pat. Nos. 5,585,089, 5,693,761 and 5,693,762, the contents of all of which are hereby incorporated by reference. Those methods include isolating, manipulating, and expressing the nucleic acid sequences that encode all or part of immunoglobulin Fv variable regions from at least one of a heavy or light chain. Sources of such nucleic acids are known to those skilled in the art and, for example, may be obtained from a

hybridoma producing an antibody against a predetermined target. The recombinant DNA encoding the recombinant antibody, or fragment thereof, can then be cloned into an appropriate expression vector.

Assays for Identifying Test Compounds that Modulate Formation of the Complex

[0174] In another aspect, the invention provides a method, or an assay, for identifying a test compound that modulates, e.g., inhibits or increases, the formation and/or stability of a complex that includes the test compound, an immunophilin, and a calcium channel subunit. The method, or the assay, includes: contacting a sample that includes an immunophilin or a functional variant thereof, and β subunit or a functional variant thereof with a test compound under conditions that allow the formation of the complex; detecting the presence of the complex in the sample contacted with the test compound relative to a reference sample (e.g., a control sample not exposed to the test agent, or a control sample exposed to rapamycin). A change (e.g., an increase or a decrease) in the level of the complex in the presence of the test compound, relative to the level of the complex in the reference sample, indicates that said test compound affects (e.g., increases or decreases) the formation and/or stability of said complex. Test compounds that increase complex formation by, e.g., about 1.5, 2, 5, 10 fold or higher, relative to a reference sample are preferred.

[0175] Test compounds can be obtained, for example, from bacteria, actinomycetes (e.g., *S. hygroscopicus*), yeast or other organisms (e.g., natural products), produced chemically (e.g., small molecules, including peptidomimetics), or produced recombinantly. For example, polyketides can be produced from naturally occurring or genetically modified *Streptomyces* species, as for example, described in U.S. 2005/0272133, U.S. 2005/0197379. Modified forms of the rapamycin and meridamycin analogues disclosed herein can be alternatively by chemical synthesis.

[0176] The complex of the invention allows for the generation of new modified macrolides, e.g., modified forms of the rapamycin and meridamycin analogues disclosed herein. The purified complex can be used for determination of a three-dimensional crystal structure, which can be used for modeling intermolecular interactions. For example, crystal structures of the complex can be determined and modifications of the structure can be generated by performing rational drug design using techniques known in the art. Numerous computer programs are available for rational drug design, computer modeling, model building as described in U.S. 2005/0288489A1, the contents of which are incorporated by reference herein.

[0177] A variety of assay formats will suffice and, in light of the present disclosure, those not expressly described herein will nevertheless be comprehended by one of ordinary skill in the art. Assay formats which approximate such conditions as formation of protein complexes, enzymatic activity, and may be generated in many different forms, and include assays based on cell-free systems, e.g., purified proteins or cell lysates, as well as cell-based assays which utilize intact cells. Simple binding assays can be used to detect compounds that inhibit or potentiate the interaction between components of the complex, or the binding of the complex to a substrate.

[0178] In certain embodiments, the present invention provides reconstituted protein preparations including a polypeptide of the complex, and one or more interacting polypeptides of the complex. In one embodiments, all components or the

complex are added simultaneously in a reaction mixture. In other embodiments, the reaction mixture is prepared by adding the components sequentially, e.g., forming a mixture of the immunophilin and the calcium channel, and adding the immunophilin ligand. Alternatively, the immunophilin ligand can be added to the immunophilin or the calcium channel. Any order or combination of the components can be used. Assays of the present invention include labeled in vitro protein-protein binding assays, immunoassays for protein binding, and the like. In one embodiment, the sample is a cell lysate or a reconstituted system. The reconstituted complex can comprise a reconstituted mixture of at least semi-purified proteins. By semi-purified, it is meant that the proteins utilized in the reconstituted mixture have been previously separated from other cellular proteins. For instance, in contrast to cell lysates, proteins involved in the complex formation are present in the mixture to at least 50% purity relative to all other proteins in the mixture, and more preferably are present at 90-95% purity. In certain embodiments, the reconstituted protein mixture is derived by mixing highly purified proteins such that the reconstituted mixture substantially lacks other proteins (such as of cellular origin) which might interfere with or otherwise alter the ability to measure the complex assembly and/or disassembly. In certain embodiments, assaying in the presence and absence of a candidate compound, can be accomplished in any vessel suitable for containing the reactants. Examples include microtitre plates, test tubes, and micro-centrifuge tubes.

[0179] In certain embodiments, drug screening assays can be generated which detect test compounds on the basis of their ability to interfere with assembly, stability, or function of a complex of the invention. Detection and quantification of the complex provide a means for determining the compound's efficacy at inhibiting (or potentiating) interaction between the components. The efficacy of the compound can be assessed by generating dose response curves from data obtained using various concentrations of the test compound. Moreover, a control assay can also be performed to provide a baseline for comparison. In the control assay, the formation of complexes is quantitated in the absence of the test compound.

[0180] In certain embodiments, association between any two polypeptides in a complex or between the complex and a substrate polypeptide, may be detected by a variety of techniques, many of which are effectively described above. For instance, modulation in the formation of complexes can be quantitated using, for example, detectably labeled proteins (e.g., radiolabeled, fluorescently labeled, or enzymatically labeled), by immunoassay, or by chromatographic detection. Surface plasmon resonance systems, such as those available from Biacore International AB (Uppsala, Sweden), may also be used to detect protein-protein interaction.

[0181] In certain embodiments, one of the polypeptides of a complex can be immobilized to facilitate separation of the complex from uncomplexed forms of one of the polypeptides, as well as to accommodate automation of the assay. Affinity matrices or beads are described herein that contain the immunophilin ligand (or other components of the complex) that permits other components of the complex to be bound to an insoluble matrix. Test compound are incubated under conditions conducive to complex formation. Following incubation, the beads are washed to remove any unbound interacting protein, and the matrix bead-bound radiolabel determined directly (e.g., beads placed in scintillant), or in the supernatant after the complexes are dissociated, e.g., when microtitre

plate is used. Alternatively, after washing away unbound protein, the complexes can be dissociated from the matrix, separated by SDS-PAGE gel, and the level of interacting polypeptide found in the matrix-bound fraction quantitated from the gel using standard electrophoretic techniques.

[0182] Alternatively, the assays can be performed using cells in culture, e.g., purified cultured or recombinant cells. For example, a two-hybrid assay (also referred to as an interaction trap assay) can be used for detecting the interaction of any two polypeptides in the complex, and for subsequently detecting test compounds which inhibit or potentiate binding of the proteins to one and other (see also, U.S. Pat. No. 5,283,317; WO94/10300; Zervos et al. (1993) *Cell* 72: 223-232; Madura et al. (1993) *J. Biol. Chem.* 268: 12046-12054; Bartel et al. (1993) *Biotechniques* 14: 920-924; and Iwabuchi et al. (1993) *Oncogene* 8: 1693-1696), the contents of all of which are incorporated by reference.

[0183] In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Assays of the present invention which are performed in cell-free systems, such as may be developed with purified or semi-purified proteins or with lysates, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound. Moreover, the effects of cellular toxicity and/or bioavailability of the test compound can be generally ignored in the in vitro system, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest in an alteration of binding affinity with other proteins or changes in enzymatic properties of the molecular target.

[0184] In certain embodiments, activities of a protein complex may include, without limitation, a protein complex formation, which may be assessed by immunoprecipitation and analysis of co-immunoprecipitated proteins or affinity purification and analysis of co-purified proteins. Fluorescence Resonance Energy Transfer (FRET)-based assays may also be used to determine complex formation. Fluorescent molecules having the proper emission and excitation spectra that are brought into close proximity with one another can exhibit FRET. The fluorescent molecules are chosen such that the emission spectrum of one of the molecules (the donor molecule) overlaps with the excitation spectrum of the other molecule (the acceptor molecule). The donor molecule is excited by light of appropriate intensity within the donor's excitation spectrum. The donor then emits the absorbed energy as fluorescent light. The fluorescent energy it produces is quenched by the acceptor molecule. FRET can be manifested as a reduction in the intensity of the fluorescent signal from the donor, reduction in the lifetime of its excited state, and/or re-emission of fluorescent light at the longer wavelengths (lower energies) characteristic of the acceptor. When the fluorescent proteins physically separate, FRET effects are diminished or eliminated. FRET-based assays are described in U.S. Pat. No. 5,981,200, the contents of which are incorporated by reference.

[0185] In general, where a screening assay is a binding assay (whether protein-protein binding, compound-protein binding, etc.), one or more of the molecules may be joined to a label, where the label can directly or indirectly provide a detectable signal. Various labels include radioisotopes, fluorescers, chemiluminescers, enzymes, specific binding mol-

ecules, particles, e.g., magnetic particles, and the like. Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin etc. For the specific binding members, the complementary member would normally be labeled with a molecule that provides for detection, in accordance with known procedures.

[0186] A variety of other reagents may be included in the screening assay. These include reagents like salts, neutral proteins, e.g., albumin, detergents, etc that are used to facilitate optimal protein-protein binding and/or reduce nonspecific or background interactions. Reagents that improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial compounds, etc. may be used. The mixture of components are added in any order that provides for the requisite binding. Incubations are performed at any suitable temperature, typically between 4 and 40° C. Incubation periods are selected for optimum activity, but may also be optimized to facilitate rapid high-throughput screening.

[0187] In certain embodiments, the test compounds can be further assayed to identify compounds that modulate calcium channel activity. For example, the effect of a test compound can be measured by testing calcium channel activity of a eukaryotic cell having a functional calcium channel (e.g., a heterologous channel) when such cell is exposed to a solution containing the test compound and a calcium channel selective ion, and comparing the measured calcium channel activity to the calcium channel activity of the same cell or a substantially identical control cell in a solution not containing the test compound. The cell is maintained, in one embodiment, in a solution having a concentration of calcium channel selective ions sufficient to provide an inward current when the channels open. Methods for practicing such assays are known to those of skill in the art. For example, for similar methods applied with *Xenopus laevis* oocytes and acetylcholine receptors, see, Mishina et al. (1985) *Nature* 313:364; Noda et al. (1986) *Nature* 322:826-828; Claudio et al. (1987) *Science* 238:1688-1694.

[0188] The assays are based on cells that express functional calcium channels and measure functionally, such as electrophysiologically, the ability of a test compound to potentiate, antagonize or otherwise modulate the magnitude and duration of the flow of calcium channel selective ions, such as Ca^{++} or Ba^{++} , through the heterologous functional channel. The amount of current, which flows though the recombinant calcium channels of a cell may be determined, in one embodiment, directly, such as electrophysiologically, or, in another embodiment, by monitoring an independent reaction which occurs intracellularly and which is directly influenced in a calcium (or other) ion dependent manner.

[0189] Any method for assessing the activity of a calcium channel may be used in conjunction with the methods described herein. For example, in one embodiment of the method for testing a compound for its ability to modulate calcium channel activity, the amount of current is measured by its modulation of a reaction which is sensitive to calcium channel selective ions and uses a eukaryotic cell which expresses a heterologous calcium channel and also contains a transcriptional control element operatively linked for expression to a structural gene that encodes an indicator protein. The transcriptional control element used for transcription of the indicator gene is responsive in the cell to a calcium channel selective on, such as Ca^{2+} and Ba^{+} . The details of such transcriptional based assays are described, for example, in PCT International Patent Application No. PCT/US91/5625.

[0190] In other embodiments, electrophysiological methods for measuring calcium channel activity, which are known to those of skill in the art and exemplified herein may be utilized for the indicated purposes. Any such methods may be used in order to detect the formation of functional calcium channels and to characterize the kinetics and other characteristics of the resulting currents. Pharmacological studies may be combined with the electrophysiological measurements, in other embodiments, in order to further characterize the calcium channels.

[0191] In general, activity of a given test compound in the nervous system can be assayed by detecting the compound's ability to affect one of more of: promote neurite outgrowth, protect neurons from damage by chemical treatments, promote the growth of neurons or neuronal cells, recover lost or damaged motor, functional or cognitive ability associated with nervous tissue or organs of the nervous system, or regenerate neurons. For example, isolated neuronal cell cultures (e.g., dopaminergic, cortical, DRG cell cultures) can be isolated and cultured by methods known in the art (see e.g., Pong et al. (1997) *J. Neurochem.* 69:986-994; Pong et al. (2001) *Exp Neurol.* 171(1):84-97). Changes in neuronal activity, differentiation, survival can be detected and quantified using art recognized techniques as described in, e.g., US 2005/0197356 (describing examples showing measuring changes in 3H-dopamine uptake and neurofilament content in cultured dopaminergic neurons and cortical neurons, respectively). Alternatively, neuronal activities can be characterized in cultured neural cell lines, e.g., neuroblastoma cell lines, pheochromocytoma cells (PC12 cells), F11. Activities in vitro can be useful in identifying agents that can be used to treat and/or ameliorate a number of human neurodegenerative conditions, including but not limited to, Parkinson's disease; Alzheimer's disease; amyotrophic lateral sclerosis (ALS); traumatic injury; spinal cord injury; multiple sclerosis; diabetic neuropathy; neuropathy associated with medical treatments such as chemotherapy; ischemia or ischemia-induced injury; stroke, among others.

[0192] Methods for detecting neuronal activity include, for example, neuroprotective assays where a compound is tested for its ability to protect against glutamate neurotoxicity. Sensory neuronal cultures (DRG) can also be assayed for neurite outgrowth, and assayed for neurotrophic activity. Cultured cells are treated with an immunophilin ligand and later assayed for the presence of new neurite fibers. Immunohistochemistry can aid in the visualization and quantitation of neurites as compared to control.

[0193] A number of animal models and cell culture assays have been developed and can be relied on for their clinical relevance to disease treatments, including the human diseases noted above. Each of the following references can be used as a source for these assays, and all of them are specifically incorporated herein by reference in their entirety for that purpose: Steiner, et al., *Proc. Natl. Acad. Sci. U.S.A.* 94: 2019-2024 (1997); Hamilton, et al., *Bioorgan. Med. Chem. Lett.* 7:1785-1790 (1997); McMahon, et al., *Curr. Opin. Neurobiol.* 5:616-624 (1995); Gash, et al., *Nature* 380:252-255 (1996); Gerlach, et al., *Eur. J. Pharmacol.-Mol. Pharmacol.* 208:273-286 (1991); Apfel, et al., *Brain Res.* 634:7-12 (1994); Wang, et al., *J. Pharmacol. Exp. Therap.* 282:1084-1093 (1997); Gold, et al., *Exp. Neurol.* 147:269-278 (1997);

Hoffer et al., *J. Neural Transm. /Suppl.* 49:1-10 (1997); and Lyons, et al., *PNAS* 91:3191-3195 (1994).

Therapeutic and Prophylactic Uses

[0194] In yet another aspect, the invention provides methods for modulating a function (e.g., calcium channel activity (e.g., voltage-gated calcium channel activity), in a cell (e.g., a mammalian cell) that expresses an immunophilin, e.g., an FKBP52 or a functional variant thereof and a subunit of the voltage gated L-type calcium channel, e.g., a β 1 subunit, or a functional variant thereof. In one embodiment, the calcium channel or FKBP52 activity or expression is inhibited. In those embodiments where calcium channel activity is inhibited, neurite outgrowth and/or survival is preferably stimulated. Typically, the cell used in the methods of the invention is a mammalian cell, e.g., a human cell (e.g., a neuronal or a cardiovascular cell). In some embodiments, the methods include contacting the cell with an immunophilin ligand, e.g., a rapamycin or a meridamycin analogue as described herein, under conditions that allow the formation of a complex described herein to occur, thereby inhibiting the calcium channel activity.

[0195] In related embodiments, the methods include contact the cell (e.g., a dopaminergic, cholinergic, cortical, and spinal cord neuronal cell) with an antagonist of a calcium channel β subunit, e.g., a β 1 subunit of the voltage gated L-type calcium channel. The antagonist can also be an inhibitor of activity and/or expression of the calcium channel β subunit. The term "antagonist" as used herein refers to an agent which reduces, inhibits or otherwise diminishes one or more biological activities of a calcium channel β subunit (e.g., β 1 subunit). Antagonism does not necessarily indicate a total elimination of the calcium channel β subunit biological activity. In one embodiment, the antagonist is an immunophilin ligand, e.g., a rapamycin or meridamycin analogue as described herein. Typically, the immunophilin ligand is administered in an amount sufficient to form and/or stabilize a complex that includes the ligand, an immunophilin or a functional variant thereof, and a calcium channel subunit or a functional variant thereof. In other embodiment, the antagonist is an inhibitor of transcription of the calcium channel β subunit, e.g., a nucleic acid inhibitor (e.g., RNAi) as described in more detail herein.

[0196] The methods of the invention can be performed in cells in cultured medium. Alternatively, the method can be performed on cells (e.g., neuronal or cardiovascular cells) present in a subject, e.g., as part of an in vivo (e.g., therapeutic or prophylactic) protocol, or in an animal subject (e.g., an in vivo animal model).

[0197] Accordingly, methods of treating or preventing, in a subject, a disorder associated with calcium channel dysfunction, are encompassed by the present invention. The method includes administering to a subject an immunophilin ligand, e.g., a rapamycin or meridamycin analogue, in an amount sufficient to form and/or stabilize a complex that includes the ligand, an immunophilin or a functional variant thereof, and a calcium channel subunit or a functional variant thereof, thereby treating or preventing the disorder. The method can, optionally, include the step(s) of identifying (e.g., evaluating, diagnosing, screening, and/or selecting) a subject at risk of having, or having, one or more symptoms associated with a disorder involving calcium channel dysfunction. The subject can be a mammal, e.g., a human suffering from, e.g., a neurodegenerative or a cardiovascular disorder. For example, the

subject is a human (e.g., a human patient) suffering from a disorder chosen from one or more of stroke, Parkinson's disease, migraine, cerebellar ataxia, angina, epilepsy, hypertension, ischemia, or cardiac arrhythmias.

[0198] As used herein, the term "subject" is intended to include human and non-human animals. Preferred human animals include a human patient having a disorder characterized by abnormal calcium channel activity. The term "non-human animals" includes vertebrates, e.g., mammals and non-mammals, such as non-human primates, rodents, sheep, dog, cow, chickens, amphibians, reptiles, etc. The subject can be, for example, a mammal, e.g., a human suffering from, e.g., a neurodegenerative or a cardiovascular disorder.

[0199] The phrase "therapeutically effective amount" of an immunophilin ligand refers to an amount of an agent which is effective, upon single or multiple dose administration to a subject, e.g., a human patient, at treating the subject. The term "treating" or "treatment" includes curing, reducing the severity of, ameliorating one or more symptoms of a disorder, or in prolonging the survival of the subject beyond that expected in the absence of such treatment. Similarly, the phrase "a prophylactically effective amount" of an immunophilin ligand refers to an amount of an agent which is effective, upon single- or multiple-dose administration to a subject, e.g., a human patient, in preventing or delaying the occurrence of the onset or recurrence of a disorder, e.g., a disorder as described herein.

[0200] The immunophilin ligand, e.g., the rapamycin analogue, can be administered alone, or in combination with one or more agents, e.g., therapeutic agents. The term "in combination" in this context means that the agents are given substantially contemporaneously, either simultaneously or sequentially. If given sequentially, at the onset of administration of the second compound, the first of the two compounds is preferably still detectable at effective concentrations at the site of treatment. In one embodiment, the second agent is a calcium channel antagonist, e.g., an antagonists of an L-type calcium channel. Examples of antagonists of L-type calcium channels include dihydropyridines; phenylalkylamines (e.g., verapamil, gallpamil, and thiapamil); benzothiazepines; diphenylbutylpiperidine class of antischizophrenic neuroleptic drugs (e.g., pimozide, fluspiridine, penfluridol and clopimozide); as well as nifedipine, carbamazepine, diltiazem, nicardipine, nimodipine, and nitredipine.

[0201] Exemplary disorders associated with calcium channel dysfunction include stroke; Parkinson's disease; migraine (e.g., congenital migraine); cerebellar ataxia; angina; epilepsy; hypertension; ischemia (e.g., cardiac ischemia); cardiac arrhythmias; stroke; head trauma or spinal injury, or other injuries to the brain, peripheral nervous, central nervous, or neuromuscular system; chronic, neuropathic and acute pain; mood disorders; schizophrenia; depression; anxiety; psychoses; drug addiction; alcohol dependence and urinary incontinence.

[0202] Examples of other conditions associated with dysfunction of calcium (Ca^{2+}) ion channels, include, but not limited to, malignant hyperthermia, central core disease, cathecolaminergic polymorphic ventricular tachycardia, and arrhythmogenic right ventricular dysplasia type 2 (ARVD-2). Examples of neurological disorders that can be treated using the methods of the invention include Alzheimer's disease; Huntington's disease; spinal cord injury; traumatic brain injury; Lewy body dementia; Pick's disease; Niewmann-Pick disease; amyloid angiopathy; cerebral amyloid angiopathy;

systemic amyloidosis; hereditary cerebral hemorrhage with amyloidosis of the Dutch type; inclusion body myositis; mild cognitive impairment; Down's syndrome; and neuromuscular disorders, including amyotrophic lateral sclerosis (ALS), multiple sclerosis, and muscular dystrophies including Duchenne dystrophy, Becker muscular dystrophy, Facioscapulohumeral (Landouzy-Dejerine) muscular dystrophy, and limb-girdle muscular dystrophy (LGMD). The immunophilin ligands are also useful as neuroprotective and/or neuroregenerative agents, e.g., in restoring some neurological and/or neuromuscular or other function following onset of one of the above conditions and/or injury, stroke, or other trauma.

[0203] Examples of additional cardiovascular disorders that can be treated include, but not limited to, congestive heart failure; arrhythmogenic syndromes, including paroxysmal tachycardia, delayed after depolarizations, ventricular tachycardia, sudden tachycardia, exercise-induced arrhythmias, long QT syndromes, and bidirectional tachycardia; thromboembolic disorders, including arterial cardiovascular thromboembolic disorders, venous cardiovascular thromboembolic disorders, and thromboembolic disorders in the chambers of the heart; atherosclerosis; restenosis; peripheral arterial disease; coronary bypass grafting surgery; carotid artery disease; arteritis; myocarditis; cardiovascular inflammation; vascular inflammation; coronary heart disease (CHD); unstable angina (UA); unstable refractory angina; stable angina (SA); chronic stable angina; acute coronary syndrome (ACS); first or recurrent myocardial infarction; acute myocardial infarction (AMI); myocardial infarction; non-Q wave myocardial infarction; non-STE myocardial infarction; coronary artery disease; ischemic heart disease; ischemic sudden death; transient ischemic attack; stroke; peripheral occlusive arterial disease; venous thrombosis; deep vein thrombosis; thrombophlebitis; arterial embolism; coronary arterial thrombosis; cerebral arterial thrombosis; cerebral embolism; kidney embolism; pulmonary embolism; thrombosis resulting from (a) prosthetic valves or other implants, (b) indwelling catheters, (c) stents, (d) cardiopulmonary bypass, (e) hemodialysis, or (f) other procedures in which blood is exposed to an artificial surface that promotes thrombosis; thrombosis resulting from atherosclerosis, surgery or surgical complications, prolonged immobilization, arterial fibrillation, congenital thrombophilia, cancer, diabetes, effects of medications or hormones, and complications of pregnancy; cardiac arrhythmias including supraventricular arrhythmias, atrial arrhythmias, atrial flutter, atrial fibrillation; other diseases listed in *Heart Disease: A Textbook of Cardiovascular Medicine*, 2 Volume Set, 6th Edition, 2001, Eugene Braunwald, Douglas P. Zipes, Peter Libby, Douglas D. Zipes; and in the preparation of medicaments therefor.

[0204] In a further embodiment, the cardiovascular disease is chosen from one or more of: atherosclerosis; coronary heart disease (CHD); restenosis; peripheral arterial disease; coronary bypass grafting surgery; carotid artery disease; arteritis; myocarditis; cardiovascular inflammation; vascular inflammation; unstable angina (UA); unstable refractory angina; stable angina (SA); chronic stable angina; acute coronary syndrome (ACS); myocardial infarction; or acute myocardial infarction (AMI), including first or recurrent myocardial infarction, non-Q wave myocardial infarction, non-ST-segment elevation myocardial infarction and ST-segment elevation myocardial infarction.

[0205] The amount or dosage requirements of the immunophilin ligands can vary depending on the condition, sever-

ity of the symptoms presented and the particular subject being treated. One of skill in the art would readily be able to determine the amount of the immunophilin ligand required following the methods described herein. Preferably, the dosage of the immunophilin ligand is such that it is sufficient to form and/or stabilize a complex that includes the ligand, an immunophilin or a functional variant thereof, and a calcium channel subunit or a functional variant thereof. In some embodiments, the dosage can be tested in vitro following the teachings of the invention. In one embodiment, about 0.5 to 200 mg, about 0.5 to 100 mg, about 0.5 to about 75 mg is administered. In yet a further embodiment, about 1 to about 25 mg is administered. In another embodiment, about 0.5 to about 10 mg is administered, particularly when used in combination with another agent. In yet a further embodiment, about 2 to about 5 mg is administered. In yet another embodiment, about 5 to about 15 mg is administered.

[0206] Treatment can be initiated with dosages of the immunophilin ligand lower than those required to produce a desired effect and generally less than the optimum dose of the ligand. Thereafter, the dosage can be increased until the optimum effect under the circumstances is reached. Precise dosages will be determined by the administering physician based on experience with the individual subject being treated. In general, the compositions are most desirably administered at a concentration that will generally afford effective results without causing any harmful or deleterious side effects.

[0207] In certain embodiments, nucleic acid antagonists are used to decrease expression of an endogenous gene encoding the calcium channel β subunit (e.g., the $\beta 1$ subunit). In one embodiment, the nucleic acid antagonist is an siRNA that targets mRNA encoding the calcium channel β subunit. Other types of antagonistic nucleic acids can also be used, e.g., a dsRNA, a ribozyme, a triple-helix former, or an antisense nucleic acid. In some embodiments, nucleic acid antagonists can be directed to downstream effector targets of the calcium channel β subunit.

[0208] siRNAs are small double stranded RNAs (dsRNAs) that optionally include overhangs. For example, the duplex region of an siRNA is about 18 to 25 nucleotides in length, e.g., about 19, 20, 21, 22, 23, or 24 nucleotides in length. Typically, the siRNA sequences are exactly complementary to the target mRNA. dsRNAs and siRNAs in particular can be used to silence gene expression in mammalian cells (e.g., human cells). siRNAs also include short hairpin RNAs (shRNAs) with 29-base-pair stems and 2-nucleotide 3' overhangs. See, e.g., Clemens et al. (2000) *Proc. Natl. Acad. Sci. USA* 97:6499-6503; Billy et al. (2001) *Proc. Natl. Sci. USA* 98:14428-14433; Elbashir et al. (2001) *Nature* 411:494-8; Yang et al. (2002) *Proc. Natl. Acad. Sci. USA* 99:9942-9947; Siolas et al. (2005), *Nat. Biotechnol.* 23(2):227-31; 20040086884; U.S. 20030166282; 20030143204; 20040038278; and 20030224432.

[0209] Anti-sense agents can include, for example, from about 8 to about 80 nucleobases (i.e. from about 8 to about 80 nucleotides), e.g., about 8 to about 50 nucleobases, or about 12 to about 30 nucleobases. Anti-sense compounds include ribozymes, external guide sequence (EGS) oligonucleotides (oligozymes), and other short catalytic RNAs or catalytic oligonucleotides which hybridize to the target nucleic acid and modulate its expression. Anti-sense compounds can include a stretch of at least eight consecutive nucleobases that are complementary to a sequence in the target gene. An oligonucleotide need not be 100% complementary to its target

nucleic acid sequence to be specifically hybridizable. An oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target interferes with the normal function of the target molecule to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of in vivo assays or therapeutic treatment or, in the case of in vitro assays, under conditions in which the assays are conducted.

[0210] Hybridization of antisense oligonucleotides with mRNA (e.g., an mRNA encoding the calcium channel β subunit) can interfere with one or more of the normal functions of mRNA. The functions of mRNA to be interfered with include all key functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in by the RNA. Binding of specific protein(s) to the RNA may also be interfered with by antisense oligonucleotide hybridization to the RNA.

[0211] Exemplary antisense compounds include DNA or RNA sequences that specifically hybridize to the target nucleic acid, e.g., the mRNA encoding the calcium channel β subunit. The complementary region can extend for between about 8 to about 80 nucleobases. The compounds can include one or more modified nucleobases. Modified nucleobases may include, e.g., 5-substituted pyrimidines such as 5-iodouracil, 5-iodocytosine, and C5-propynyl pyrimidines such as C5-propynylcytosine and C5-propynyluracil. Other suitable modified nucleobases include N⁴-(C₁-C₁₂) alkylaminocytosines and N⁴, N⁴-(C₁-C₁₂) dialkylaminocytosines. Modified nucleobases may also include 7-substituted-8-aza-7-deazapurines and 7-substituted-7-deazapurines such as, for example, 7-ido-7-deazapurines, 7-cyano-7-deazapurines, 7-aminocarbonyl-7-deazapurines. Examples of these include 6-amino-7-ido-7-deazapurines, 6-amino-7-cyano-7-deazapurines, 6-amino-7-aminocarbonyl-7-deazapurines, 2-amino-6-hydroxy-7-ido-7-deazapurines, 2-amino-6-hydroxy-7-cyano-7-deazapurines, and 2-amino-6-hydroxy-7-aminocarbonyl-7-deazapurines. Furthermore, N⁶-(C₁-C₁₂) alkylaminopurines and N⁶, N⁶-(C₁-C₁₂) dialkylaminopurines, including N⁶-methylaminoadenine and N⁶, N⁶-dimethylaminoadenine, are also suitable modified nucleobases. Similarly, other 6-substituted purines including, for example, 6-thioguanine may constitute appropriate modified nucleobases. Other suitable nucleobases include 2-thiouracil, 8-bromoadenine, 8-bromoguanine, 2-fluoroadenine, and 2-fluoroguanine. Derivatives of any of the aforementioned modified nucleobases are also appropriate. Substituents of any of the preceding compounds may include C₁-C₃₀ alkyl, C₂-C₃₀ alketyl, C₂-C₃₀ alkynyl, aryl, aralkyl, heteroaryl, halo, amino, amido, nitro, thio, sulfonyl, carboxyl, alkoxy, alkylcarbonyl, alkoxy carbonyl, and the like.

[0212] Descriptions of other types of nucleic acid agents are also available. See, e.g., U.S. Pat. Nos. 4,987,071; 5,116,742; and 5,093,246; Woolf et al. (1992) *Proc Natl Acad Sci USA; Antisense RNA and DNA*, D. A. Melton, Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1988); 89:7305-9; Haselhoff and Gerlach (1988) *Nature* 334:585-59; Helene, C. (1991) *Anticancer Drug Des.* 6:569-84; Helene (1992) *Ann. N.Y. Acad. Sci.* 660:27-36; and Maher (1992) *Bioassays* 14:807-15.

Pharmaceutical Compositions

[0213] In one aspect, the present invention includes methods of preparing a pharmaceutical composition containing

one or more immunophilin ligands. In other embodiments, pharmaceutical compositions containing the complexes described herein are disclosed. As used herein, compositions containing "an immunophilin ligand" or "the immunophilin ligand" are intended to encompass compositions containing one or more immunophilin ligands. The composition can be administered to a mammalian subject by several different routes and is desirably administered orally in solid or liquid form.

[0214] Solid forms, including tablets, capsules, and caplets, containing the immunophilin ligand can be formed by blending the immunophilin ligand with one or more of the components described above. In one embodiment, the components of the composition are dry or wet blended. In another embodiment, the components are dry granulated. In a further embodiment, the components are suspended or dissolved in a liquid and added to a form suitable for administration to a mammalian subject.

[0215] Liquid forms containing the immunophilin ligand can be formed by dissolving or suspending the immunophilin ligand in a liquid suitable for administration to a mammalian subject.

[0216] The compositions described herein containing the immunophilin ligand can be formulated in any form suitable for the desired route of delivery using a pharmaceutically effective amount of the immunophilin ligand. For example, the compositions of the invention can be delivered by a route such as oral, dermal, transdermal, intrabronchial, intranasal, intravenous, intramuscular, subcutaneous, parenteral, intraperitoneal, intranasal, vaginal, rectal, sublingual, intracranial, epidural, intratracheal, or by sustained release. Preferably, delivery is oral.

[0217] The oral dosage tablet composition of this invention can also be used to make oral dosage tablets containing derivatives of the immunophilin ligand, including, but not limited to, esters, carbamates, sulfates, ethers, oximes, carbonates, and the like which are known to those of skill in the art.

[0218] A pharmaceutically effective amount of the immunophilin ligand can vary depending on the specific compound (s), mode of delivery, severity of the condition being treated, and any other active ingredients used in the composition. The dosing regimen can also be adjusted to provide the optimal therapeutic response. Several divided doses can be delivered daily, e.g., in divided doses 2 to 4 times a day, or a single dose can be delivered. The dose can however be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. In one embodiment, the delivery is on a daily, weekly, or monthly basis. In another embodiment, the delivery is on a daily delivery. However, daily dosages can be lowered or raised based on the periodic delivery.

[0219] The immunophilin ligands can be combined with one or more pharmaceutically acceptable carriers or excipients including, without limitation, solid and liquid carriers which are compatible with the compositions of the present invention. Such carriers include adjuvants, syrups, elixirs, diluents, binders, lubricants, surfactants, granulating agents, disintegrating agents, emollients, metal chelators, pH adjustors, surfactants, fillers, disintegrants, and combinations thereof, among others. In one embodiment, the immunophilin ligand is combined with metal chelators, pH adjustors, surfactants, fillers, disintegrants, lubricants, and binders. Adjuvants can include, without limitation, flavoring agents, coloring agents, preservatives, and supplemental antioxidants,

which can include vitamin E, ascorbic acid, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA).

[0220] Binders can include, without limitation, cellulose, methylcellulose, hydroxymethylcellulose, carboxymethylcellulose calcium, carboxymethylcellulose sodium, hydroxypropylcellulose, hydroxypropylmethylcellulose phthalate, microcrystalline cellulose, noncrystalline cellulose, polypropylpyrrolidone, polyvinylpyrrolidone (povidone, PVP), gelatin, gum arabic and acacia, polyethylene glycols, starch, sugars such as sucrose, kaolin, dextrose, and lactose, cholesterol, tragacanth, stearic acid, gelatin, casein, lecithin (phosphatides), cetostearyl alcohol, cetyl alcohol, cetyl esters wax, dextrates, dextrin, glyceryl monooleate, glyceryl monostearate, glyceryl palmitostearate, polyoxyethylene alkyl ethers, polyoxyethylene castor oil derivatives, polyoxyethylene stearates, polyvinyl alcohol, and gelatin, among others. In one embodiment, the binder is povidone, hydroxypropylmethylcellulose, carboxymethylcellulose, or gelatin. In another embodiment, the binder is povidone.

[0221] Lubricants can include magnesium stearate, light anhydrous silicic acid, talc, stearic acid, sodium lauryl sulfate, and sodium stearyl furamate, among others. In one embodiment, the lubricant is magnesium stearate, stearic acid, or sodium stearyl furamate. In another embodiment, the lubricant is magnesium stearate.

[0222] Granulating agents can include, without limitation, silicon dioxide, microcrystalline cellulose, starch, calcium carbonate, pectin, crospovidone, and polyplasdone, among others.

[0223] Disintegrating agents or disintegrants can include croscarmellose sodium, starch, carboxymethylcellulose, substituted hydroxypropylcellulose, sodium bicarbonate, calcium phosphate, calcium citrate, sodium starch glycolate, pregelatinized starch or crospovidone, among others. In one embodiment, the disintegrand is croscarmellose sodium.

[0224] Emollients can include, without limitation, stearyl alcohol, mink oil, cetyl alcohol, oleyl alcohol, isopropyl laurate, polyethylene glycol, olive oil, petroleum jelly, palmitic acid, oleic acid, and myristyl myristate.

[0225] Surfactants can include polysorbates, sorbitan esters, poloxamer, or sodium lauryl sulfate. In one embodiment, the surfactant is sodium lauryl sulfate.

[0226] Metal chelators can include physiologically acceptable chelating agents including edetic acid, malic acid, or fumaric acid. In one embodiment, the metal chelator is edetic acid.

[0227] pH adjusters can also be utilized to adjust the pH of a solution containing the immunophilin ligand to about 4 to about 6. In one embodiment, the pH of a solution containing the immunophilin ligand is adjusted to a pH of about 4.6. pH adjustors can include physiologically acceptable agents including citric acid, ascorbic acid, fumaric acid, or malic acid, and salts thereof. In one embodiment, the pH adjuster is citric acid.

[0228] Fillers that can be used according to the present invention include anhydrous lactose, microcrystalline cellulose, mannitol, calcium phosphate, pregelatinized starch, or sucrose. In one embodiment, the filler is anhydrous lactose. In another embodiment, the filler is microcrystalline cellulose.

[0229] In one embodiment, compositions containing the immunophilin ligand are delivered orally by tablet, caplet or capsule, microcapsules, dispersible powder, granule, suspension, syrup, elixir, and aerosol. Desirably, when compositions

containing the immunophilin ligand are delivered orally, delivery is by tablets and hard- or liquid-filled capsules. In another embodiment, the compositions containing the immunophilin ligand can be delivered intravenously, intramuscularly, subcutaneously, parenterally and intraperitoneally in the form of sterile injectable solutions, suspensions, dispersions, and powders which are fluid to the extent that easy syringe ability exists. Such injectable compositions are sterile and stable under conditions of manufacture and storage, and free of the contaminating action of microorganisms such as bacteria and fungi. In a further embodiment, compositions containing the immunophilin ligand can be delivered rectally in the form of a conventional suppository. In another embodiment, compositions containing the immunophilin ligand can be delivered vaginally in the form of a conventional suppository, cream, gel, ring, or coated intrauterine device (IUD).

[0230] In another embodiment, compositions containing the immunophilin ligand can be delivered via coating or impregnating of a supporting structure, i.e., a framework capable of containing of supporting pharmaceutically acceptable carrier or excipient containing a compound of the invention, e.g., vascular stents or shunts, coronary stents, peripheral stents, catheters, arterio-venous grafts, by-pass grafts, and drug delivery balloons for use in the vasculature. In one embodiment, coatings suitable for use include, but are not limited to, polymeric coatings composed of any polymeric material in which the compound of the invention is substantially soluble. Supporting structures and coating or impregnating methods, e.g., those described in U.S. Pat. No. 6,890,546, are known to those of skill in the art and are not a limitation of the present invention.

[0231] In yet another embodiment, compositions containing the immunophilin ligand can be delivered intranasally or intrabronchially in the form of an aerosol.

[0232] Solutions or suspensions of these active compounds as a free base or pharmacologically acceptable salt are prepared in water suitably mixed with a surfactant such as hydroxypropylcellulose. Dispersions are also prepared in glycerol, liquid, polyethylene glycols and mixtures thereof in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

[0233] The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form is sterile and fluid to the extent that easy syringe ability exists. It is stable under conditions of manufacture and storage and is preserved against the contaminating action of microorganisms such as bacterial and fungi. The carrier is a solvent or dispersion medium containing, for example, water, ethanol (e.g., glycerol, propylene glycol and liquid polyethylene glycol), suitable mixtures thereof, and vegetable oil.

[0234] The present invention also provides kits or packages containing the immunophilin ligands. Kits of the present invention can include the ligand and a carrier suitable for administration to a mammalian subject as discussed above. The kits can also contain the reagents required to prepare the immunophilin ligands. Also within the scope of the invention are kits comprising the complexes, components thereof, and/or reagents and instructions for use.

[0235] The following examples are provided to illustrate the invention and do not limit the scope thereof. One skilled in the art will appreciate that although specific reagents and

conditions are outlined in the following examples, modifications can be made which are meant to be encompassed by the spirit and scope of the invention.

Example 1

Synthesis of Rapamycin Analogues I and II

[0236] The complexes of FK506 and rapamycin with their respective protein targets result in immunosuppressive activity that may be undesirable in the context of a therapy for chronic neurodegeneration (Lam et al, *J. Biol. Chem.* 270, 26511-22 (1995)). Therefore, to develop non-immunosuppressive immunophilin ligands, rapamycin analogues I and II were prepared from rapamycin via a [4+2] cycloaddition reaction with nitrosobenzene at the C1,C3 diene in order to disrupt the interaction with mTOR while leaving the FKBP binding portion intact (FIG. 1A) as described in more detail below.

Synthesis of Rapamycin Analogue I

[0237] Chemicals were purchased from Sigma-Aldrich (St. Louis, Mo.).

[0238] Rapamycin (0.3 g, 0.328 mmol) was dissolved in 5 mL toluene with gentle heating. To this solution was added, dropwise, a solution of nitrosobenzene (0.1 g, 3 eq) in 5 mL toluene. The reaction mixture was stirred at 70° C. for 16 hours, and then the products were chromatographed via reversed-phase high performance liquid chromatography (HPLC) (column: 250×20 mm YMC ODS-A with 50×20 guard, mobile phase: 80 to 85% methanol:water in 40 minutes, flow=20 mL/min) to yield 0.139 g of the product (42% yield, $t_R=12.1$ min, analytical HPLC conditions: column=YMC ODS-A S-3 120 Å, mobile phase/gradient: 95% water (+0.025% formic acid)/acetonitrile (+0.025% formic acid) to 5% water in 6 minutes, hold at 5% for 9 minutes, flow=0.30 mL/min). ¹H-NMR (500 MHz, CD₃CN): δ 87.29 (m, 2H, H57), 7.02 (m, 2H, H56), 6.90 (m, 1H, H58), 6.25 (m, 1H, H2), 5.65 (m, 1H, H43), 5.25 (m, 1H, H29), 5.16 (m, 1H, H5), 5.12 (m, 1H, H25), 5.07 (m, 1H, H4), 4.37 (m, 1H, H22), 4.09 (m, 1H, H31), 3.95 (m, 1H, H32), 3.74 (m, 1H, H9), 3.69 (m, 1H, H1), 3.59 (m, 1H, 31-OH), 3.44 (m, 1H, H28), 3.44 (m, 1H, H28), 3.33 (s, 3H, Me54), 3.29 (m, 3H, Me53), 3.27 (m, H4, H42), 3.07 (m, 1H, H34), 3.06 (s, 3H, Me52), 2.94 (m, 1H, H18), 2.86 (m, 1H, H41), 2.84 (m, 1H, H26), 2.64 (m, 1H, H26'), 2.14 (m, H4, H21), 2.09 (m, 1H, H12), 2.05 (m, 1H, H40), 2.01 (m, 1H, H36), 2.00 (m, 1H, H35), 1.87 (m, 1H, H37), 1.85 (m, 1H, H43), 1.81 (m, 1H, H21'), 1.78 (s, 3H, Me48), 1.74 (m, 1H, H19'), 1.74 (m, 1H, H20), 1.69 (m, 1H, H8), 1.64 (m, 1H, H44), 1.63 (m, 1H, H8'), 1.60 (m, 1H, H11), 1.55 (m, 1H, H44'), 1.51 (s, 3H, Me45), 1.43 (m, 2H, H10), 1.42 (m, 1H, H19'), 1.39 (m, 1H, H20'), 1.37 (m, 1H, H39), 1.27 (m, 1H, H38), 1.12 (d, 3H, Me50), 1.06 (d, 3H, Me47), 1.04 (m, 1H, H38'), 1.03 (d, 3H, Me49), 0.89 (d, 3H, Me51), 0.83 (d, 3H, Me46), 0.63 (m, 1H, H40'); ¹³C-NMR (125 MHz, CD₃CN): δ 215.4 (s, C33), 209.5 (s, C27), 198.5 (s, C15), 170.6 (s, C23), 166.4 (s, C16), 149.2 (s, C55), 139.9 (s, C6), 138.7 (s, C30), 130.0 (d, C57), 128.0 (d, C3), 127.9 (d, C29), 127.2 (d, C5), 127.0 (d, C2), 121.5 (d, C58), 116.1 (d, C56), 99.5 (s, C13), 87.3 (d, C32), 85.2 (d, C41), 84.8 (d, C7), 78.2 (d, C31), 77.0 (d, C25), 74.5 (d, C42), 68.4 (d, C4), 68.3 (d, C9), 60.3 (d, C1), 58.6 (q, C53), 57.4 (d, C22), 56.9 (q, C54), 56.1 (q, C52), 46.9 (d, C28), 42.8 (d, C34), 41.7 (t, C26), 39.5 (t, C18), 39.5 (t, C8), 38.6 (t, C35), 38.5 (t, C38), 37.5 (d, C36), 35.6 (d, C12), 35.3 (t, C40), 33.9 (d, C37), 33.8

(d, C39), 32.9 (t, C43), 32.2 (t, C10), 32.2 (t, C44), 28.2 (t, C21), 27.7 (t, C11), 25.1 (t, C19), 21.6 (t, C20), 18.5 (q, C50), 18.0 (q, C49), 16.7 (q, C51), 16.3 (q, C46), 16.0 (q, C47), 12.4 (q, C48), 10.8 (q, C45); FT-ICRMS (m/z): [M+H]⁺ calc for C₅₇H₈₈N₂O₁₄, 1021.59954; found, 1021.59780.

[0239] Synthesis of Rapamycin Analogue II

[0240] Rapamycin analogue I (0.29 g, 0.284 mmol) was dissolved in 7 mL methanol in an 18 mm test-tube, and a spatula tip of Pd/C catalyst (Aldrich) was added. The mixture was hydrogenated on a Parr apparatus for 15 minutes at 2.0 atmosphere H₂. The products were chromatographed via reversed-phase HPLC (column 250×20 mm YMC ODS-A with 50×20 guard, mobile phase: 80% methanol:water for 15 minutes, then to 85% in 5 minutes, then held at 85% for 20 minutes, flow=20 mL/min) to yield 0.089 g of the product (31% yield, t_R=12.6 min, analytical HPLC conditions: column=YMC ODS-A S-3 120 Å, mobile phase/gradient: 95% water (+0.025% formic acid)/acetonitrile (+0.025% formic acid) to 5% water in 6 minutes, hold at 5% for 9 minutes, flow=0.30 mL/min). ¹H-NMR (500 MHz, CD₃CN): 87.25 (m, 2H, H57), 6.91 (m, 2H, H56), 6.79 (m, 1H, H58), 5.44 (m, 1H, H29), 5.35 (m, 1H, H5), 5.24 (m, 1H, H25), 5.11 (m, 1H, H22), 4.50 (m, 1H, H4), 4.42 (m, 1H, 13-OH), 4.00 (m, 1H, H31), 3.80 (m, 1H, H9), 3.77 (m, 1H, H32), 3.67 (m, 1H, H7), 3.57 (m, 1H, 31-OH), 3.43 (m, 1H, H28), 3.35 (m, 1H, H18), 3.35 (s, 3H, Me54), 3.34 (m, 1H, H1), 3.32 (m, 1H, H18'), 3.32 (s, 3H, Me53), 3.27 (m, 1H, H42), 3.16 (m, 1H, H34), 3.08 (s, 3H, Me52), 3.00 (m, 1H, 42-OH), 2.87 (m, 1H, H41), 2.79 (m, 1H, H26), 2.71 (m, 1H, H26'), 2.29 (m, 1H, H21), 2.18 (m, 1H, H36), 2.10 (m, 1H, H40), 1.95 (m, 1H, H35), 1.95 (m, 1H, H37), 1.86 (m, 1H, H43), 1.85 (m, 1H, H2), 1.85 (m, 1H, H3), 1.82 (m, 1H, H12), 1.79 (m, 1H, H2'), 1.77 (m, 1H, H₂O), 1.71 (m, 1H, H8), 1.69 (m, 1H, H19), 1.68 (m, 1H, H21'), 1.66 (s, 3H, Me48), 1.64 (m, 1H, H44), 1.63 (m, 1H, H8'), 1.61 (m, 1H, H10), 1.60 (m, 2H, H11), 1.50 (m, 1H, Me45), 1.46 (m, 1H, H3'), 1.43 (m, 1H, H19'), 1.39 (m, 1H, H20), 1.39 (m, 1H, H39), 1.35 (m, 1H, H10'), 1.29 (m, 1H, H38), 1.26 (m, 1H, H43'), 1.13 (d, 3H, Me47), 1.12 (m, 1H, H38'), 1.07 (d, 3H, Me49), 1.03 (m, 1H, H35'), 1.03 (d, 3H, Me46), 1.00 (m, 1H, H44'), 0.97 (d, 3H, Me50), 0.91 (d, 3H, Me51), 0.66 (m, 1H, H40'); ¹³C-NMR (125 MHz, CD₃CN): δ216.1 (s, C33), 210.3 (s, C27), 198.3 (s, C15), 170.3 (s, C23), 168.3 (s, C16), 149.9 (s, C55), 139.9 (s, C30), 139.4 (s, C6), 130.2 (d, C57), 129.4 (d, C5), 128.1 (d, C29), 119.7 (d, C58), 114.2 (d, C56), 98.4 (s, C13), 88.5 (d, C32), 85.4 (d, C41), 85.0 (d, C7), 77.7 (d, C31), 76.3 (d, C25), 74.8 (d, C42), 72.3 (d, C4), 68.5 (d, C9), 60.0 (d, C1), 59.2 (q, C53), 57.1 (q, C54), 56.0 (q, C52), 52.0 (d, C22), 46.5 (d, C28), 45.1 (t, C18), 42.7 (d, C34), 42.1 (t, C26), 40.8 (t, C35), 39.1 (t, C38), 38.3 (t, C8), 35.7 (t, C40), 35.0 (d, C12), 34.3 (d, C37), 34.1 (d, C39), 33.1 (t, C43), 32.5 (t, C44), 32.1 (t, C10), 32.0 (d, C36), 29.1 (t, C11), 28.0 (t, C21), 26.8 (t, C3), 25.9 (t, C19), 21.7 (t, C20), 20.6 (t, C2), 19.0 (q, C49), 17.5 (q, C47), 17.4 (q, C50), 16.8 (q, C46), 16.4 (q, C51), 13.1 (q, C48), 10.4 (q, C45); FT-ICRMS (m/z): [M+H]⁺ calc for C₅₇H₈₇N₂O₁₄, 1023.61519; found, 1023.61722.

Biological Activities of Rapamycin Analogues I and II

Methods

Neurite Outgrowth Measurements

[0241] Cortical neurons were fixed using 2% paraformaldehyde for 5 min followed by 4% paraformaldehyde for 5 min. Cells were incubated in blocking solution (0.2% Triton-

X+1.5% normal goat serum in PBS) followed by primary (anti-neuronal class III β-tubulin (TUJ1) (Covance Innovative Antibodies, Berkeley, Calif.) and secondary antibody (Alexa Fluor 488 goat anti-mouse) (Molecular Probes, Carlsbad, Calif.). Each step was performed at room temperature for 1 hr. Total neurite outgrowth for each condition was analyzed using the Neuronal Profiling Bioapplication on an ArrayScan HCS Reader (Cellomics, Pittsburgh, Pa.).

Neuronal Survival Assay (Neurofilament ELISA)

[0242] Cultures were fixed for 30 min with 4% paraformaldehyde at 37° C. Nonspecific binding was blocked by incubating with PBS containing 0.3% Triton X-100 and 5% fetal bovine serum (FBS) for 45 min. Cultures were then incubated overnight at 4° C. with an anti-neurofilament (200 kD) monoclonal antibody (1:1000, clone RT-97, Chemicon, Temecula, Calif.). After washing, a peroxidase-conjugated secondary antibody (1:1000, Vector Labs, Burlingame, Calif.) was applied for 2 h. After three washes, the peroxidase substrate K-BlueMax (Neogen, Lexington, Ky.; Young et al., 1999) was added to the cultures and incubated for 10 min on an orbital shaker. The peroxidase substrate is highly soluble in the K-BlueMax solution. Optical density is then readily measured using a Molecular Devices Spectramax Plus colorimetric plate reader at 650

Immunosuppression Assay

[0243] Human CD4⁺ T cells were purified by negative selection from peripheral blood lymphocytes using RosetteSep as per manufacturer's instructions (StemCell Technologies, Inc. Vancouver, British Columbia). Tosyl-activated magnetic microspheres (Dynal, Great Neck, N.Y.) were coated with anti-CD3 Ab (1 µg/10⁷ microspheres), and anti-CD28Ab (0.5 m/10⁷ microspheres) as described in Blair et al. J. Immunol., 160:12, 1998. Murine IgG was used to saturate the binding capacity of the microspheres (total protein=5 µg/10⁷ microspheres). Protein-coated microspheres were added to purified CD4+T cells (2×10⁶cells/mL, ratio 1 bead: 1 cell) and activated for 72 hours in RPMI, 10% fetal calf serum, 2 mM glutamine media. Cells were harvested, washed, and cultured overnight in fresh media and re-stimulated with IL-2 as described in Bennett et al., J. Immunol. 170:711, 2003. Briefly, overnight rested cells were recounted, plated (10⁵ cells/well) in flat-bottomed 96 well microtiter plates and stimulated with 1 ng/mL human IL-2 (R&D Systems, Minneapolis, Minn.) in the presence of increasing concentrations of compound. Seventy-two hours after culture re-stimulation, plates were pulsed with 1 µCi/well tritiated thymidine and incubated for a 6-16 hour period.

Results

[0244] As described above, rapamycin analogs I and II were prepared from rapamycin via a [4+2] cycloaddition reaction with nitrosobenzene at the C1, C3 diene in order to disrupt the interaction with mTOR while leaving the FKBP binding portion of the compound intact. (FIG. 1A). Compound II showed no detectable inhibition of IL-2 stimulated CD4+T-cell proliferation up to 1 µM, in contrast to rapamycin (IC₅₀=0.005 µM). Moreover, Compound I was found to promote neuronal survival, as measured by neurofilament ELISA, in cultured rat cortical neurons (FIG. 1B), and to promote neurite outgrowth in both cortical neurons (FIG. 1C) and F-11 cells (FIG. 1D). Importantly, 10 and 30 mg/kg of

Compound 2 significantly reduced infarct volume by 24% and 23%, respectively, in a transient mid-cerebral artery occlusion model for ischemic stroke (see Example 9 of U.S. Ser. No. 06/0135549). Given the therapeutic potential of these compounds, the cellular target(s) of these compounds were identified to evaluate their roles in promoting neuronal survival and neurite outgrowth.

Example 2

Chemical Synthesis and Preparation of Affinity Matrix

[0245] To identify the target proteins, affinity matrices containing rapamycin analogue I, rapamycin analogue II and the meridamycin analogue were prepared by linking the compound to Affi-Gel 10 resin through amino-phenyl-butyric acid (FIG. 2) according to the methods published by Fretz et al. supra. Briefly, the amino group of amino-phenyl-butyric acid (1200 mg) was protected with an allyloxycarbonyl group by treating with diallyldicarbonate (1200 μ M) in dioxane: water (3:1; 50 ml) for 3 h at room temperature.

[0246] The acid group of the resulting 4-(para-N-Allocaminophenyl) butanylester (80 mg) was activated by PhOP (O)Cl₂.DMF complex in CH₂Cl₂ (1 ml) at 4° C., and reacted with the 42-hydroxyl group of the rapamycin analogue I (80 mg) in the presence of pyridine (90 μ M) at room temperature for 30 min. The reaction was quenched with methanol and the ester product was purified by HPLC with a purity of 99% and characterized by MS and NMR. After removing the allyloxy-carbonyl group of the ester product (40 mg) by treatment with Pd(PPh₃)₄ (2 mg) and dimedone (7 mg) in THF (1.2 ml), the amino group of the product was linked to Affigel-10 matrix (4 ml) in the presence of 2% pyridine in THF. The resulting Affi-Gel-rapamycin analogue I affinity matrix was washed with ethanol, water and ethanolamine 50 mM Hepes pH 8.0 buffer and stored in 40% ethanol.

[0247] Similar approaches were used to prepare affinity matrix containing rapamycin analogue II, a meridamycin analogue disclosed as compound I in U.S. 2005/0197379, FK506 and rapamycin.

Example 3

Affinity Precipitation of Target Proteins

[0248] The matrices prepared in Example 2 were used to precipitate target proteins from the lysates of F-11 (a hybrid of rat dorsal root ganglia neurons (DRG) and mouse neuroblastoma) cells (Platika, D. et al. (1985) *Proc. Natl. Acad. Sci. USA* 82:3499-3503).

[0249] Experiment A.

[0250] F11 cells were grown in culture medium, DMEM supplemented with 10% FBS and 1% pen/Strep, in 75 cm² vented flasks in 37° C. incubator with 5% CO₂. Cells were harvested at 80% confluence and washed with PBS buffer. Lysis buffer (6 ml; 50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na₃VO₄, 1% Nonidet P40, 0.1% mercaptoethanol and 2% protease inhibitor cocktails) was added to 10⁹ cells. Cells were broken by forcing them through a 26-gauge needle, and S-100 supernatant was collected after 15 min centrifugation at 4° C. Aliquots (2 ml) were mixed with affinity beads (100-150 μ l; such as Affigel10, Affigel10-FK506 and Affigel10-rapamycin analogue I) at 4° C. overnight. After washes with lysis buffer (2 ml) and then PBS (2 ml), the beads were analyzed on 4-20%

SDS-PAGE gel. FIG. 2 shows the following lanes: lysate of F11 cells, blank (proteins bind to Affigel-10 beads), FK506 (proteins bind to Affigel-10-FK506 beads), rapamycin analogue II (proteins bind to Affigel-10-rapamycin analogue I beads), marker (protein standards). The protein bands (FIG. 3) were cut out and digested with trypsin (0.3 ng) in digestion buffer (30 μ l; 0.2% NH₄HCO₃) at 30° C. overnight. The resulting peptides were purified on C18-resin and submitted for FT-ICR-MS analysis. The FT-ICR-MS data was manually edited and used to search protein databases. The results are shown in FIG. 4 and have the following scores. FK506-binding protein (FKBP52) (P30416, score: 94, expect: 9.6e-05); MS Data of the 59 kDa band: 2753.35; 1710.94; 2215.13; 2363.15; 1298.71; 1215.59; 1000.51; 1000.46; 1790.93; 1381.70; 2746.36; 1316.71; and 1171.60. Voltage dependent L-type calcium channel β 1 subunit (Q8R3Z5-03-00-00, score: 133, expect: 1e-09); MS Data of the 52 kDa band: 651.38; 663.39; 779.54; 853.55; 1014.50; 1347.75; 1217.78; 1346.67; 1297.75; 1231.77; 877.52; 853.47; 919.48; 1014.56; 1041.63; 1217.74; 1231.74; 1296.84; 869.58 (major).

[0251] Thirteen fragments of the ~60 kDa band matched the partial sequence of the FKBP52 protein with a p-value of 9.6e-5, and 19 fragments of the ~50 kDa band matched the partial sequence of the β 1 subunit of the voltage gated L-type calcium channel (CACB1). Other minor components were skeleton proteins (actin and myosin).

[0252] Therefore, immunophilin FKBP52 and CACB1 were identified as binding candidates for rapamycin analogue I.

[0253] Experiment B.

[0254] In another experiment, F11 cells were grown in culture medium, DMEM supplemented with 10% FBS and 1% Pen/Strep, in 75 cm² vented flasks in a 37° C. incubator with 5% CO₂. Cells were harvested at 80% confluence and washed with PBS buffer. To 3×10⁸ cells, lysis buffer (2 ml; 50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na₃VO₄, 1% Nonidet P40, 0.1% mercaptoethanol and 2% protease inhibitor cocktails) was added, and its S-100 supernatant was collected after 15 min centrifugation at 4° C. Aliquots (2 ml) were incubated with affinity beads (100-150 μ l) at 4° C. After wash with lysis buffer (2 ml) and then PBS buffer (2 ml), beads were analyzed by SDS-PAGE. The protein bands were cut and digested with trypsin (0.3 μ g) in digestion buffer (30 μ l; 0.2% NH₄HCO₃) at 30° C. The resulting peptides (2 μ l) were loaded into a nanoelectrospray tip of FT-ICR-MS and mixed with 1% formic acid in methanol (2 μ l). A high voltage about -800 V was applied between the nanoelectrospray tip and the glass capillary. The resulting mass spectra data were externally calibrated using HP tuning mix, and used for Mascot search in NCBI protein databases. Reasonable protein candidates were selected based on confident scores (p value). For Western analysis, the precipitated proteins were separated on by SDS-PAGE, transferred to PVDF membranes by electroblotting (100V, 1 hr), immunoblotted with the anti-CACB1 or anti-FKBP4 antibody, and visualized by 3,3',5,5'-tetramethylbenzidine (TMB) staining.

Results

[0255] As shown in FIG. 5A, three strong bands (220 kDa, 60 kDa and 50 kDa) and two very weak bands (25 kDa and 12 kDa) were found in both rapamycin analogue I and II pull-down fractions. FT-ICR-MS spectra of each band were used for Mascot search in the NCBI database (see Table 1 below). FKBP52 (Gold, B. G. *Drug Metab. Rev.* 31, 649-663 (1999))

and the $\beta 1$ subunit (CACNB1) of the voltage gated L-type calcium channel (VGCC) (Opatowsky, Y. et al. *Neuron* 42, 387-399 (2004)). Structural analysis of the voltage-dependent calcium channel beta subunit functional core and its complex with the alpha 1 interaction domain were identified as major targets of rapamycin analogue I and II, and their presence was confirmed by Western analysis (FIG. 5B). FKBP25 and FKBP12 were identified in the weak bands, whereas myosin and actin were found in all fractions, indicating non-specific binding to the resin.

Tween-20, (0-10 μ M) immunophilin ligands, 3 nM [3 H]-FK506 (87 Ci/mmol), and (5 nM) enzyme. Reactions were carried out in triplicate at 25° C. for 30 min. K_d were calculated using methods described by Carreras (*Anal. Biochem.* 298, 57-61 (2001)).

[0259] The following materials used in the examples described herein were obtained from the following commercially available sources: Antibodies were from Abcam (Cambridge, Mass.). Media, human ORF clones (cacnb1, cacnb4, fkbp3, fkbp4, fkbp8, ppiF, and ppiD), plasmids (pDEST17),

TABLE 1

FT-ICR-MS analysis of proteins that bind to rapamycin analogues I and II

MS Data	Identified protein
2753.35, 1710.94, 2215.13, 2363.15, 1298.71, 1215.59, 1000.51, 1000.46, 1790.93, 1381.70, 2746.36, 1316.71, 1171.60	Major band, 59 kDa FKBP52, P = 1e-09
651.38, 663.39, 779.54, 853.55, 1014.50, 1347.75, 869.58 (major), 877.52, 853.47, 919.48, 1014.56, 1041.63, 1217.74, 1231.74, 1296.84, 1346.67	Major band, 52 kDa CACNB1, P = 1e-09
616.32, 701.42, 802.42, 888.15, 908.97, 980.48, 1010.56, 1132.55, 1405.67, 1424.71, 1611.90, 1764.81, 2328.12, 2366.15, 2342.22, 2365.15, 2442.22, 2458.17, 2493.20, 2525.20, 3101.49, 3277.66, 3235.66, 3275.65, 739.47, 881.58, 1190.68, 1314.66, 1011.69, 1533.69, 903.6	Minor band (<5%), 25 kDa, FKBP25, P = 3.8e-12
	Minor band (<5%), 12 kDa, FKBP12, P = 0.03

Example 4

Characterization of the Precipitated Targets by Western and Kinetic Analysis

Methods

[0256] Cloning and Expressing Recombinant Genes and Binding Assays.

[0257] Using Gateway cloning methods developed by Invitrogen (Carlsbad, Calif.), cacnb1/CACNB 1, cacnb4/CACNB4fkbp3/FKBP25, fkbp4/FKBP52, ppid, ppif and fkbp8/FKBP38 genes were cloned into the pDEST17 (N-His₆ tag) vector. The His₆-CACNB1: TGG548TAA were generated from pDEST17-CACNB1 using QuikChange site-directed mutagenesis kit (Stratagene, LaJolla, Calif.). The His₆ tagged protein was purified on a Ni-NTA column (Qiagen, Valencia, Calif.). Proteins showed above 95% purity by SDS-PAGE analysis, and were used fresh. FKBP38 was tested in the presence of 2 mM Ca²⁺ and 5 μ M CaM (Edlich, F. et al. *J. Biol. Chem.* 281, 14961-14970 (2006)). The binding to rapamycin analogue II was measured by SDS-PAGE based on the amount of proteins retained on rapamycin analogue II matrix in comparison with blank Affi-Gel 10 beads. The binding of rapamycin analogue I was measured by quantifying the 14 C radioactivity coeluted with the protein through TopTip P-4 column, after reacting each purified protein (10 μ M) with [14 C]-rapamycin analogue I (10 μ M, 241 Ci/mol) at 37° C. The protein fluorescent quenching induced by rapamycin analogue I was measured by titrating His₆-CACNB1: TGG548TAA protein (0.8 μ M) with rapamycin analogue I (1 μ M).

Kinetic Analysis

[0258] Binding of immunophilin ligands to His₆-tagged FKBP12 and FKBP52 proteins was measured by quantitation of 3 H FK506 retained on Ni-chelated FLASH plate in 0.1 ml reaction mixtures containing 50 mM Hepes, pH 7.4, 0.1%

and SUPERSCRIPT® System were from Invitrogen (Carlsbad, Calif.). Protein purification kits were from Pierce (Rockford, Ill.) or Qiagen (Valencia, Calif.). TOPTip P-4 column was from Glygen (Columbia, Md.). Ni-chelated Flash plates and [3 H]-FK506 were from PerkinElmer Life Science (Boston, Mass.). PCR reagents and Affi-Gel 10 were from BioRad (Hercules, Calif.). Rat Genome 230 2.0 GENECHIP® is from AFFYMETRIX® (Santa Clara, Calif.). FT-ICR-MS analysis was carried out on a Bruker (Billerica, Mass.) APEXII FT-ICR mass spectrometer equipped with an actively shielded 9.4 Tesla superconducting magnet (Magnex Scientific Ltd., UK), and an external Bruker APOLLO ESI source.

Results

[0260] Table 2 shows that both FK506 and rapamycin bind to FKBP12 and FKBP52 with comparable affinities (K_d (FKBP12)/ K_d (FKBP52)=0.46 and 0.23 respectively). In contrast, Compound 2 showed a marked preference of binding to FKBP52 relative to FKBP12 (K_d (FKBP12)/ K_d (FKBP52)=229). This is unexpected because Compound 2 has the same piperolate moiety for FKBP binding as rapamycin, and the site of modification is distant. X-ray structures have shown that the isomerase domains of FKBP52 and FKBP12 are very similar (Wu et al., *Proc Natl Acad Sci USA*. 101, 8348-53 (2004)), and sequence alignment of their active site residues showed only one amino acid difference (His87 in FKBP12 versus Ser118 in FKBP52) (Dornan et al., *Curr. Top. Med. Chem.* 3, 1392-1409 (2003)). Rapamycin and its analogs are known to exist as a set of major and minor solution conformers, due to rotation about the amide bond (Kessler et al., *Helv. Chim. Acta* 76, 117-130 (1993)). The additional moiety NO-phenyl moiety affects the overall global population of macrocyclic conformers, which in turn affects immunophilin selectivity. This observation appears consistent with the dramatic differences in binding affinities for Compound 2

towards different yet homologous immunophilins appears consistent with the dramatic differences in binding affinity reported for FKBP25 between rapamycin and FK506 (Galat et al., *Biochemistry* 31, 2427-2434 (1992)). This shows non-scaffold modifications to rapamycin that enhance binding to specific FKPs.

[0261] To further validate the specificity of the compounds for immunophilins and the related cyclophilins, the binding of Compound 1 and Compound 2 to purified recombinant FKBP25, FKBP38, cyclophilin F (PPIF), cyclophilin D (PPIF) was measured. These targets were chosen, in light of the in vivo activity of Compound 2 (vide infra), because of their reported importance in stroke models (Edlich et al., *J. Biol. Chem.* 281, 14961-14970 (2006); Baines et al., *Nature* 434, 658-662 (2005); Edlich et al., *EMBO J.* 24, 2688-2699 (2005)). The binding results of ^{14}C -1 to the various putative targets are shown in FIG. 5C. At a 10 μM concentration, ^{14}C -1 binds to FKPs well, PPIF weakly, and PPIF and FKBP38/ Ca^{2+} /CaM negligibly. Compound 2 also binds to FKBP52, FKBP25 and FKBP12 with a similar selectivity profile (FIG. 5D, Table 2).

TABLE 2

Binding of immunophilin ligands to FKBP12 and FKBP52		
Compounds	FKBP 12 (K_d , nM)	FKBP 52 (K_d , nM)
FK506	0.33 ± 0.03 (Lit. 0.4 ³⁰)	0.72 ± 0.07
Rapamycin analogue II	110 ± 11	0.48 ± 0.04
Rapamycin analogue I	4.7 ± 0.4	0.55 ± 0.05
Rapamycin	0.33 ± 0.03	1.4 ± 0.1
GPI-1046	>110	>12

[0262] The other major binding protein identified in the affinity purification and confirmed by Western analysis (FIG. 6A), CACNB1, is one of the β subunits associated with the L-type Ca^{2+} channels in primary neurons. To further validate this specific subunit as a binding partner for Compounds 1 and 2, binding to the P4 subunit (CACNB4) of the VGCC and C-terminal truncated CACNB1 was determined (FIG. 6B). Recombinant His₆-CACNB1:TGG548TAA protein was prepared by removing 51 C-terminal residues from CACNB1. Binding to full length CACNB4 was also tested because of its sequence homology to CACNB1 (Opatowsky, Y. et al. *Neuron* 42, 387-399 (2004)). At a 10 μM concentration, ^{14}C -Compound 1 binds to the mutant His₆-CACNB1:TGG548TAA weakly and CACNB4 negligibly. To further confirm the binding of Compound 1 to the His₆-CACNB1:TGG548TAA mutant, we measured protein fluorescent quenching induced by Compound 1. FIG. 6C shows a linear dose response curve, indicating binding of Compound 1 to CACNB1. Compound 2 also binds to CACNB1 with a similar selectivity profile (FIG. 6D).

[0263] The existence of the drug targets or binding candidates for rapamycin analogue I (immunophilin FKBP52 and CACB1) was also confirmed by Western blotting using the corresponding antibodies. The proteins on the affinity beads were separated by 4-20% SDS-PAGE gel, and transferred to PVDF membrane at 100 V for 1 h. The membranes were blotted with blocking solution, primary antibody (anti-FKBP52 or anti- Ca^{2+} channel- β 1 subunit antibodies; 1:200 dilution), and secondary antibody (peroxidase conjugated anti-rabbit IgG antibody; 1:1000 dilution). The existence of the target proteins was visualized after TMB staining, as shown in FIG. 8.

[0264] Western analysis demonstrated the binding of both proteins to rapamycin analogue I, but not to the blank beads. FKBP52 was detected in the fractions of rapamycin analogue I beads and FK506 beads, but not in the blank beads. The voltage dependent L-type calcium channel β 1 subunit was only detected in the fraction of the rapamycin analogue I beads. This indicates that rapamycin analogue I specifically bound to FKBP52 and the β 1 subunit of the voltage gated L-type calcium channel.

Example 5

Formation of a Novel Complex, FKBP52-Rapamycin Analogue 1- Ca^{2+} Channel β 1 Subunit

[0265] Co-immunoprecipitation was used to investigate the complex formation among FKBP52, rapamycin analogue I and the voltage gated calcium channel β 1 subunit. Briefly, aliquots (1.8 ml) of F11 cell lysate were mixed with 0, 5, and 50 μM rapamycin analogue I, respectively, at 4° C. for 5 h. Anti-FKBP52 antibody was added at 1:200 dilution to each aliquot and incubated at 4° C. for 5 h. Protein A beads (50-100 μM) were then added to precipitate the anti-FKBP52-antibody-associated complex. The proteins immunoprecipitated on the beads were washed with PBS buffer, separated on 4-20% SDS-PAGE gel, transferred to PVDF, and immunoblotted with anti- Ca^{2+} channel β 1 subunit antibody (1:500 dilution) to detect the β 1 subunit.

Results

[0266] The results are shown in FIG. 7. The Ca^{2+} channel β 1 subunit did not precipitate with FKBP52 in the absence of rapamycin analogue I, indicating that the Ca^{2+} channel β 1 subunit does not associate with FKBP52. In the presence of rapamycin analogue I (5 μM), a large amount of Ca^{2+} channel β 1 subunit was co-immunoprecipitated with FKBP52, indicating a complex formation. However, an excess amount of rapamycin analogue I (50 μM) reduced the amount of precipitated β 1 subunit, indicating lower amount of complex formation, which may be caused by saturation of the compound binding sites on both FKBP52 and β 1 subunit in a limited amount of lysate.

Example 6

Complex Formation Correlates with Neurite Outgrowth

[0267] Neurofilament ELISA was used to measure the neurite outgrowth of F11 cells grown in the absence or presence of rapamycin analogue I. Briefly, F11 cells were grown in DMEM supplemented with 10% FBS, 1% pen/Strep, and rapamycin analogue I (0, 5, or 50 μM) for 96 hrs. Cells were fixed with 4% paraformaldehyde for 30 min at 37° C. Non-specific binding was blocked by incubating with PBS containing 0.3% Triton X-100 and 5% fetal bovine serum (FBS) for 45 min. Cultures were then incubated overnight at 4° C. with an anti-neurofilament (200 kD) monoclonal antibody (1:1000). After washing, a peroxidase-conjugated anti-mouse secondary antibody (1:1000) was applied for 2 h. After three washes, the peroxidase substrate K-BlueMax was added to the cultures and incubated for 10 min. Optical density was determined at 650 nm.

Results

[0268] The results are shown in FIG. 8. The cells treated with 5 μM rapamycin analogue I showed 4-5 fold higher

neurofilament content than those treated with 50 μ M rapamycin analogue I or no compound control, indicating strong neurite outgrowth at 5 μ M rapamycin analogue I. This directly correlated with the complex formation in the presence of the identical concentration of rapamycin analogue I.

Example 7

Evaluation of the Electrophysiological Properties of the Calcium Channel in F-11 Cells, Following Treatment with Rapamycin Analogs

[0269] Methods. Whole-Cell Patch Clamp Recordings
[0270] The whole-cell configuration of the patch-clamp technique was used to record calcium currents from the cells at room temperature using an EPC-9 amplifier (HEKA, Instrutech Corp.) with the acquisition and analysis program Pulse-PulseFit from HEKA (Lambrecht, Germany). Electrodes were fabricated using a P-87 puller (Sutter Instrument). Electrodes had a resistance of 2-5 M Ω when filled with recording solution (140 mM CsCl, 10 mM EGTA, 10 mM HEPES, 5 mM MgCl₂, 2 M ATP, 1 mM cAMP, pH 7.2). The standard bath recording solution is Ca²⁺ and Mg²⁺ free HBSS (pH 7.4) containing 10 mM HEPES, 10 mM dextrose, and 4 mM BaCl₂. Currents were filtered at 3 kHz, and the inward Ca²⁺ currents were recorded from cells held at -90 mV with 10 mV depolarizing steps from -80 mV to 60 mV for 50 ms.

Results

[0271] CACNB1, is one of the β subunits associating with the L-type Ca²⁺ channels in primary neuron (Pichler et al., *J. Biol. Chem.* 272, 13877-13882 (1997)). The β 1b, β 3 and β 4 subunits are known to enhance L-type Ca²⁺ channel current, whereas the O₂ subunit plays a negative role (Opatowsky et al., *Neuron* 42, 387-399 (2004); Schjott et al., *J. Biol. Chem.* 278, 33936-33942 (2003)). If binding of our rapalogs to β 1b subunit inhibits the function of this subunit, the L-type Ca²⁺ current is expected to be reduced. Therefore, the electrophysiological properties of the Ca²⁺ channel in F-11 cells following treatment with rapamycin analogue I were measured.

[0272] Whole-cell Ca²⁺ currents recorded in F-11 cells was not affected by bath application of Compound 1 for short time periods (10 min application; FK506 inhibited the Ca²⁺ current within this time period), so cells were exposed to 5 μ M 1 for 2 hrs and then compared to vehicle treated controls. This treatment paradigm strongly reduced the Ca²⁺ currents detected in the cells, reducing the current density from 6.5+/-0.5 pA/pF to 3.2+/-0.3 pA/pF, a 49% decrease (FIG. 9A). FK506 also was found to produce a similar effect on the Ca²⁺ currents (current was reduced to 2.9+/-0.1 pA/pF, a 55% decrease), as has been described for calcineurin dependent action on Ca²⁺ currents ((Yasutsune, et al. *British Journal of Pharmacology* 126(3), 717-729 (1999); Fauconnier, J., et al. *Am J Physiol Heart Circ Physiol.* 288, H778-H786 (2005)). This, combined with the large size of Compound 1, required that for subsequent experiments, the compound be added into the cell directly by way of the recording patch pipette.

[0273] Internal application of Compound 1 via diffusion into the cell beginning when the whole-cell configuration was achieved (time 0) produced an inhibition of Ca²⁺ current immediately, reaching a steady state level of current block within several minutes. Interestingly, the compound's effect in F-11 was quite variable, but as a hybrid of DRG and neuroblastoma cells, the expression profiles of N- and L-type Ca²⁺ channels are known to differ among individual F-11

cells (Boland, L M. et al. *Journal of Physiology* 420, 223-245 (1990)). Some cells (FIG. 9C) contained predominantly the L-type Ca²⁺ channel as determined by inhibition with BAY-K5552 (L-type blocker), while others (FIG. 9D) contained mainly the N-type channel that was inhibited by ω CTX MVIIA (N-type blocker). FIG. 9C shows that treatment with Compound 1 reduces the Ca²⁺ current, in cells responding to BAY-K5552, while FIG. 9D illustrates how cells not responding to Compound 1 contained Ca²⁺ current sensitive to ω CTX MVIIA. In the former case, internal application of Compound 1 (10 μ M) reduced the Ca²⁺ current by an average of 46+/-1.8% within 10 min. (FIG. 9C). No significant current reduction was found in cells responding significantly to ω CTX MVIIA (FIG. 9D). Further validation of rapalog effects on Ca²⁺ currents was performed on cultured rat hippocampal neurons. When N-type Ca²⁺ channels were blocked and Compound 2 (10 μ M) was added to the internal pipette solution, the current was slowly inhibited by 74.5+/-8.8 after 10 min (FIG. 9E,F). This effect was due at least partly to an inhibition of L-type channels, as block of both N- and L-type Ca²⁺ channels reduced the inhibition to only 21.3+/-4.4% of the remaining current in the cells (FIG. 9F).

Example 8

Transcriptional Profiling Following Treatment with Rapamycin Analogs

Methods

Transcriptional Profiling

[0274] Cortical neuron cultures were prepared from E16 rat embryos. After plating for 24 hrs, cultures were treated with 10 μ M immunophilin ligands and the corresponding vehicle. After treatment for 4 hrs, 12 hrs, 24 hrs and 48 hrs, cells were lysed. Total RNA from each sample was extracted with the RNEASY® Mini Kit (QIAGEN®). Double stranded cDNA was synthesized from 2 μ g of each RNA sample using the SUPERSCRIPT® System (INVITROGEN®), purified, transcribed in vitro to prepare biotinylated cRNA using T7 RNA polymerase in the presence of biotin labeled UTP and CTP. The fragmented cRNAs were hybridized to a Rat Genome 230 2.0 GENECHIP® (AFFYMETRIX®, Santa Clara, Calif.) as recommended by the manufacturer. Hybridized arrays were stained according to manufacture protocols on a Fluidics Station 450 and subsequently scanned on an AFFYMETRIX® scanner 3000. The raw data was generated using AFFYMETRIX® MAS 5.0 Software. Transcriptional profiling data were analyzed in Ingenuity.

Results

[0275] To further analyze downstream consequences of rapamycin analogue binding, transcriptional profiling data of rat cortical neuron cultures treated with 10 μ M of rapamycin analogue I or II were obtained.

[0276] Transcriptional profiling revealed overall down-regulation of Ca²⁺ signaling pathways after rapamycin analogue I or II treatment (see Table 3A). Rapamycin analogue I caused down-regulation of major plasma membrane Ca²⁺ influx channels, such as VGCC, transient receptor potential channels, N-methyl D-aspartate subtype of glutamate receptors (NMDA), and SHT3R channels. Among these channels, Ca²⁺ influx through the NMDA channel is a major event leading to apoptosis (Ghosh et al., *Science* 268, 239-247 (1995)). Plasminogen activator (PLAU), known to cleave the

NMDA peptide and activate Ca²⁺ influx (Traynelis et al., *Nat. Med.* 7, 17-18 (2001)), was significantly down regulated (~40 fold by rapamycin analogue I, ~10 fold by rapamycin analogue II); this is likely to reduce the Ca²⁺ influx through NMDA channels. Also, down regulation of IP3 receptor might reduce Ca²⁺ release from internal storage, and down regulation of calmodulin and calmodulin kinases (e.g. PNCK, ~20 fold) would reduce the cytosolic Ca²⁺ signaling. The observed attenuation of Ca²⁺ influx and Ca²⁺ signaling pathways may be critical for the treatment of stroke and traumatic brain injury, because Ca²⁺ overload of neurons is generally considered the critical event triggering the Ca²⁺ dependent processes that eventually lead to neuronal death (Ghosh et al., *Science* 268, 239-247 (1995)). In addition, lowering cellular

Ca²⁺ levels may suppress apoptosis by FKBP38/Ca²⁺/CaM activation of Bcl2 (Edlich et al., *J. Biol. Chem.* 281, 14961-14970 (2006)), or PPID associated mitochondrial permeability transition pore (Baines et al., *Nature* 434, 658-662 (2005)).

[0277] Significant upregulation of cholesterol biosynthesis genes (e.g. LSS, +13 fold) was observed, indicating activation of steroid receptors (Wang et al., *J. Lipid Res.* 47, 778-786 (2006)) (see Table 3B). Because activation of steroid receptors by FK506, steroid hormones or geldanamycin has been reported to stimulate neurite outgrowth, it is possible that binding of rapamycin analogue I and II to FKBP52 activates steroid receptors and promotes neurite outgrowth.

TABLE 3A

Calcium signaling pathway genes							
Gene Symbol	Gene Name	fold change 2 vs DMSO	p-value 2 vs DMSO	fold change 1 vs DMSO	p-value 1 vs DMSO	location	family
ACTA1	actin, alpha 1, skeletal muscle	-1.6	<0.001	-1.53	<0.001	Cytoplasm	other
ACTA2	actin, alpha 2, smooth muscle, aorta	1.37	0.089	1.41	<0.001	Cytoplasm	other
AKAP5	—	-1.5	0.17	-2.56	0.003	Plasma Membrane	other
ASPH	aspartate beta-hydroxylase	-3.27	<0.001	-3.19	<0.001	Cytoplasm	enzyme
ATP2A2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	2	<0.001	1.85	<0.001	Cytoplasm	transporter
ATP2B1	ATPase, Ca++ transporting, plasma membrane 1	1.81	0.003	1.53	0.016	Plasma Membrane	transporter
ATP2B3	ATPase, Ca++ transporting, plasma membrane 3	-1.45	0.019	-1.54	0.009	Plasma Membrane	transporter
ATP2C1	ATPase, Ca++ transporting, type 2C, member 1	-1.32	0.002	-1.38	<0.001	Cytoplasm	transporter
CABIN1	calcineurin binding protein 1	1.34	0.01	1.33	0.011	Nucleus	other
CACNA1B	calcium channel, voltage-dependent, L type, alpha 1B subunit	-1.45	0.001	-1.77	<0.001	Plasma Membrane	ion channel
CACNA1C	calcium channel, voltage-dependent, L type, alpha 1C subunit	1.82	0.001	1.48	0.001	Plasma Membrane	ion channel
CACNA1D	calcium channel, voltage-dependent, L type, alpha 1D subunit	-1.28	0.21	-1.72	0.017	Plasma Membrane	ion channel
CACNA2 D1	calcium channel, voltage-dependent, alpha 2/delta subunit 1	5.73	0.009	3.47	0.03	Plasma Membrane	ion channel
CACNB1	calcium channel, voltage-dependent, beta 1 subunit	-1.14	0.051	-1.32	0.026	Plasma Membrane	ion channel

TABLE 3A-continued

Calcium signaling pathway genes							
Gene Symbol	Gene Name	fold change 2 vs DMSO	p-value 2 vs DMSO	fold change 1 vs DMSO	p-value 1 vs DMSO	location	family
CACNG2	calcium channel, voltage-dependent, gamma subunit 2	-2	<0.001	-1.8	<0.001	Plasma Membrane	ion channel
CACNG3	calcium channel, voltage-dependent, gamma subunit 3	-2.46	0.001	-3.73	<0.001	Plasma Membrane	ion channel
CALM1	calmodulin 1 (phosphorylase kinase, delta)	-1.63	<0.001	-1.74	<0.001	Plasma Membrane	other
CALM2	calmodulin 2 (phosphorylase kinase, delta)	-1.33	0.007	-1.22	0.028	Plasma Membrane	other
CALM3	calmodulin 3 (phosphorylase kinase, delta)	-1.52	0.005	-1.34	0.01	Plasma Membrane	other
CALR	calreticulin	1.19	0.016	1.18	0.023	Nucleus	transcription regulator
CAMK1	calcium/calmodulin-dependent protein kinase I	-1.43	0.006	-1.6	0.001	Cytoplasm	kinase
CAMK4	calcium/calmodulin-dependent protein kinase IV	-1.33	0.46	-1.42	0.049	Nucleus	kinase
CAMK1G	calcium/calmodulin-dependent protein kinase IG	-2.26	<0.001	-2.47	<0.001	Plasma Membrane	kinase
CAMK2A	calcium/calmodulin-dependent protein kinase (CaM kinase) II alpha	-4.13	0.06	-2.03	0.004	Cytoplasm	kinase
CAMK2B	calcium/calmodulin-dependent protein kinase (CaM kinase) II beta	-1.88	<0.001	-1.82	<0.001	Cytoplasm	kinase
CAMK2D	calcium/calmodulin-dependent protein kinase (CaM kinase) II delta	-1.48	0.002	-1.45	0.004	Cytoplasm	kinase
CHP	calcium binding protein P22	1.54	0.001	1.65	0.001	Cytoplasm	transporter
CREBBP	CREB binding protein (Rubinstein-Taybi syndrome)	5.84	0.004	4.14	0.009	Nucleus	transcription regulator
DSCR1	Down syndrome critical region gene 1	-1.82	<0.001	-1.93	<0.001	Nucleus	transcription regulator
DSCR1L1	Down syndrome critical region gene 1-like 1	-4.73	<0.001	-4.44	<0.001	Unknown	other
GRIA1	glutamate receptor, ionotropic, AMPA 1	-2.16	0.001	-2.78	0.008	Plasma Membrane	ion channel
GRIA2	glutamate receptor, ionotropic, AMPA 2	2.71	0.002	2.74	<0.001	Plasma Membrane	ion channel
GRIN1	glutamate receptor, ionotropic, N-methyl D-aspartate 1	-2.8	<0.001	-2.41	<0.001	Plasma Membrane	ion channel
GRIN2B	glutamate receptor, ionotropic, N-methyl D-aspartate 2B	-1.57	0.052	-1.94	0.008	Plasma Membrane	ion channel
GRIN3A	glutamate receptor, ionotropic, N-methyl-D-aspartate 3A	-2.94	0.063	-1.84	0.013	Plasma Membrane	ion channel

TABLE 3A-continued

Calcium signaling pathway genes							
Gene Symbol	Gene Name	fold change 2 vs DMSO	p-value 2 vs DMSO	fold change 1 vs DMSO	p-value 1 vs DMSO	location	family
GRINA	glutamate receptor, ionotropic, N-methyl D-aspartate-associated protein 1 (glutamate binding)	-1.2	0.009	-1.21	0.007	Unknown	ion channel
HDAC5	histone deacetylase 5	1.8	0.014	1.78	0.016	Nucleus	transcription regulator
HDAC6	histone deacetylase 6	-1.13	0.015	-1.18	0.005	Nucleus	transcription regulator
HDAC7A	histone deacetylase 7A	1.27	0.007	1.24	0.011	Nucleus	transcription regulator
HTR3A	5-hydroxytryptamine (serotonin) receptor 3A	-1.64	0.005	-1.57	0.005	Plasma Membrane	ion channel
ITPR3	inositol 1,4,5-triphosphate receptor, type 3	-1.93	0.002	-1.85	0.019	Cytoplasm	ion channel
MAPK1	mitogen-activated protein kinase 1	1.38	0.009	1.29	0.029	Cytoplasm	kinase
MAPK3	mitogen-activated protein kinase 3	-1.19	0.046	-1.31	0.01	Cytoplasm	kinase
MYH1	myosin, heavy polypeptide 1, skeletal muscle, adult	-2.17	0.008	-2.18	0.006	Cytoplasm	enzyme
MYH6	myosin, heavy polypeptide 6, cardiac muscle, alpha (cardiomyopathy, hypertrophic 1)	-5.93	0.017	-7.18	0.02	Cytoplasm	other
MYH7	myosin, heavy polypeptide 7, cardiac muscle, beta	-5.88	<0.001	-6.11	<0.001	Cytoplasm	other
MYL6B	myosin, light polypeptide 6B, alkali, smooth muscle and non-muscle	-1.68	<0.001	-1.69	0.001	Cytoplasm	other
PPP3CB	protein phosphatase 3 (formerly 2B), catalytic subunit, beta isoform (calcineurin A beta)	-1.27	<0.001	-1.3	<0.001	Unknown	phosphatase
PPP3CC	protein phosphatase 3 (formerly 2B), catalytic subunit, gamma isoform (calcineurin A gamma)	-1.27	0.007	-1.18	0.037	Unknown	phosphatase
PPP3R1	protein phosphatase 3 (formerly 2B), regulatory subunit B, 19 kDa, alpha isoform (calcineurin B, type I)	-1.44	0.039	-1.49	0.031	Cytoplasm	phosphatase
PRKAG1	protein kinase, AMP-activated, gamma 1 non-catalytic subunit	-1.24	0.005	-1.21	0.012	Unknown	kinase
PRKAR1A	protein kinase, cAMP-dependent, regulatory, type I alpha (tissue specific extinguisher 1)	-1.1	0.001	1.08	0.015	Cytoplasm	kinase
PRKAR1B	protein kinase, cAMP-dependent, regulatory, type I, beta	-3.18	<0.001	-3.36	<0.001	Cytoplasm	kinase

TABLE 3A-continued

<u>Calcium signaling pathway genes</u>							
Gene Symbol	Gene Name	fold change 2 vs DMSO	p-value 2 vs DMSO	fold change 1 vs DMSO	p-value 1 vs DMSO	location	family
PRKAR2A	protein kinase, cAMP-dependent, regulatory, type II, alpha	-1.23	0.26	-1.56	0.047	Cytoplasm	kinase
PRKAR2B	protein kinase, cAMP-dependent, regulatory, type II, beta	-1.5	<0.001	-1.56	<0.001	Cytoplasm	kinase
RAP1B	RAP1B, member of RAS oncogene family	-1.33	0.002	-1.29	<0.001	Cytoplasm	enzyme
TPM1	tropomyosin 1 (alpha)	-2.83	0.002	-2.55	<0.001	Cytoplasm	other
TPM3	tropomyosin 3	-1.99	0.001	-2.04	<0.001	Cytoplasm	other
TRPC1	transient receptor potential cation channel, subfamily C, member 1	-1.08	0.33	-1.21	0.033	Plasma Membrane	ion channel
TRPC3	transient receptor potential cation channel, subfamily C, member 3	-1.95	0.003	-2.4	0.015	Plasma Membrane	ion channel
TRPC4	transient receptor potential cation channel, subfamily C, member 4	-5.42	0.023	-4.56	0.005	Plasma Membrane	ion channel
TRPV6	transient receptor potential cation channel, subfamily V, member 6	1.73	<0.001	1.65	<0.001	Plasma Membrane	ion channel

TABLE 3B

<u>Sterol biosynthesis pathway genes</u>							
Gene Symbol	Gene Name	fold change 2 vs DMSO	p-value 2 vs DMSO	fold change 1 vs DMSO	p-value 1 vs DMSO	location	family
CYP27B1	cytochrome P450, family 27, subfamily B, polypeptide 1	-1.38	0.008	-1.27	0.055	Cytoplasm	enzyme
DHCR7	7-dehydrocholesterol reductase	1.73	0.001	1.84	<0.001	Cytoplasm	enzyme
EBP	empapamil binding protein (sterol isomerase)	1.32	0.034	1.45	0.012	Cytoplasm	enzyme
FDFT1	farnesyl-diphosphate farnesyl-transferase 1	1.89	<0.001	1.94	<0.001	Cytoplasm	enzyme
FDPS	farnesyl diphosphate synthase (farnesyl pyrophosphate synthetase, dimethylallyl-transferring, geranyltransferase)	2.07	<0.001	2.34	<0.001	Cytoplasm	enzyme
HMGCR	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	2.26	<0.001	2.24	<0.001	Cytoplasm	enzyme
IDI1	isopentenyl-diphosphate delta isomerase 1	2.85	<0.001	3.24	<0.001	Cytoplasm	enzyme

TABLE 3B-continued

Sterol biosynthesis pathway genes							
Gene Symbol	Gene Name	fold change 2 vs DMSO	p-value 2 vs DMSO	fold change 1 vs DMSO	p-value 1 vs DMSO	location	family
LSS	lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)	13.19	0.009	13.3	0.009	Cytoplasm	enzyme
MVD	mevalonate (diphospho) decarboxylase	1.81	0.005	1.94	0.004	Cytoplasm	enzyme
MVK	mevalonate kinase (mevalonic aciduria)	1.6	0.001	1.6	<0.001	Cytoplasm	kinase
NQO1	NAD(P)H dehydrogenase, quinone 1	1.64	0.001	1.8	<0.001	Cytoplasm	enzyme
PMVK	phosphomevalonate kinase	1.25	0.024	1.33	0.009	Cytoplasm	kinase
SC5DL	sterol-C5-desaturase (ERG3 delta-5-desaturase homolog, fungal)-like	1.94	<0.001	2.01	0.001	Cytoplasm	enzyme
SQLE	squalene epoxidase	1.9	<0.001	1.92	<0.001	Cytoplasm	enzyme

Example 10

Reduction of Subunit of Voltage-Gated L-Type Calcium Channel Stimulates Neurite Outgrowth

[0278] RNAi technology was used to reduce the transcription levels of the CACB1 (Ca²⁺ channel β1 subunit) and the FKBP4 (FKBP52) genes, and the biological effect was examined by growth phenotype.

Methods.

Neuronal Cultures

[0279] Briefly, cortical neuron cultures were prepared from embryonic day 15 (E15) rat embryos (Sprague-Dawley, Charles River Laboratories, Wilmington, Mass.). The embryos were collected, their brains were removed, and the cortices were dissected out in ice-cold phosphate-buffered saline (PBS) without Ca²⁺ and Mg²⁺. Dissected pieces of cortical tissue were pooled together and transferred to an enzymatic dissociation media containing 20 IU/ml papain in Earle's balanced salt solution (Worthington Biochemical, Freehold, N.J.) and incubated for 30 min at 37° C. After enzymatic dissociation, the papain solution was aspirated and the tissue mechanically triturated with a fire-polished Pasteur pipette in complete media [Neurobasal Medium with B-27 supplement (Gibco, Grand Island, N.Y.), 100 IU/ml penicillin, 100 µg/ml streptomycin, 3.3 µg/ml aphidicolin, 0.5 mM glutamate] containing 2,000 IU/ml DNase and 10-mg/ml ovomucoid protease inhibitor.

Transient Transfection of siRNA into Primary Cortical Neurons

[0280] For each condition, 5×10⁵ cortical neurons were transfected with 200 ng of siGLO Lamin A/C siRNA (Dharmacon RNA Technologies, Boulder, Colo.), L-type calcium channel β1 subunit siRNA (GGAGAAGUA-

CAAUAUAGACTT (SEQ ID NO:15) (sense) and GUCA-UUUAUUGUACUUUCUCCCTT (SEQ ID NO:16) (antisense)) or FKBP4 siRNA (CCUAGCUAUGC UUUUGGCATT (SEQ ID NO:17) (sense) AND UGCCAAAGCAUAGC-UAGGTT (SEQ ID NO:18)(antisense) (Ambion, Inc., Austin, Tex.) using program DC-104 on the 96-well shuttle (amaxa biosystems, Gaithersburg, Md.). 25 µl from each transfection reaction were added to a poly-D-lysine-coated 96 well ((4 wells per experiment). Transfected cortical neurons were maintained in culture for 24 h.

Western Blotting

[0281] Cortical neurons treated with scrambled siRNA, lamin A/C, CACNB1, or FKBP52 siRNA were lysed in RIPA buffer containing protease inhibitor cocktail and phosphatase inhibitors and protein concentrations were measured using a Bradford assay (Bio-Rad Laboratories, Hercules, Calif.). 2 µg of protein per condition were loaded into each well and separated via SDS-PAGE. Proteins were transferred onto nitrocellulose and incubated with an antibody against lamin A/C (Upstate), CACNB1 (abcam, Cambridge, Mass.), or FKBP52 (Santa Cruz Biotechnology, Inc.) and actin (Sigma) as a loading control. Bands were developed and quantified using an Odyssey Infrared Imaging System and Odyssey software (LiCor Biosciences, Lincoln, Nebr.). Protein expression knock down was calculated as the ratio to actin as a percentage of scrambled siRNA expression.

Results

[0282] To further demonstrate that inhibition of both FKBP52 and CACNB1 by rapamycin analogue I or II contributes to the neurite outgrowth and neuronal survival, we transfected rat cortical neurons with siRNA against lamin A/C (to serve as a control), FKBP52, CACNB1, or FKBP52+

CACNB1 and measured total neurite outgrowth after 24 h. Total neurite outgrowth compared to control was essentially unchanged in CACNB1 siRNA-treated neurons, but significantly increased in FKBP52 siRNA- ($125\pm12\%$ of control) and FKBP52+CACNB1 siRNA-treated ($126\pm14\%$ of control) neurons (FIG. 10A), indicating inhibition of FKBP52 stimulates neurite outgrowth. In parallel, we assessed the effects of siRNA on neuronal survival by an ELISA assay to quantify neurofilament expression. Percent neuronal survival compared to control was decreased in CACNB1 siRNA-treated cells ($80\pm3\%$ of control) and mildly increased in FKBP52 siRNA- ($112\pm2\%$ of control) and significantly in FKBP52+CACNB1 siRNA-treated ($152\pm2\%$ of control) cells (FIG. 10B), indicating that reducing both FKBP52 and CACNB1 promotes neuronal survival. Western blots were performed to verify that siRNA treatment reduced lamin A/C, CACNB1 or FKBP52 protein expression in cortical neurons after 24 h. A representative blot is shown in FIG. 10C. Lamin A/C expression was reduced by $79.21\pm13.68\%$, CACNB1 expression was reduced by $70.79\pm20.79\%$ and FKBP52 expression was reduced by $86.83\pm7.03\%$ (n=3).

[0283] These experiments demonstrate that rapamycin analogue I forms a novel complex with FKBP52 and the voltage gated L-type calcium channel $\beta 1$ subunit. The complex formation inhibited the activity of the $\beta 1$ subunit, and stimulated neurite outgrowth. They also demonstrate that two substantially non-immunosuppressive immunophilin ligands, rapamycin analogues I and II, prepared by modification of rapamycin at the mTOR binding region (Abraham et al., *Annu.*

Rev. Immunol. 14, 483-510 (1996)), demonstrated potent neurite outgrowth activity. Affinity purification revealed that both bound to the immunophilin FKBP52 and the $\beta 1$ -subunit of L-type voltage dependent Ca^{2+} channels (CACNB1). Rapamycin analogue II showed 687-fold higher binding selectivity for FKBP52 versus FKBP12 than that of rapamycin. Further more, rat cortical neurons treated with the compounds demonstrated an overall down regulation of Ca^{2+} signaling pathways, and partial inhibition of L-type Ca^{2+} channel was observed in treated F-11 cells. Genetic reduction of FKBP52 and/or CACNB1 in rat cortical neurons promoted neurite outgrowth and neuronal survival. Without being bound to theory, Applicants believe that immunophilin ligands can potentially protect neurons from Ca^{2+} induced cell death by modulating Ca^{2+} signaling, and promote neurite outgrowth by activation of steroid receptors via FKBP52 binding. This novel mechanism of neuroprotective action provides valuable insights for the treatment of many diseases.

[0284] The contents of all references, pending patent applications and published patents, cited throughout this application are hereby expressly incorporated by reference.

EQUIVALENTS

[0285] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

SEQUENCE LISTING

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35          40          45

Thr Thr Ser Asn Ser Phe Val Arg Gln Gly Ser Ala Glu Ser Tyr Thr
50          55          60

Ser Arg Pro Ser Asp Ser Asp Val Ser Leu Glu Glu Asp Arg Glu Ala
65          70          75          80

Leu Arg Lys Glu Ala Glu Arg Gln Ala Leu Ala Gln Leu Glu Lys Ala
85          90          95

Lys Thr Lys Pro Val Ala Phe Ala Val Arg Thr Asn Val Gly Tyr Asn
100         105         110

Pro Ser Pro Gly Asp Glu Val Pro Val Gln Gly Val Ala Ile Thr Phe
115         120         125

Glu Pro Lys Asp Phe Leu His Ile Lys Glu Lys Tyr Asn Asn Asp Trp
130         135         140

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-continued

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																175	
Arg	Gln	Asn	Arg	Leu	Gly	Ser	Ser	Lys	Ser	Gly	Asp	Asn	Ser	Ser	Ser		
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Ser	Leu	Gly	Asp	Val	Val	Thr	Gly	Thr	Arg	Arg	Pro	Thr	Pro	Pro	Ala		
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Ser	Ala	Lys	Gln	Lys	Gln	Lys	Ser	Thr	Glu	His	Val	Pro	Pro	Tyr	Asp		
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Lys	His	Arg	Phe	Asp	Gly	Arg	Ile	Ser	Ile	Thr	Arg	Val	Thr	Ala	Asp		
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Leu	Asp	Ala	Asp	Thr	Ile	Asn	His	Pro	Ala	Gln	Leu	Ser	Lys	Thr	Ser		
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Leu	Ala	Pro	Ile	Ile	Val	Tyr	Ile	Lys	Ile	Thr	Ser	Pro	Lys	Val	Leu		
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Gln	Arg	Leu	Ile	Lys	Ser	Arg	Gly	Lys	Ser	Gln	Ser	Lys	His	Leu	Asn		
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Val	Gln	Ile	Ala	Ala	Ser	Glu	Lys	Leu	Ala	Gln	Cys	Pro	Pro	Glu	Met		
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Phe	Asp	Ile	Ile	Leu	Asp	Glu	Asn	Gln	Leu	Glu	Asp	Ala	Cys	Glu	His		
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Leu	Ala	Glu	Tyr	Leu	Glu	Ala	Tyr	Trp	Lys	Ala	Thr	His	Pro	Pro	Ser		
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Ser	Thr	Pro	Pro	Asn	Pro	Leu	Leu	Asn	Arg	Thr	Met	Ala	Thr	Ala	Ala		
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Leu	Ala	Ala	Ser	Pro	Ala	Pro	Val	Ser	Asn	Leu	Gln	Gly	Pro	Tyr	Leu		
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Ala	Ser	Gly	Asp	Gln	Pro	Leu	Glu	Arg	Ala	Thr	Gly	Glu	His	Ala	Ser		
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Met	His	Glu	Tyr	Pro	Gly	Glu	Leu	Gly	Gln	Pro	Pro	Gly	Leu	Tyr	Pro		
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Ser	Ser	His	Pro	Pro	Gly	Arg	Ala	Gly	Thr	Leu	Arg	Ala	Leu	Ser	Arg		
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Gln	Asp	Thr	Phe	Asp	Ala	Asp	Thr	Pro	Gly	Ser	Arg	Asn	Ser	Ala	Tyr		
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Thr	Glu	Leu	Gly	Asp	Ser	Cys	Val	Asp	Met	Glu	Thr	Asp	Pro	Ser	Glu		
																525	
Gly	Pro	Gly	Leu	Gly	Asp	Pro	Ala	Gly	Gly	Thr	Pro	Pro	Ala	Arg			
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Gln	Gly	Ser	Trp	Glu	Asp	Glu	Glu	Glu	Asp	Tyr	Glu	Glu	Lys	Leu	Thr		

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545	550	555	560
Asp Asn Arg Asn Arg Gly Arg Asn Lys Ala Arg Tyr Cys Ala Glu Gly			
565	570	575	
Gly Gly Pro Val Leu Gly Arg Asn Lys Asn Glu Leu Glu Gly Trp Gly			
580	585	590	
Arg Gly Val Tyr Ile Arg			
595			

<210> SEQ ID NO 2

<211> LENGTH: 3687

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 2

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aaatacagca	agaggaaagg	gcgattcaaa	cggtcagatg	ggagcacgtc	ctcgatatacc	300
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acgctacggg	cactgtcccg	ccaagacact	tttgtatggc	acaccccccgg	cagccgaaac	1680
tctgcctaca	cgagctggg	agactcatgt	gtggacatgg	agactgaccc	ctcagagggg	1740

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gcattccctt gccttggcat ccctgttagcc cagcaaccct gcccctcccc agcatccca	3060
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gcttctttgtt cctcgccaca tagtcgtcag cgtaggcacc tgggagctgc tgatatgcac	3420
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aagatgtatcc ttgtatggt gcccctgtat gtattgtgcc caagccagga gctgcttgg	3540
cagtcctccagc tccacactgg ccctgagcccc cttcacttac ctgtctctcc acaagtagag	3600
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<210> SEQ ID NO 3
<211> LENGTH: 523
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 3

Met Val Gln Lys Thr Ser Met Ser Arg Gly Pro Tyr Pro Pro Ser Gln

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			20			25					30				
Lys	Arg	Lys	Gly	Arg	Phe	Lys	Arg	Ser	Asp	Gly	Ser	Thr	Ser	Ser	Asp
		35				40					45				
Thr	Thr	Ser	Asn	Ser	Phe	Val	Arg	Gln	Gly	Ser	Ala	Glu	Ser	Tyr	Thr
			50			55					60				
Ser	Arg	Pro	Ser	Asp	Ser	Asp	Val	Ser	Leu	Glu	Glu	Asp	Arg	Glu	Ala
	65				70			75				80			
Leu	Arg	Lys	Glu	Ala	Glu	Arg	Gln	Ala	Leu	Ala	Gln	Leu	Glu	Lys	Ala
		85				90					95				
Lys	Thr	Lys	Pro	Val	Ala	Phe	Ala	Val	Arg	Thr	Asn	Val	Gly	Tyr	Asn
			100				105			110					
Pro	Ser	Pro	Gly	Asp	Glu	Val	Pro	Val	Gln	Gly	Val	Ala	Ile	Thr	Phe
		115				120			125						
Glu	Pro	Lys	Asp	Phe	Leu	His	Ile	Lys	Glu	Lys	Tyr	Asn	Asn	Asp	Trp
		130				135			140						
Trp	Ile	Gly	Arg	Leu	Val	Lys	Glu	Gly	Cys	Glu	Val	Gly	Phe	Ile	Pro
	145				150			155			160				
Ser	Pro	Val	Lys	Leu	Asp	Ser	Leu	Arg	Leu	Leu	Gln	Glu	Gln	Lys	Leu
		165				170			175						
Arg	Gln	Asn	Arg	Leu	Gly	Ser	Ser	Lys	Ser	Gly	Asp	Asn	Ser	Ser	Ser
		180				185			190						
Ser	Leu	Gly	Asp	Val	Val	Thr	Gly	Thr	Arg	Arg	Pro	Thr	Pro	Pro	Ala
	195				200			205							
Ser	Gly	Asn	Glu	Met	Thr	Asn	Leu	Ala	Phe	Glu	Leu	Asp	Pro	Leu	Glu
	210				215			220							
Leu	Glu	Glu	Glu	Ala	Glu	Leu	Gly	Glu	Gln	Ser	Gly	Ser	Ala	Lys	
	225				230			235			240				
Thr	Ser	Val	Ser	Val	Thr	Thr	Pro	Pro	Pro	His	Gly	Lys	Arg	Ile	
	245				250			255							
Pro	Phe	Phe	Lys	Lys	Thr	Glu	His	Val	Pro	Pro	Tyr	Asp	Val	Val	Pro
	260				265			270							
Ser	Met	Arg	Pro	Ile	Ile	Leu	Val	Gly	Pro	Ser	Leu	Lys	Gly	Tyr	Glu
	275				280			285							
Val	Thr	Asp	Met	Met	Gln	Lys	Ala	Leu	Phe	Asp	Phe	Leu	Lys	His	Arg
	290				295			300							
Phe	Asp	Gly	Arg	Ile	Ser	Ile	Thr	Arg	Val	Thr	Ala	Asp	Ile	Ser	Leu
	305				310			315			320				
Ala	Lys	Arg	Ser	Val	Leu	Asn	Asn	Pro	Ser	Lys	His	Ile	Ile	Glu	
	325				330			335							
Arg	Ser	Asn	Thr	Arg	Ser	Ser	Leu	Ala	Glu	Val	Gln	Ser	Glu	Ile	Glu
	340				345			350							
Arg	Ile	Phe	Glu	Leu	Ala	Arg	Thr	Leu	Gln	Leu	Val	Ala	Leu	Asp	Ala
	355				360			365							
Asp	Thr	Ile	Asn	His	Pro	Ala	Gln	Leu	Ser	Lys	Thr	Ser	Leu	Ala	Pro
	370				375			380							
Ile	Ile	Val	Tyr	Ile	Lys	Ile	Thr	Ser	Pro	Lys	Val	Leu	Gln	Arg	Leu
	385				390			395			400				
Ile	Lys	Ser	Arg	Gly	Lys	Ser	Gln	Ser	Lys	His	Leu	Asn	Val	Gln	Ile
	405				410			415							

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Ala Ala Ser Glu Lys Leu Ala Gln Cys Pro Pro Glu Met Phe Asp Ile
420 425 430

Ile Leu Asp Glu Asn Gln Leu Glu Asp Ala Cys Glu His Leu Ala Glu
435 440 445

Tyr Leu Glu Ala Tyr Trp Lys Ala Thr His Pro Pro Ser Ser Thr Pro
450 455 460

Pro Asn Pro Leu Leu Asn Arg Thr Met Ala Thr Ala Ala Leu Ala Ala
465 470 475 480

Ser Pro Ala Pro Val Ser Asn Leu Gln Val Gln Val Leu Thr Ser Leu
485 490 495

Arg Arg Asn Leu Gly Phe Trp Gly Gly Leu Glu Ser Ser Gln Arg Gly
500 505 510

Ser Val Val Pro Gln Glu Gln Glu His Ala Met
515 520

<210> SEQ ID NO 4
<211> LENGTH: 1847
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 4

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ccggccggc	ggcggggaggg	gaggctcctc	tccatggtcc	agaagaccag	catgtcccg	180
ggcccttacc	caccctccca	ggagatcccc	atggaggct	tcgacccca	ccgcagggc	240
aaatacagca	agaggaaagg	gcgattcaaa	cggtcagatg	ggagcacgtc	ctcgatacc	300
acatccaaca	gtttgtccg	ccagggctca	goggagtcct	acaccagccg	tccatcagac	360
tctgtatgtat	tcctggagga	ggaccggaa	gccttaagga	aggaagcaga	gcgccaggca	420
ttagcgcagc	tcgagaaggc	caagaccaag	ccagtggcat	ttgctgtgcg	gacaaatgtt	480
ggctacaatc	cgtctccagg	ggatgaggtg	cctgtgcagg	gagtggccat	cacccatcgag	540
cccaaact	tcctgcacat	caaggagaaa	tacaataatg	actgggtgat	cggcgccgt	600
gtgaaggagg	gctgtgaggt	tggttcatt	cccaagcccg	tcaaactgga	cagecctcg	660
ctgctgcagg	aacagaagct	gcccggagaac	cgcctcggt	ccagcaatc	aggcgataac	720
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gctgagcttgc	gtgagcagag	tggctctgac	aagacttagtgc	ttagcagtgt	caccaccccg	900
ccacccatgc	gcaaacgcac	ccccttcttt	aagaagacag	agcatgtgcc	cccctatgac	960
gtgggtccctt	ccatgaggcc	catcatctgc	gtgggaccgt	cgtcaagggt	ctacgagggt	1020
acagacatga	tgcagaaagc	tttatttgac	ttcttgaago	atcggttga	tggcaggatc	1080
tccatcactc	gtgtgacggc	agatatttcc	ctggctaagc	gctcagttct	caacaacccc	1140
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gaaatcgagc	aatcttcga	gctggcccg	acccttcagt	tggtcgtct	ggatgctgac	1260
accatcaatc	acccagccca	gctgtccaaag	acctcgctgg	ccccatcat	tgtttacatc	1320
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<210> SEQ ID NO 5

<211> LENGTH: 478

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 5

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Glu Ile Pro Met Glu Val Phe Asp Pro Ser Pro Gln Gly Lys Tyr Ser
20 25 30

Lys Arg Lys Gly Arg Phe Lys Arg Ser Asp Gly Ser Thr Ser Ser Asp
35 40 45

Thr Thr Ser Asn Ser Phe Val Arg Gln Gly Ser Ala Glu Ser Tyr Thr
50 55 60

Ser Arg Pro Ser Asp Ser Asp Val Ser Leu Glu Glu Asp Arg Glu Ala
65 70 75 80

Leu Arg Lys Glu Ala Glu Arg Gln Ala Leu Ala Gln Leu Glu Lys Ala
85 90 95

Lys Thr Lys Pro Val Ala Phe Ala Val Arg Thr Asn Val Gly Tyr Asn
100 105 110

Pro Ser Pro Gly Asp Glu Val Pro Val Gln Gly Val Ala Ile Thr Phe
115 120 125

Glu Pro Lys Asp Phe Leu His Ile Lys Glu Lys Tyr Asn Asn Asp Trp
130 135 140

Trp Ile Gly Arg Leu Val Lys Glu Gly Cys Glu Val Gly Phe Ile Pro
145 150 155 160

Ser Pro Val Lys Leu Asp Ser Leu Arg Leu Leu Gln Glu Gln Lys Leu
165 170 175

Arg Gln Asn Arg Leu Gly Ser Ser Lys Ser Gly Asp Asn Ser Ser Ser
180 185 190

Ser Leu Gly Asp Val Val Thr Gly Thr Arg Arg Pro Thr Pro Pro Ala
195 200 205

Ser Ala Lys Gln Lys Gln Lys Ser Thr Glu His Val Pro Pro Tyr Asp
210 215 220

Val Val Pro Ser Met Arg Pro Ile Ile Leu Val Gly Pro Ser Leu Lys
225 230 235 240

Gly Tyr Glu Val Thr Asp Met Met Gln Lys Ala Leu Phe Asp Phe Leu
245 250 255

Lys His Arg Phe Asp Gly Arg Ile Ser Ile Thr Arg Val Thr Ala Asp
260 265 270

Ile Ser Leu Ala Lys Arg Ser Val Leu Asn Asn Pro Ser Lys His Ile

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275	280	285
Ile Ile Glu Arg Ser Asn Thr Arg Ser Ser Leu Ala Glu Val Gln Ser		
290	295	300
Glu Ile Glu Arg Ile Phe Glu Leu Ala Arg Thr Leu Gln Leu Val Ala		
305	310	315
Leu Asp Ala Asp Thr Ile Asn His Pro Ala Gln Leu Ser Lys Thr Ser		
325	330	335
Leu Ala Pro Ile Ile Val Tyr Ile Lys Ile Thr Ser Pro Lys Val Leu		
340	345	350
Gln Arg Leu Ile Lys Ser Arg Gly Lys Ser Gln Ser Lys His Leu Asn		
355	360	365
Val Gln Ile Ala Ala Ser Glu Lys Leu Ala Gln Cys Pro Pro Glu Met		
370	375	380
Phe Asp Ile Ile Leu Asp Glu Asn Gln Leu Glu Asp Ala Cys Glu His		
385	390	395
Leu Ala Glu Tyr Leu Glu Ala Tyr Trp Lys Ala Thr His Pro Pro Ser		
405	410	415
Ser Thr Pro Pro Asn Pro Leu Leu Asn Arg Thr Met Ala Thr Ala Ala		
420	425	430
Leu Ala Ala Ser Pro Ala Pro Val Ser Asn Leu Gln Val Gln Val Leu		
435	440	445
Thr Ser Leu Arg Arg Asn Leu Gly Phe Trp Gly Gly Leu Glu Ser Ser		
450	455	460
Gln Arg Gly Ser Val Val Pro Gln Glu Gln Glu His Ala Met		
465	470	475

<210> SEQ ID NO: 6
<211> LENGTH: 1700
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<400> SEQUENCE: 6

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ccggggccggc	ggggggagggg	gaggctctc	tccatggtc	agaagaccag	catgtcccc	180
ggcccttacc	caccctccca	ggagatcccc	atggaggct	tgcacccca	cccgcagggg	240
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ttagcgcagc	tcgagaaggc	caagaccaag	ccagtggcat	ttgctgtgcg	gacaaatgtt	480
ggctacaatc	cgtctccagg	ggatgagggt	cctgtgcagg	gagtggccat	caccttcgag	540
cccaaagact	tcctgcacat	caaggagaaa	tacaataatg	actggtgat	cgggcggtcg	600
gtgaaggagg	gctgtgaggt	tggcttcatt	cccagcccc	tcaaactgga	cageccttcg	660
ctgctgcagg	aacagaagct	gcccagaac	cgccctcggt	ccagcaatc	aggcgataac	720
tccagttcca	gtctgggaga	tgtggtgact	ggcacccgcc	gccccacacc	ccctgccagt	780
gccaaacaga	agcagaagtc	gacagagcat	gtgccccct	atgacgtgg	gccttccatg	840
aggcccatca	tcctggtggg	accgtcgctc	aagggtacg	aggttacaga	catgtgcag	900

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attgagcgct ccaacacacg ctccagcctg gctgagggtgc agagtgaat cgagcgaatc 1080
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caaatacgccg cctcgaaaaa gctggcacag tgccccctg aaatgttga catcatcctg 1320
gatgagaacc aattggagga tgcctgcgag catctggcgg agtacttggaa agcctattgg 1380
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gtgccccagg agcaggaaca tgccatgttag tgggcgcctt gcccgtcttc ctcctgtctc 1620
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<210> SEQ ID NO 7

<211> LENGTH: 524

<212> TYPE: PRT

<213> ORGANISM: Mus musculus

<400> SEQUENCE: 7

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20 25 30

Lys Arg Lys Gly Arg Phe Lys Arg Ser Asp Gly Ser Thr Ser Ser Asp
35 40 45

Thr Thr Ser Asn Ser Phe Val Arg Gln Gly Ser Ala Glu Ser Tyr Thr
50 55 60

Ser Arg Pro Ser Asp Ser Asp Val Ser Leu Glu Asp Arg Glu Ala
65 70 75 80

Leu Arg Lys Glu Ala Glu Arg Gln Ala Leu Ala Gln Leu Glu Lys Ala
85 90 95

Lys Thr Lys Pro Val Ala Phe Ala Val Arg Thr Asn Val Gly Tyr Asn
100 105 110

Pro Ser Pro Gly Asp Glu Val Pro Val Gln Gly Val Ala Ile Thr Phe
115 120 125

Glu Pro Lys Asp Phe Leu His Ile Lys Glu Lys Tyr Asn Asn Asp Trp
130 135 140

Trp Ile Gly Arg Leu Val Lys Glu Gly Cys Glu Val Gly Phe Ile Pro
145 150 155 160

Ser Pro Val Lys Leu Asp Ser Leu Arg Leu Leu Gln Glu Gln Thr Leu
165 170 175

Arg Gln Asn Arg Leu Ser Ser Ser Lys Ser Gly Asp Asn Ser Ser Ser
180 185 190

Ser Leu Gly Asp Val Val Thr Gly Thr Arg Arg Pro Thr Pro Pro Ala
195 200 205

Ser Gly Asn Glu Met Thr Asn Phe Ala Phe Glu Leu Asp Pro Leu Glu

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210	215	220	
Leu Glu Glu Glu Ala Glu Leu Gly Glu His Gly Ser Ala Lys			
225	230	235	240
Thr Ser Val Ser Ser Val Thr Pro Pro Pro His Gly Lys Arg Ile			
245	250	255	
Pro Phe Phe Lys Lys Thr Glu His Val Pro Pro Tyr Asp Val Val Pro			
260	265	270	
Ser Met Arg Pro Ile Ile Leu Val Gly Pro Ser Leu Lys Gly Tyr Glu			
275	280	285	
Val Thr Asp Met Met Gln Lys Ala Leu Phe Asp Phe Leu Lys His Arg			
290	295	300	
Phe Asp Gly Arg Ile Ser Ile Thr Arg Val Thr Ala Asp Ile Ser Leu			
305	310	315	320
Ala Lys Arg Ser Val Leu Asn Asn Pro Ser Lys His Ile Ile Ile Glu			
325	330	335	
Arg Ser Asn Thr Arg Ser Ser Leu Ala Glu Val Gln Ser Glu Ile Glu			
340	345	350	
Arg Ile Phe Glu Leu Ala Arg Thr Leu Gln Leu Val Ala Leu Asp Ala			
355	360	365	
Asp Thr Ile Asn His Pro Ala Gln Leu Ser Lys Thr Ser Leu Ala Pro			
370	375	380	
Ile Ile Val Tyr Ile Lys Ile Thr Ser Pro Lys Val Leu Gln Arg Leu			
385	390	395	400
Ile Lys Ser Arg Gly Lys Ser Gln Ser Lys His Leu Asn Val Gln Ile			
405	410	415	
Ala Ala Ser Glu Lys Leu Ala Gln Cys Pro Pro Glu Met Phe Asp Ile			
420	425	430	
Ile Leu Asp Glu Asn Gln Leu Glu Asp Ala Cys Glu His Leu Ala Glu			
435	440	445	
Tyr Leu Glu Ala Tyr Trp Lys Ala Thr His Pro Pro Ser Ser Thr Pro			
450	455	460	
Pro Asn Pro Leu Leu Asn Arg Thr Met Ala Thr Ala Ala Leu Ala Ala			
465	470	475	480
Ser Pro Ala Pro Val Ser Asn Leu Gln Val Gln Val Leu Thr Ser Leu			
485	490	495	
Arg Arg Asn Leu Ser Phe Trp Gly Gly Leu Glu Ala Ser Pro Arg Gly			
500	505	510	
Gly Asp Ala Val Ala Gln Pro Gln Glu His Ala Met			
515	520		

<210> SEQ ID NO 8

<211> LENGTH: 1892

<212> TYPE: DNA

<213> ORGANISM: Mus musculus

<400> SEQUENCE: 8

ttccggcggc	ggggggggcg	acggcgccag	cggccgcaga	gagcacagcg	cgagccggga	60
gggcaagcaa	ggcgccgagc	gtgcagccgg	aggtcagct	gggagactgc	accgggtgt	120
ggctgcgcga	cgccgcgcgt	ctctgggtctc	ggacggccctc	tcccatgcgc	tgagagcgcc	180
cggctgggt	gggagggcgg	ccggacccgga	ggatcctctc	catggtccag	aagagcggca	240
tgtccccgggg	cccttaccca	ccttcccaag	agatccctat	ggaggtcttc	gaccccagcc	300

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cacaggcaa	gtacagcaag	aggaaaggc	ggttcaaaag	gtcagacggg	agtacgtct	360
cggataacaac	atccaacacgc	ttcgtccgcc	agggctcago	agagtcctac	acgagccgac	420
catcagactc	tgatgtgtct	ctggaggagg	accgggaago	cttaaggaaag	gaggcagagc	480
gccaggcctt	agcccagctc	gagaagcca	agaccaaacc	agtggcttt	gctgttcgga	540
caaatgttgg	ctacaatccg	tctccagggg	atgaggtgc	tgtacaggga	gtggccatca	600
ccttgagcc	caaggacttc	ctacacatca	aggagaagta	caataatgac	tggtgattg	660
ggcggcttgt	gaaggaaggc	tgcgagggtt	gcttcatccc	cagcccggtc	aaactggaca	720
gccttcgtct	gctgcaggaa	cagaccctgc	gccagaaccg	cctcagtc	agcaagtca	780
gtgacaactc	cagttccagt	ctgggagatg	tggtgactgg	cacccggcc	cccacacccc	840
ctgcccagtgg	taatgaaaatg	actaacttttgc	cctttgagct	agaccccta	gagtttagagg	900
aggaggaggc	agagctaggg	gagcacggcg	gctcagccaa	gactagcgtg	agcagtgtca	960
ccacgcgccc	acccccacggc	aagegcaccc	ccttctttaa	gaagacagag	cacgtgcccc	1020
cctatgacgt	ggtgcccttcc	atgaggccca	tcatctgtt	gggaccgtcg	ctcaaggggct	1080
atgaggtgac	agacatgtat	cagaaagcgt	tgtttgactt	cctcaagcat	cggtttgatg	1140
gcaggatttc	catcacccgg	gtaacagctg	acattccct	ggccaaacgc	tccgtccctca	1200
acaacccacag	caaacacatc	atcattgagc	gctccaacac	gcgttccagc	ctggctgagg	1260
tacagagtga	aattgaggg	atcttcgagc	tggccoggac	cttgcagctg	gtcgcccttg	1320
acgctgacac	catcaaccac	ccagcccagc	tctctaaaac	gtcgctggcc	cccatcattg	1380
tttacatcaa	gatcacatct	cccaaggatc	tgcagaggct	catcaaattcc	cgaggaaagt	1440
ctcaatccaa	acacctcaat	gtccaaatag	cagcctcgga	gaagctggca	cagtgtcccc	1500
ccgaaatgtt	tgacataatc	ctggacgaga	accaatttgg	agatgcctgc	gagcacctgg	1560
ctgagttactt	ggaaggctac	tggaggcaca	cacatccgccc	tagcagcacg	ccacccaatc	1620
cgctgctgaa	ccgcaccatg	gctaccgcag	ctctggctgc	cagccctgcc	cccgctctca	1680
acctccaggt	acaggtgctc	acctcgctca	ggagaaatct	cagttctgg	ggcgggctgg	1740
aggcctcacc	ggggggaggc	gacgcgggtgg	cccagcctca	ggagcacgcc	atgtagccg	1800
tgtccctctg	gtcttccttc	ccaccctgg	gtgcaggaa	catgaggaag	gaagggaaaga	1860
gctttatttt	gtaaaaaacg	tggtgagcgg	ca			1892

<210> SEQ ID NO 9

<211> LENGTH: 597

<212> TYPE: PRT

<213> ORGANISM: Mus musculus

<400> SEQUENCE: 9

Met	Val	Gln	Lys	Ser	Gly	Met	Ser	Arg	Gly	Pro	Tyr	Pro	Pro	Ser	Gln
1				5			10							15	

Glu	Ile	Pro	Met	Glu	Val	Phe	Asp	Pro	Ser	Pro	Gln	Gly	Lys	Tyr	Ser
			20			25							30		

Lys	Arg	Lys	Gly	Arg	Phe	Lys	Arg	Ser	Asp	Gly	Ser	Thr	Ser	Ser	Asp
					35			40				45			

Thr	Thr	Ser	Asn	Ser	Phe	Val	Arg	Gln	Gly	Ser	Ala	Glu	Ser	Tyr	Thr
					50			55				60			

Ser	Arg	Pro	Ser	Asp	Ser	Asp	Val	Ser	Leu	Glu	Glu	Asp	Arg	Glu	Ala
-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----

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65	70	75	80
Leu Arg Lys Glu Ala Glu Arg Gln Ala Leu Ala Gln Leu Glu Lys Ala			
85	90	95	
Lys Thr Lys Pro Val Ala Phe Ala Val Arg Thr Asn Val Gly Tyr Asn			
100	105	110	
Pro Ser Pro Gly Asp Glu Val Pro Val Gln Gly Val Ala Ile Thr Phe			
115	120	125	
Glu Pro Lys Asp Phe Leu His Ile Lys Glu Lys Tyr Asn Asn Asp Trp			
130	135	140	
Trp Ile Gly Arg Leu Val Lys Glu Gly Cys Glu Val Gly Phe Ile Pro			
145	150	155	160
Ser Pro Val Lys Leu Asp Ser Leu Arg Leu Leu Gln Glu Gln Thr Leu			
165	170	175	
Arg Gln Asn Arg Leu Ser Ser Ser Lys Ser Gly Asp Asn Ser Ser Ser			
180	185	190	
Ser Leu Gly Asp Val Val Thr Gly Thr Arg Arg Pro Thr Pro Pro Ala			
195	200	205	
Ser Ala Lys Gln Lys Gln Lys Ser Thr Glu His Val Pro Pro Tyr Asp			
210	215	220	
Val Val Pro Ser Met Arg Pro Ile Ile Leu Val Gly Pro Ser Leu Lys			
225	230	235	240
Gly Tyr Glu Val Thr Asp Met Met Gln Lys Ala Leu Phe Asp Phe Leu			
245	250	255	
Lys His Arg Phe Asp Gly Arg Ile Ser Ile Thr Arg Val Thr Ala Asp			
260	265	270	
Ile Ser Leu Ala Lys Arg Ser Val Leu Asn Asn Pro Ser Lys His Ile			
275	280	285	
Ile Ile Glu Arg Ser Asn Thr Arg Ser Ser Leu Ala Glu Val Gln Ser			
290	295	300	
Glu Ile Glu Arg Ile Phe Glu Leu Ala Arg Thr Leu Gln Leu Val Ala			
305	310	315	320
Leu Asp Ala Asp Thr Ile Asn His Pro Ala Gln Leu Ser Lys Thr Ser			
325	330	335	
Leu Ala Pro Ile Ile Val Tyr Ile Lys Ile Thr Ser Pro Lys Val Leu			
340	345	350	
Gln Arg Leu Ile Lys Ser Arg Gly Lys Ser Gln Ser Lys His Leu Asn			
355	360	365	
Val Gln Ile Ala Ala Ser Glu Lys Leu Ala Gln Cys Pro Pro Glu Met			
370	375	380	
Phe Asp Ile Ile Leu Asp Glu Asn Gln Leu Glu Asp Ala Cys Glu His			
385	390	395	400
Leu Ala Glu Tyr Leu Glu Ala Tyr Trp Lys Ala Thr His Pro Pro Ser			
405	410	415	
Ser Thr Pro Pro Asn Pro Leu Leu Asn Arg Thr Met Ala Thr Ala Ala			
420	425	430	
Leu Ala Ala Ser Pro Ala Pro Val Ser Asn Leu Gln Gly Pro Tyr Leu			
435	440	445	
Ala Ser Gly Asp Gln Pro Leu Asp Arg Ala Thr Gly Glu His Ala Ser			
450	455	460	
Val His Glu Tyr Pro Gly Glu Leu Gly Gln Pro Pro Gly Leu Tyr Pro			
465	470	475	480

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Ser Asn His Pro Leu Gly Arg Ala Gly Thr Leu Arg Ala Leu Ser Arg
485 490 495

Gln Asp Thr Phe Asp Ala Asp Thr Pro Gly Ser Arg Asn Ser Ala Tyr
500 505 510

Thr Glu Pro Gly Asp Ser Cys Val Asp Met Glu Thr Asp Pro Ser Glu
515 520 525

Gly Pro Gly Pro Gly Asp Pro Ala Gly Gly Thr Pro Pro Ala Arg
530 535 540

Gln Gly Ser Trp Glu Asp Glu Glu Asp Tyr Glu Glu Glu Met Thr Asp
545 550 555 560

Asn Arg Asn Arg Gly Arg Asn Lys Ala Arg Tyr Cys Ala Glu Gly Gly
565 570 575

Gly Pro Val Leu Gly Arg Asn Lys Asn Glu Leu Glu Gly Trp Gly Gln
580 585 590

Gly Val Tyr Thr Arg
595

<210> SEQ ID NO 10

<211> LENGTH: 1794

<212> TYPE: DNA

<213> ORGANISM: Mus musculus

<400> SEQUENCE: 10

atggtccaga agagcggcat	gtccccgggc ccttacccac	cttcccaaga gatccctatg	60
gagggtttcg accccagccc	acagggaag tacagcaaga	ggaaaggcg gttcaaaaagg	120
tca gagcggga	gtacgttcctc ggataacaaca	tccaacagct tctgtccgcca	180
gagtcctaca cgagccgacc	atcagactct gatgtgtctc	tggaggagga ccgcgaagcc	240
ttaaggaaagg aggca gagcgc	ccaggccta gcccagctcg	agaaagccaa gaccaa accca	300
gtggcttttg ctgttcggac	aaatgttgc tacaatccgt	ctccaggggg tgaggtgcct	360
gtacagggag tggccatcac	ctttgagecc aaggacttcc	tacacatcaa ggagaagtac	420
aataatgact ggtggattgg	gccccgttgc aactggacag	cgagggatgg cttcatcccc	480
agcccggtca aactggacag	ccttcgtctg ctgcaggaac	agaccctgcg ccagaaccgc	540
ctcagctcca gcaagtcagg	tgacaactcc agttccagtc	tgggagatgt ggtgactggc	600
acccgcggcc ccacaccccc	tgcccaatgccc aaacagaagc	agaaatcgac agacacgttg	660
cccccttatg acgtggtgcc	ttccatgagg cccatcatcc	tggggacc gtcgctcaag	720
ggctatgagg tgacagacat	gatgcagaaa gcttttttg	acttcctcaa gcatcggtt	780
gatggcagga ttccatcac	ccgggtaaca gctgacattt	ccctggccaa acgtccgtc	840
ctcaacaacc ccagcaaaca	catcatcatt gagcgttcca	acacgcgttc cagectggct	900
gaggatcaga gtgaaattga	gaggatctc gagctggccc	ggaccttgca gctggtcgoc	960
ttggacgctg acaccatcaa	ccacccagcc cagctctcta	aaacgtcgct ggccccatc	1020
attgtttaca tcaagatcac	atctcccaag gtactgcaga	ggctcatcaa atcccgagg	1080
aagtctcaat ccaaacaccc	aatgtccaa atagcagcc	cgagaaagct ggcacagtgt	1140
ccccccgaaa tggttgcacat	aatctggac gagaaccaat	tggaagatgc ctgcgagcac	1200
ctggctgagt acttggaaagc	ctactggaaag gccacacato	cgccctagcag cacgccaccc	1260
aatccgctgc tgaaccgcac	catggctacc gcaagctctgg	ctgcccagccc tgccccgtc	1320

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tccaaacctcc agggacccta ccttgcttcc ggggaccaga cgctggaccg ggccactggg 1380
gaacatgcca gtgtgcacga gtaccccggg gaattgggcc agccccagg cctttacccc 1440
agcaaccacc cacttggccg ggcaggcacc ctgcgggcgc tatccgcca agacaccttt 1500
gatgctgaca ccccccggcag ccgaaattct gcctaacacgg agccgggaga ctcgtgtgtg 1560
gacatggaga cagaccctc agagggccca gggcctggag accctgcagg gggaggcaca 1620
ccaccagccc ggcagggctc ctggaaagac gaggaagact atgaggagga gatgaccgac 1680
aacaggaacc gggccggaa taaggccgc tactgtgcgg agggtgtgg gccggttctg 1740
gggcgcaata agaatgagct ggagggctgg ggacaaggcg tctacactcg ctga 1794
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<210> SEQ ID NO 11

<211> LENGTH: 457

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 11

Met	Thr	Thr	Asp	Glu	Gly	Ala	Lys	Asn	Asn	Glu	Glu	Ser	Pro	Thr	Ala
1				5		10							15		

Thr	Val	Ala	Glu	Gln	Gly	Glu	Asp	Ile	Thr	Ser	Lys	Lys	Asp	Arg	Gly
	20					25							30		

Val	Leu	Lys	Ile	Val	Lys	Arg	Val	Gly	Asn	Gly	Glu	Glu	Thr	Pro	Met
		35			40								45		

Ile	Gly	Asp	Lys	Val	Tyr	Val	His	Tyr	Lys	Gly	Lys	Leu	Ser	Asn	Gly
50			55									60			

Lys	Lys	Phe	Asp	Ser	Ser	His	Asp	Arg	Asn	Glu	Pro	Phe	Val	Phe	Ser
65							70			75			80		

Leu	Gly	Lys	Gly	Gln	Val	Ile	Lys	Ala	Trp	Asp	Ile	Gly	Val	Ala	Thr
					85				90				95		

Met	Lys	Lys	Gly	Ile	Cys	His	Leu	Leu	Cys	Lys	Pro	Glu	Tyr	Ala
				100			105				110			

Tyr	Gly	Ser	Ala	Gly	Ser	Leu	Pro	Lys	Ile	Pro	Ser	Asn	Ala	Thr	Leu
115						120						125			

Phe	Phe	Glu	Ile	Glu	Leu	Leu	Asp	Phe	Lys	Gly	Glu	Asp	Leu	Phe	Glu
130						135						140			

Asp	Gly	Gly	Ile	Ile	Arg	Arg	Thr	Lys	Arg	Lys	Gly	Glu	Gly	Tyr	Ser
145					150			155					160		

Asn	Pro	Asn	Glu	Gly	Ala	Thr	Val	Glu	Ile	His	Leu	Glu	Gly	Arg	Cys
					165			170				175			

Gly	Gly	Arg	Met	Phe	Asp	Cys	Arg	Asp	Val	Ala	Phe	Thr	Val	Gly	Glu
			180			185					190				

Gly	Glu	Asp	His	Asp	Ile	Pro	Ile	Gly	Ile	Asp	Lys	Ala	Leu	Glu	Lys
195					200			205				205			

Met	Gln	Arg	Glu	Glu	Gln	Cys	Ile	Leu	Tyr	Leu	Gly	Pro	Arg	Tyr	Gly
					210		215		220						

Phe	Gly	Glu	Ala	Gly	Lys	Pro	Lys	Phe	Gly	Ile	Glu	Pro	Asn	Ala	Glu
225					230			235			240				

Leu	Ile	Tyr	Glu	Val	Thr	Leu	Lys	Ser	Phe	Glu	Lys	Ala	Lys	Glu	Ser
				245			250			255			255		

Trp	Glu	Met	Asp	Thr	Lys	Glu	Lys	Leu	Glu	Gln	Ala	Ala	Ile	Val	Lys
				260			265			270			270		

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Glu	Lys	Gly	Thr	Val	Tyr	Phe	Lys	Gly	Gly	Lys	Tyr	Met	Gln	Ala	Val
275				280						285					
Ile	Gln	Tyr	Gly	Lys	Ile	Val	Ser	Trp	Leu	Glu	Met	Glu	Tyr	Gly	Leu
290					295				300						
Ser	Glu	Lys	Glu	Ser	Lys	Ala	Ser	Glu	Ser	Phe	Leu	Leu	Ala	Ala	Phe
305					310				315				320		
Leu	Asn	Leu	Ala	Met	Cys	Tyr	Leu	Lys	Leu	Arg	Glu	Tyr	Thr	Lys	Ala
325							330			335					
Val	Glu	Cys	Cys	Asp	Lys	Ala	Leu	Gly	Leu	Asp	Ser	Ala	Asn	Glu	Lys
340							345			350					
Gly	Leu	Tyr	Arg	Arg	Gly	Glu	Ala	Gln	Leu	Leu	Met	Asn	Glu	Phe	Glu
355							360			365					
Ser	Ala	Lys	Gly	Asp	Phe	Glu	Lys	Val	Leu	Glu	Val	Asn	Pro	Gln	Asn
370							375			380					
Lys	Ala	Ala	Arg	Leu	Gln	Ile	Ser	Met	Cys	Gln	Lys	Lys	Ala	Lys	Glu
385							390			395			400		
His	Asn	Glu	Arg	Asp	Arg	Arg	Ile	Tyr	Ala	Asn	Met	Phe	Lys	Lys	Phe
405								410					415		
Ala	Glu	Gln	Asp	Ala	Lys	Glu	Glu	Ala	Asn	Lys	Ala	Met	Gly	Lys	Lys
420							425			430					
Thr	Ser	Glu	Gly	Val	Thr	Asn	Glu	Lys	Gly	Thr	Asp	Ser	Gln	Ala	Met
435							440			445					
Glu	Glu	Glu	Lys	Pro	Glu	Gly	His	Val							
450							455								

<210> SEQ ID NO 12

<211> LENGTH: 3781

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 12

ggggccggctc	cgccggcgctg	ccagtcctgg	ggggcggtgt	ccggcgccgcg	ggccggccctgc	60
tgggcgggct	gaagggttag	cggagcacgg	gcaaggcgga	gagtgcacgg	gtcggcgagc	120
cccccgccgc	acagggtctc	tacttaaaag	acaatgacta	ctgtatgcgg	tgccaagaac	180
aatgaagaaa	gccccacagc	cactgttgct	gagcagggag	aggatattac	ctccaaaaaa	240
gacagggggag	tattaaagat	tgtcaaaaga	gtggggatg	gtgaggaaac	gccgatgatt	300
ggagacaaaag	tttatgtcca	ttacaaagga	aaattgtcaa	atggaaagaa	gtttgattcc	360
agtcatgata	gaaatgaacc	atttgtctt	agtcttggca	aaggccaagt	catcaaggca	420
tggggacattt	gggtggctac	catgaagaaa	ggagagatat	gccatattact	gtgcaaacc	480
gaatatgcat	atggctcgcc	tggcagtctc	cctaaaattc	cctcgaatgc	aactctcttt	540
tttgagattt	agtccttga	tttcaaaagga	gaggattat	ttgaatgg	aggcattttc	600
cgagaaacca	aacggaaagg	agagggatat	tcaaattccaa	acgaaggaggc	aacagttagaa	660
atccacctgg	aggccgctg	tggtggaaagg	atgtttgact	gcagagatgt	ggcatttact	720
gtggggcgaag	gagaagacca	cgacattcca	attggatttgc	acaagctct	ggagaaaaatg	780
cagcgggaag	aacaatgtat	tttatatctt	ggaccaagat	atggtttgg	agaggcagg	840
aagcctaaat	ttggcattga	acctaattgt	gagcttataat	atgaatgtac	acttaagaggc	900
ttcgaaaagg	ccaaagaatc	ctgggagatg	gataccaaag	aaaaatttgg	gcaggctgcc	960

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atgtcaaaag agaagggAAC cgtataactc aaggaggca aatacatgca ggccgttatt	1020
cagtagggga agatagtgtc ctggtagag atgaaatatg gtttattcaga aaaggaatcg	1080
aaagttctg aatcatttct ccttgctgcc tttctgaacc tggccatgtc ctacctgaag	1140
cttagagaat acaccaaAGC tgTTGAATGc tgtgacaagg ccctggact ggacagtGCC	1200
aatgagaaag gcttGTatAG gaggggtgaa gcccagCTGC tcatgaacGA gtttGAGTCA	1260
gccaagggtg actttgagaa agtgctggaa gtaaACCCCC agaataaggc tgcaagACTG	1320
cagatctcca tgTGCcAGAA aaaggCCAAG gagcacaACG agcgggaccG caggataAC	1380
gccaacatgt tcaagaAGTT tgCAGAGCAG gatGCCAAGG aagaggCCAA taaAGCAATG	1440
ggcaagaAGA ctTCAGAAGG ggtcactaat gaaaaAGGAAG cagacAGTCA agcaatGGAA	1500
gaagagaaAC ctgaggGCCA cgtatgacGC cacGCCAAGG agggAAAGAGT cccAGTGAAC	1560
tCGGCCCCCTC ctcaatGGGC ttTCCCCCAA ctcaggACAG aacAGTGTtT aatgtAAAGT	1620
ttgttatAGT ctatgtGATT ctggAAAGCA atggCAAAAC cAGTAGCTC CAAAAAACAG	1680
ccccCTGCT gctGCCCGGA gggTTCactG agggGTGGCA CGGGACCCT ccAGGTGGAA	1740
caaACAGAAA tgactgtGGT gtggaggGGAG tgAGCCAGCA gCTTAAGTCC agTCATTTC	1800
agTTCTATC AACCTCAAG tatCCAAATC aggGTCCCTG gagATCATCC taACAATGTG	1860
gggCTGTTAG gTTTACCTT tgaACTTTCA tagCACTGCA gaaACCTTTT aaaaaaaaaAT	1920
gCTTCATGAA ttTCTCTTT CCTACAGTTG ggtAGGGTAG gggAAAGGAGG ataAGCTTT	1980
gtTTTTAAAG tgactGAAGT gCTATAAATG tagtCTGTG tagtCTGTG catTTTAAC caACAGAAC	2040
cacAGTAGAG gggTCTCATG tCTCCCCAGT tCCACAGCAG tGTCACAGAC gtGAAAGCCA	2100
gaACCTCAGA ggCCACTTGC ttGCTGACTT AGCCTCCTC CAAAGTCCCC CTCTCAGCC	2160
AGCCTCCTTG tgAGAGTGGC ttTCTACAC ACACAGCCTG TCCCTGGGG AGTAATTCTG	2220
TCATTCTAA AACACCCCTC AGCAATGATA ATGAGCAGAT GAGAGTTCT GGATTAGTT	2280
ttcCTATTTT CGATGAAGTT CTGAGATACT GAAATGTGAA AAGAGCAATC AGAATTGTG	2340
ttttCTCCC CTCTCTATT CCTTTAGGG AATAATATTG AATAACAGT ACTTCCTCCC	2400
AGCATTGCTA CTGCTAGCT TCTTCTTCA TTCTAATCCT TGCTATTAG AATTAAAGAC	2460
ttgtGCTTAC AATATTTTG ACCTGGAGTG GATCTATTAGT CATAGTCATT TAGGATCCAT	2520
gcAGCTTTT ttGTCTTTT AAGATTATTG GCTCATAAGC ATATGTATAC TGGTTTATGG	2580
AACCTTATT ACACCTCT ATCATGCAAA AAAATTTGA CCTTTAGTA CTAAGCTAA	2640
ttttAAAAAA CAAAATCTGT AGTGTGACA AATAAATAGT TGCTCTCTA CACTAGGGT	2700
ttcacCTGCA ggtttGACAC gcAGTTGCTC gttttCCTG CCCTGTCAAG CTTCTCTGTT	2760
ctggcGTGAG ttGTGAAAGA gttGAAGACA gtttCCATG CGGGTACACA GCCAGTAGCC	2820
taaATCTCCA tGACTTGAGC tgACCATTGA ACTAGGGCAA GTCTTAAATG TGTACATGTA	2880
gttGAATTTC AGTCCTTACG ggtAAACAGA ttGAGCATGG CTCTCTATTG CCTCAGCCTA	2940
AGAAACACTC ATGGGAATGC ATTGGCAAC CCAAGGAACC ATTGCTTAA ACCTGGAAACA	3000
tctCACCTTT ttaaATCCTA AAAACACTG GCAGTTATAT tttAAATTAG tttttATTTT	3060
tatGATGGTT ttATCAAAG ACTTTTATTAG ttagATTGGG ACCCCCTCA AACCTAAAAA	3120
tcaAGTTTATT CTCTTTATA ATACCTTCTC TCCCCATGGA ACAAAATGGGA TCAATTG	3180
agTTTTTCC ttaATGATA ACTAAATCC CTCTAATTTC TCATTTATGC ttttGCTTT	3240

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tttatgaaat atttcttta aaagccccag ttcacccatc gaaatatgaa gagcaaaaagc	3300
tgattttgt tacttgcata actgttggga aagctctgta gagcatgggt ccagtggagc	3360
caagattgaa atttgatact aaaaaggcca cctagttt tgccgataac aaacaagaaa	3420
gctattccaa gactcagatg atgccagctg tctcccacgt gtgtattatg gttcaccagg	3480
gggaaactggc aaaagtgtgt gtggggaggg gaagggtgtg tgagtggttc tgagcaaata	3540
actacagggt gcccattacc actcaagaag acacttcacg tattcttgcata tcaaattcaa	3600
taatcttaaa caatttgtgt agaagtccac agacatctt caaccacctt ttaggctgca	3660
tatggattgc caagtcagca tatgaggaat taaagacatt gttttaaaaa aaaaaaaaaatc	3720
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a	3781

<210> SEQ ID NO 13

<211> LENGTH: 456

<212> TYPE: PRT

<213> ORGANISM: Mus musculus

<400> SEQUENCE: 13

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Thr Met Thr Glu Gln Gly Glu Asp Ile Thr Thr Lys Lys Asp Arg Gly			
20	25	30	

Val Leu Lys Ile Val Lys Arg Val Gly Thr Ser Asp Glu Ala Pro Met			
35	40	45	

Phe Gly Asp Lys Val Tyr Val His Tyr Lys Gly Met Leu Ser Asp Gly			
50	55	60	

Lys Lys Phe Asp Ser Ser His Asp Arg Lys Lys Pro Phe Ala Phe Ser			
65	70	75	80

Leu Gly Gln Gly Gln Val Ile Lys Ala Trp Asp Ile Gly Val Ser Thr			
85	90	95	

Met Lys Lys Gly Glu Ile Cys His Leu Leu Cys Lys Pro Glu Tyr Ala			
100	105	110	

Tyr Gly Ser Ala Gly His Leu Gln Lys Ile Pro Ser Asn Ala Thr Leu			
115	120	125	

Phe Phe Glu Ile Glu Leu Leu Asp Phe Lys Gly Glu Asp Leu Phe Glu			
130	135	140	

Asp Ser Gly Val Ile Arg Arg Ile Lys Arg Lys Gly Glu Gly Tyr Ser			
145	150	155	160

Asn Pro Asn Glu Gly Ala Thr Val Lys Val His Leu Glu Gly Cys Cys			
165	170	175	

Gly Gly Arg Thr Phe Asp Cys Arg Asp Val Val Phe Val Val Gly Glu			
180	185	190	

Gly Glu Asp His Asp Ile Pro Ile Gly Ile Asp Lys Ala Leu Val Lys			
195	200	205	

Met Gln Arg Glu Glu Gln Cys Ile Leu Tyr Leu Gly Pro Arg Tyr Gly			
210	215	220	

Phe Gly Glu Ala Gly Lys Pro Lys Phe Gly Ile Asp Pro Asn Ala Glu			
225	230	235	240

Leu Met Tyr Glu Val Thr Leu Lys Ser Phe Glu Lys Ala Lys Glu Ser			
245	250	255	

-continued

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Trp Glu Met Asp Thr Lys Glu Lys Leu Thr Gln Ala Ala Ile Val Lys
260           265           270

Glu Lys Gly Thr Val Tyr Phe Lys Gly Gly Lys Tyr Thr Gln Ala Val
275           280           285

Ile Gln Tyr Arg Lys Ile Val Ser Trp Leu Glu Met Glu Tyr Gly Leu
290           295           300

Ser Glu Lys Glu Ser Lys Ala Ser Glu Ser Phe Leu Leu Ala Ala Phe
305           310           315           320

Leu Asn Leu Ala Met Cys Tyr Leu Lys Leu Arg Glu Tyr Asn Lys Ala
325           330           335

Val Glu Cys Cys Asp Lys Ala Leu Gly Leu Asp Ser Ala Asn Glu Lys
340           345           350

Gly Leu Tyr Arg Arg Gly Glu Ala Gln Leu Leu Met Asn Asp Phe Glu
355           360           365

Ser Ala Lys Gly Asp Phe Glu Lys Val Leu Ala Val Asn Pro Gln Asn
370           375           380

Arg Ala Ala Arg Leu Gln Ile Ser Met Cys Gln Arg Lys Ala Lys Glu
385           390           395           400

His Asn Glu Arg Asp Arg Arg Val Tyr Ala Asn Met Phe Lys Lys Phe
405           410           415

Ala Glu Arg Asp Ala Lys Glu Glu Ala Ser Lys Ala Gly Ser Lys Lys
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Glu Gly Lys Ala Lys Gly His Val
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<210> SEQ ID NO 14
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<212> TYPE: PRT
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 14

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20          25           30

Val Leu Lys Ile Val Lys Arg Val Gly Thr Ser Asp Glu Ala Pro Met
35          40           45

Phe Gly Asp Lys Val Tyr Val His Tyr Lys Gly Met Leu Ser Asp Gly
50          55           60

Lys Lys Phe Asp Ser Ser His Asp Arg Lys Lys Pro Phe Ala Phe Ser
65          70           75           80

Leu Gly Gln Gly Gln Val Ile Lys Ala Trp Asp Ile Gly Val Ser Thr
85          90           95

Met Lys Lys Gly Glu Ile Cys His Leu Leu Cys Lys Pro Glu Tyr Ala
100         105          110

Tyr Gly Ser Ala Gly His Leu Gln Lys Ile Pro Ser Asn Ala Thr Leu
115         120          125

Phe Phe Glu Ile Glu Leu Leu Asp Phe Lys Gly Glu Asp Leu Phe Glu
130         135          140

Asp Ser Gly Val Ile Arg Arg Ile Lys Arg Lys Gly Glu Gly Tyr Ser
145         150          155          160

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-continued

Asn Pro Asn Glu Gly Ala Thr Val Lys Val His Leu Glu Gly Cys Cys
 165 170 175

 Gly Gly Arg Thr Phe Asp Cys Arg Asp Val Val Phe Val Val Gly Glu
 180 185 190

 Gly Glu Asp His Asp Ile Pro Ile Gly Ile Asp Lys Ala Leu Val Lys
 195 200 205

 Met Gln Arg Glu Glu Gln Cys Ile Leu Tyr Leu Gly Pro Arg Tyr Gly
 210 215 220

 Phe Gly Glu Ala Gly Lys Pro Lys Phe Gly Ile Asp Pro Asn Ala Glu
 225 230 235 240

 Leu Met Tyr Glu Val Thr Leu Lys Ser Phe Glu Lys Ala Lys Glu Ser
 245 250 255

 Trp Glu Met Asp Thr Lys Glu Lys Leu Thr Gln Ala Ala Ile Val Lys
 260 265 270

 Glu Lys Gly Thr Val Tyr Phe Lys Gly Gly Lys Tyr Thr Gln Ala Val
 275 280 285

 Ile Gln Tyr Arg Lys Ile Val Ser Trp Leu Glu Met Glu Tyr Gly Leu
 290 295 300

 Ser Glu Lys Glu Ser Lys Ala Ser Glu Ser Phe Leu Leu Ala Ala Phe
 305 310 315 320

 Leu Asn Leu Ala Met Cys Tyr Leu Lys Leu Arg Glu Tyr Asn Lys Ala
 325 330 335

 Val Glu Cys Cys Asp Lys Ala Leu Gly Leu Asp Ser Ala Asn Glu Lys
 340 345 350

 Gly Leu Tyr Arg Arg Gly Glu Ala Gln Leu Leu Met Asn Asp Phe Glu
 355 360 365

 Ser Ala Lys Gly Asp Phe Glu Lys Val Leu Ala Val Asn Pro Gln Asn
 370 375 380

 Arg Ala Ala Arg Leu Gln Ile Ser Met Cys Gln Arg Lys Ala Lys Glu
 385 390 395 400

 His Asn Glu Arg Asp Arg Arg Val Tyr Ala Asn Met Phe Lys Lys Phe
 405 410 415

 Ala Glu Arg Asp Ala Lys Glu Glu Ala Ser Lys Ala Gly Ser Lys Lys
 420 425 430

 Ala Val Glu Gly Ala Ala Gly Lys Gln His Glu Ser Gln Ala Met Glu
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 Glu Gly Lys Ala Lys Gly His Val
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<400> SEQUENCE: 15

ggagaaguac aaauaaugact t

21

<210> SEQ ID NO 16
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 <213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 16

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21

<210> SEQ ID NO 17

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<213> ORGANISM: Artificial Sequence

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21

<210> SEQ ID NO 18

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ugccaaagca uagcuagggtt

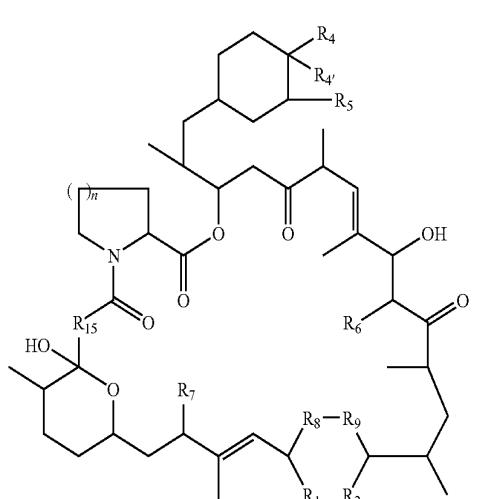
20

What is claimed is:

1. A purified complex comprising an immunophilin ligand, and one or both of (i) an immunophilin or a functional fragment thereof and/or (ii) a calcium channel subunit or a functional fragment thereof.

2. The purified complex of claim 1, wherein the immunophilin ligand is a rapamycin analogue having a heteroatom substituent at positions 1 and 4 of the rapamycin backbone.

3. The purified complex of claim 1, wherein the immunophilin ligand is a rapamycin analogue having the formula I:



wherein:

R₁ and R₂ are different, independent groups and are selected from the group consisting of OR₃ and N(R₃) (R_{3''}); or

R₁ and R₂ are different, are connected through a single bond, and are selected from the group consisting of O and NR₃;

R₃, R_{3'}, and R_{3''} are independently selected from the group consisting of H, C₁ to C₆ alkyl, C₁ to C₆ substituted alkyl, C₃ to C₈ cycloalkyl, substituted C₃ to C₈ cycloalkyl, aryl, substituted aryl, heteroaryl, and substituted heteroaryl; R₄ and R_{4'} are:

- (a) independently selected from the group consisting of H, OH, O(C₁ to C₆ alkyl), O(substituted C₁ to C₆ alkyl), O(acyl), O(aryl), O(substituted aryl), and halogen; or
- (b) taken together to form a double bond to O;

R₅, R₆, and R₇ are independently selected from the group consisting of H, OH, and OCH₃;

R₈ and R₉ are connected through a (i) single bond and are CH₂ or (ii) double bond and are CH;

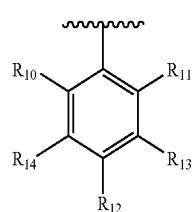
R₁₅ is selected from the group consisting of C=O, CHOH, and CH₂; n is 1 or 2; or a pharmaceutically acceptable salt thereof.

4. The purified complex of claim 3, wherein R₁ of the rapamycin analogue is O, and R₂ is NR₃.

5. The purified complex of claim 3, wherein R₁ of the rapamycin analogue is OR₃ and R₂ is N(R₃)(NR_{3''}).

6. The purified complex of claim 3, wherein R₃, R_{3'}, or R_{3''} of the rapamycin analogue is an aryl or substituted aryl.

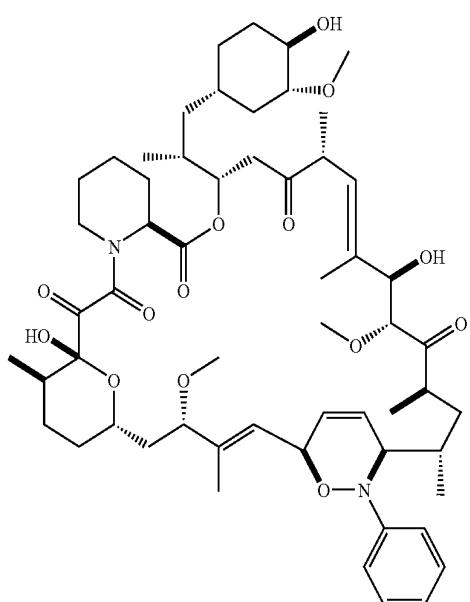
7. The purified complex of claim 6, wherein said aryl or substituted aryl of the rapamycin analogue is of the structure:



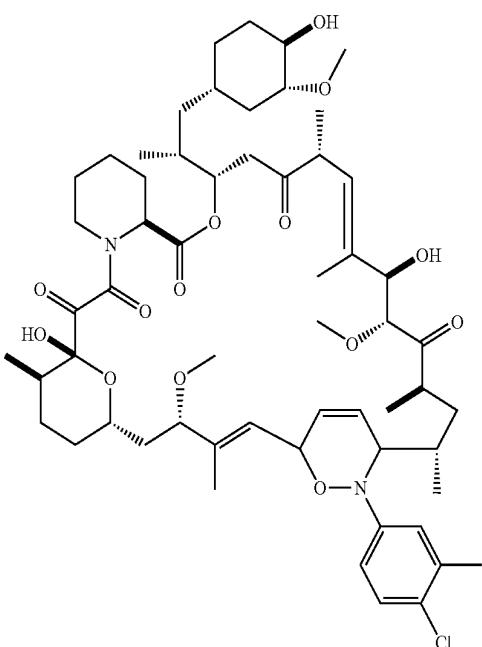
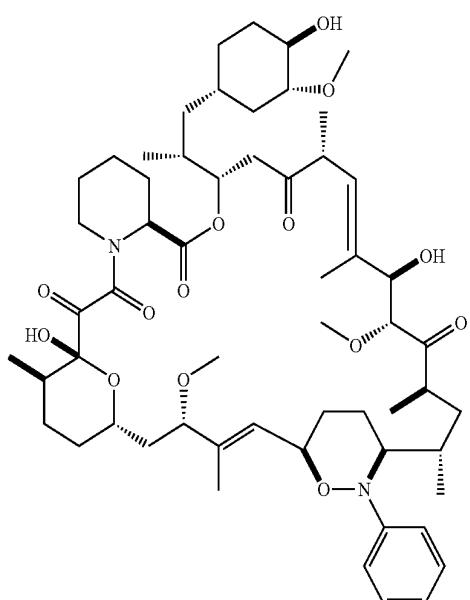
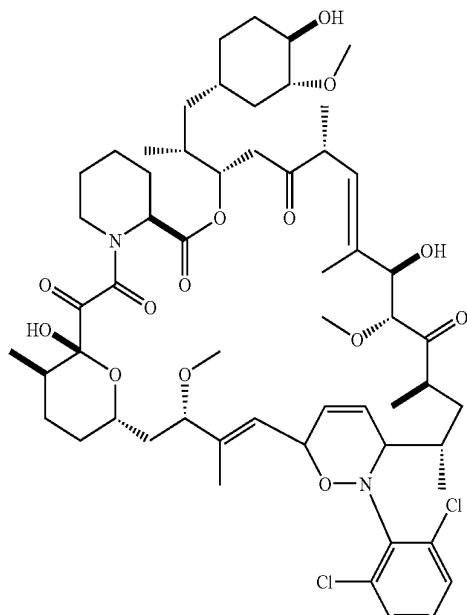
wherein:

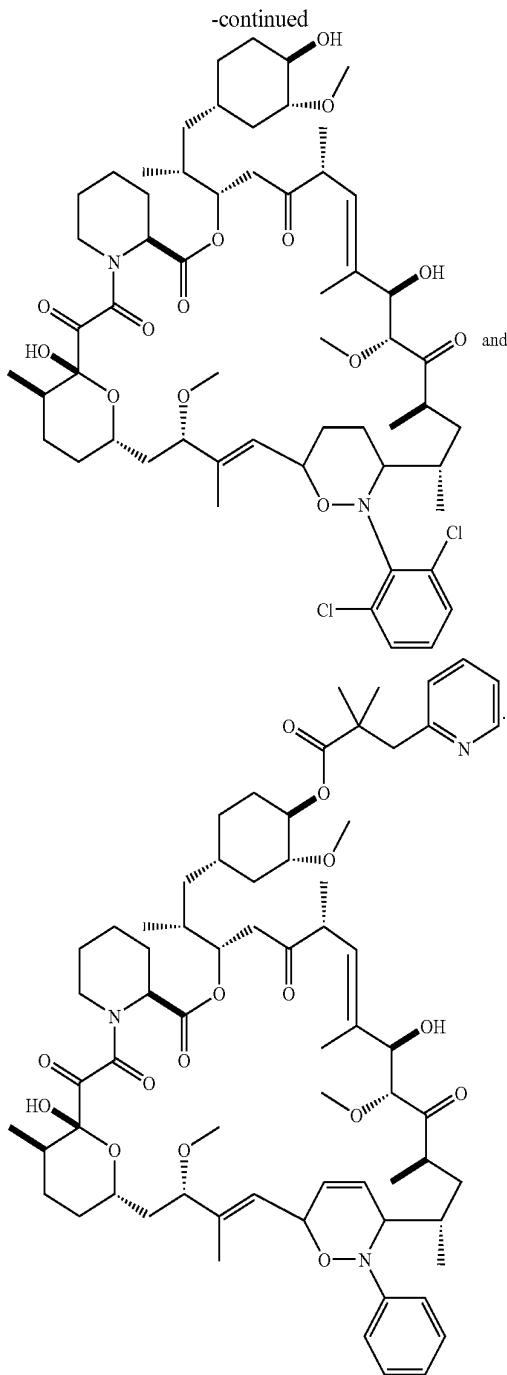
R_{10} , R_{11} , R_{12} , R_{13} , and R_{14} are independently selected from the group consisting of H, C₁ to C₆ alkyl, substituted C₁ to C₆ alkyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, halogen, acyl, OH, O(alkyl), O(substituted alkyl), O(aryl), O(substituted aryl), O(acyl), NH₂, NH(alkyl), NH(substituted alkyl), NH(aryl), NH(substituted aryl), and NH(acyl).

8. The purified complex of claim 1, wherein the immunophilin ligand is a rapamycin analogue selected from the group consisting of:



-continued





9. The purified complex of claim 1, wherein the immunophilin is FKBP52 or a functional fragment thereof having a sequence at least 95% identical, or identical, to the amino acid sequence shown in FIGS. 12A-12D (SEQ ID NO:11-14).

10. The purified complex of claim 1, wherein the calcium channel subunit is a $\beta 1$ subunit of the voltage gated L-type calcium channel, or a functional fragment thereof, having a sequence at least 95% identical, or identical, to the amino acid sequence shown in FIGS. 11A-11J (SEQ ID NO:1-10).

11. A recombinant host cell comprising a first recombinant nucleic acid that comprises a nucleotide sequence encoding an FKBP52 having the amino acid sequence shown in FIGS.

12A-12D (SEQ ID NO:11-14, and/or a second recombinant nucleic acid that comprises a nucleotide sequence encoding a $\beta 1$ subunit of the voltage gated L-type calcium channel having the amino acid sequence shown in FIGS. 11A-11J (SEQ ID NO:1-10).

12. An antibody, or antigen-binding fragment thereof, that binds to the purified complex of claim 1.

13. A method for identifying a test compound that increases the formation of a complex that includes the test compound, and one or both of (i) an immunophilin and/or (ii) a $\beta 1$ subunit of the voltage gated L-type calcium channel, comprising:

contacting an immunophilin or a functional fragment thereof, and/or a $\beta 1$ subunit or a functional fragment thereof, with a test compound under conditions that allow formation of the complex;

detecting the presence of the complex in the presence of the test compound relative to a reference;

wherein an increase in the level of the complex in the presence of the test compound, relative to the level of the complex in the reference, indicates that said test compound increases complex formation.

14. The method of claim 13, wherein the sample is a cell lysate, a reconstituted system, comprises cells in culture or in an animal subject.

15. The method of claim 13, wherein the increase in the formation of the complex is determined by detecting one or more of: an increase in the physical formation of the complex, a change in signal transduction, a decrease in calcium channel activity or a change in neuronal activity.

16. The method of claim 15, wherein the change in neuronal activity is detected as an increase in one or more of survival, differentiation or neurite outgrowth.

17. The method of claim 13, wherein the test compound is a polyketide obtained from naturally occurring or modified *S. hygroscopicus*.

18. A compound identified by the method of claim 13.

19. A method of increasing the formation of a complex that includes an immunophilin ligand, and one or both of (i) an immunophilin or a functional variant thereof and/or (ii) a calcium channel subunit or a functional variant thereof, comprising: contacting an immunophilin or a functional fragment thereof, and/or a $\beta 1$ subunit of the voltage gated L-type calcium channel or a functional fragment thereof, with an immunophilin ligand, under conditions that increase formation of the complex.

20. The method of claim 19, wherein the contacting step occurs in a cell lysate, in a reconstituted system, or cells in culture or in an animal subject.

21. A method of decreasing voltage-gated calcium channel activity, and/or FKBP52 activity, in a cell, comprising, contacting a cell that expresses one or both of an FKBP52 or a functional fragment thereof, and/or a $\beta 1$ subunit of the voltage gated L-type calcium channel or a functional fragment thereof, with an immunophilin ligand under conditions that allow binding between the immunophilin ligand, and one or both of the FKBP52 or fragment thereof, and/or the subunit or fragment thereof, to occur, thereby inhibiting the calcium channel activity.

22. The method of claim 21, wherein the contacting step comprises adding the immunophilin ligand to mammalian neuronal or cardiovascular cells in culture.

23. The method of claim 21, wherein the contacting step comprises administration to a subject the immunophilin

ligand in an amount sufficient to form a complex between the immunophilin ligand, and one or both of the FKBP52 or fragment thereof, and/or the subunit or fragment thereof.

24. The method of claim **23**, wherein the amount of the immunophilin administered to the subject is determined by testing in vitro the amount of immunophilin ligand required to induce complex formation.

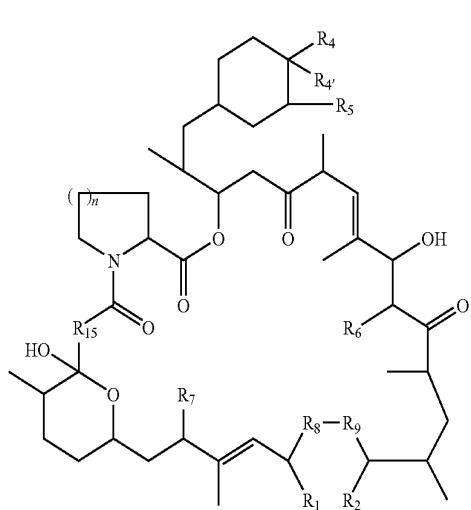
25. The method of claim **23** further comprising identifying a subject at risk of having, or having, one or more symptoms associated with a disorder involving L-type calcium channel dysfunction.

26. The method of claim **23**, wherein the subject is a mammal suffering from a neurodegenerative or a cardiovascular disorder.

27. The method of claim **23**, wherein the immunophilin ligand is administered in combination with an L-type calcium channel antagonist.

28. The method of claim **23**, wherein the immunophilin ligand is a rapamycin analogue having a heteroatom substituent at positions 1 and 4 of the rapamycin backbone.

29. The method of claim **28**, wherein the rapamycin analogue has the formula I:



wherein:

R₁ and R₂ are different, independent groups and are selected from the group consisting of OR₃ and N(R₃) (R₃); or

R₁ and R₂ are different, are connected through a single bond, and are selected from the group consisting of O and NR₃;

R₃, R₃, and R₃, are independently selected from the group consisting of H, C₁ to C₆ alkyl, C₁ to C₆ substituted alkyl, C₃ to C₈ cycloalkyl, substituted C₃ to C₈ cycloalkyl, aryl, substituted aryl, heteroaryl, and substituted heteroaryl;

R₄ and R_{4'} are:

(a) independently selected from the group consisting of H, OH, O(C₁ to C₆ alkyl), O(substituted C₁ to C₆ alkyl), O(acyl), O(aryl), O(substituted aryl), and halogen; or

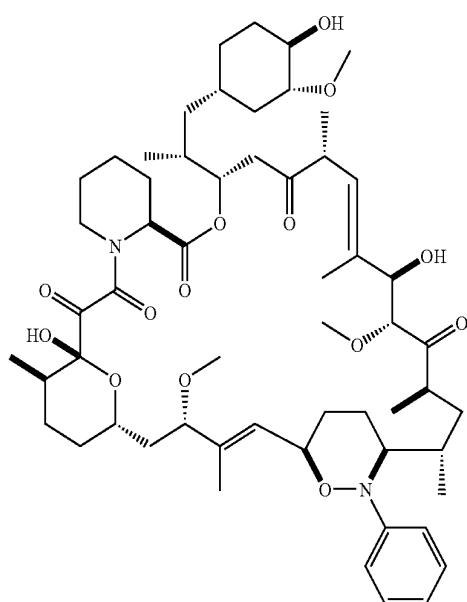
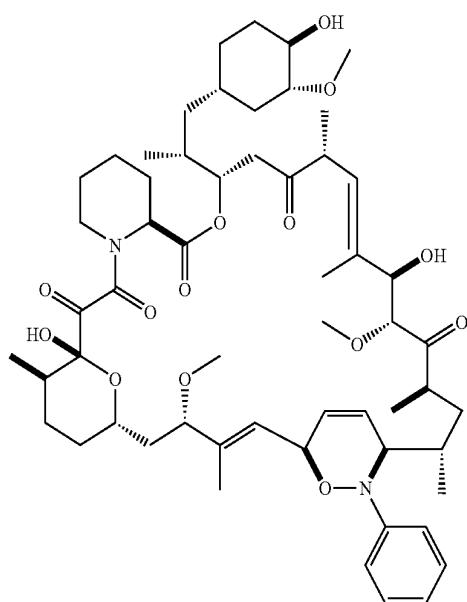
(b) taken together to form a double bond to O;

R₅, R₆, and R₇ are independently selected from the group consisting of H, OH, and OCH₃;

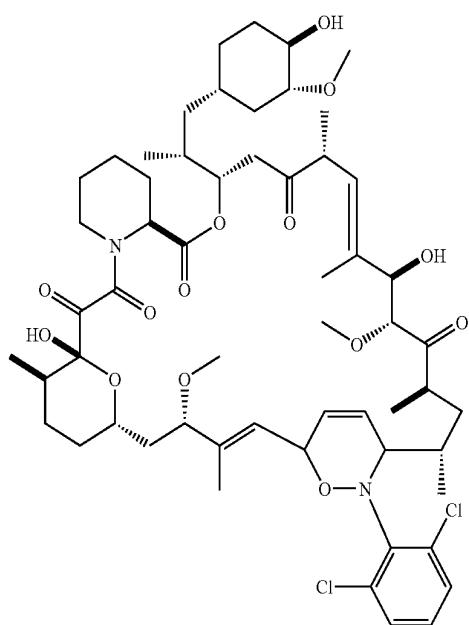
R₈ and R₉ are connected through a (i) single bond and are CH₂ or (ii) double bond and are CH;

R₁₅ is selected from the group consisting of C=O, CHOH, and CH₂; n is 1 or 2; or a pharmaceutically acceptable salt thereof.

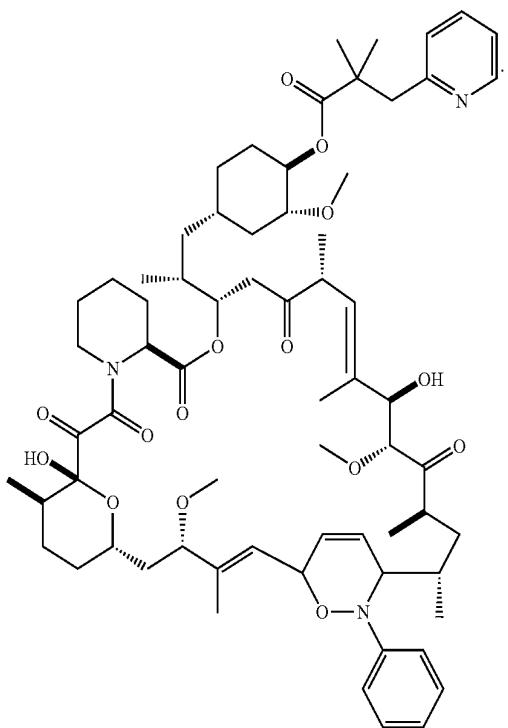
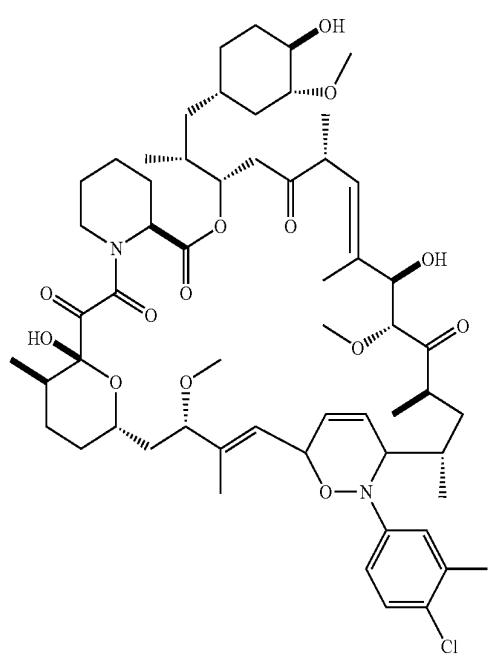
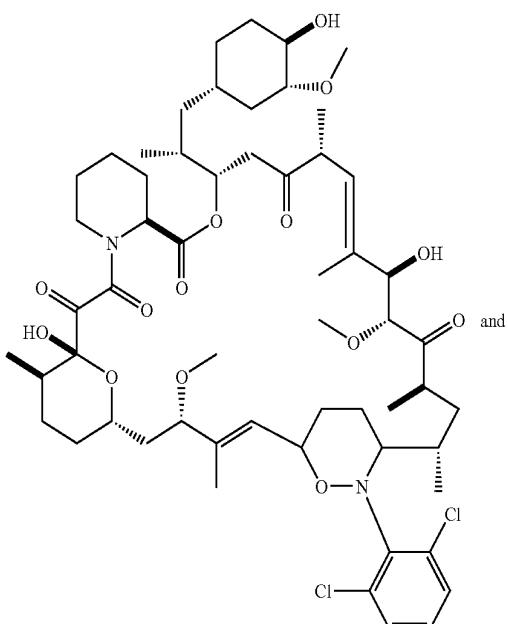
30. The method of claim **29**, wherein the rapamycin analogue is selected from the group consisting of:



-continued



-continued



31. The method of claim 21, wherein the FKBP52 or a functional fragment thereof comprises an amino acid sequence at least 95% identical, or identical, to the amino acid sequence shown in FIGS. 12A-12D (SEQ ID NOs:11-12).

32. The method of claim 21, wherein the β 1 subunit of the voltage gated L-type calcium channel, or a functional fragment thereof, comprises an amino acid sequence at least 95% identical to the amino acid sequence shown in FIGS. 11A-11J (SEQ ID NOs:1-10).

33. A method of stimulating neurite outgrowth and/or survival of a neuronal cell, comprising, contacting the neuronal cell with an immunophilin ligand, wherein the immunophilin ligand is present at a concentration that elicits one or more of the following: (i) downregulates expression or activity at least one component of the calcium signaling pathways; (ii) decreases FKBP52 activity or expression; (iii) reduces or inhibits the activity or expression of an L-type calcium channel; (iv) activates glucocorticoid receptor signaling; (v) induces formation of a complex that comprises the immunophilin ligand, FKBP52 and/or a β 1 subunit; and/or (vi) protects neurons from calcium-induced cell death.

34. The method of claim 33, wherein the contacting step comprises administration to a subject of the immunophilin ligand in an amount sufficient to form the complex that comprises the immunophilin ligand, and one or both of FKBP52 and/or a β 1 subunit.

35. The method of claim 34, wherein the amount of the immunophilin administered to the subject is determined by testing in vitro the amount of immunophilin ligand required to induce complex formation.

36. The method of claim 33 further comprising identifying a subject at risk of having, or having, one or more symptoms associated with a disorder involving L-type calcium channel dysfunction.

37. A method of treating a disorder associated with L-type calcium channel dysfunction, comprising administering to a subject an immunophilin ligand in an amount sufficient to form a complex that includes the immunophilin ligand, and one or both of an immunophilin or a functional fragment thereof, and/or a calcium channel subunit or a functional fragment thereof, thereby treating the disorder.

38. The method of claim 37, wherein the amount of the immunophilin administered to the subject is determined by testing in vitro the amount of immunophilin ligand required to induce complex formation.

39. The method of claim 37, further comprising identifying a subject at risk of having, or having, one or more symptoms associated with a disorder involving L-type calcium channel dysfunction.

40. The method of claim 37, wherein the subject is a mammal suffering from a neurodegenerative or a cardiovascular disorder.

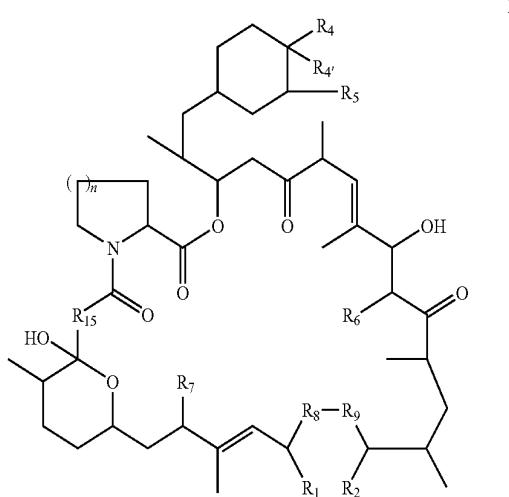
41. The method of claim 40, wherein the subject is a mammal suffering from a disorder selected from the group consisting of stroke, Parkinson's disease, epilepsy, angina, cardiac arrhythmia and ischemia.

42. The method of claim 40, wherein the subject is a mammal suffering from a disorder selected from the group consisting of migraine, neuropathic pain, acute pain, mood dis-

order, schizophrenia, depression, anxiety, cerebellar ataxia, tardive dyskinesia, hypertension and urinary incontinence.

43. The method of claim 37, wherein the immunophilin ligand is administered in combination with an L-type calcium channel antagonist.

44. The method of either claim 33 or 37, wherein the immunophilin ligand is a rapamycin analogue having the formula I:



wherein:

R₁ and R₂ are different, independent groups and are selected from the group consisting of OR₃ and N(R₃) (R_{3''}); or

R₁ and R₂ are different, are connected through a single bond, and are selected from the group consisting of O and NR₃;

R₃, R_{3'}, and R_{3''} are independently selected from the group consisting of H, C₁ to C₆ alkyl, C₁ to C₆ substituted alkyl, C₃ to C₈ cycloalkyl, substituted C₃ to C₈ cycloalkyl, aryl, substituted aryl, heteroaryl, and substituted heteroaryl;

R₄ and R_{4'} are:

(a) independently selected from the group consisting of H, OH, O(C₁ to C₆ alkyl), O(substituted C₁ to C₆ alkyl), O(acyl), O(aryl), O(substituted aryl), and halogen; or

(b) taken together to form a double bond to O;

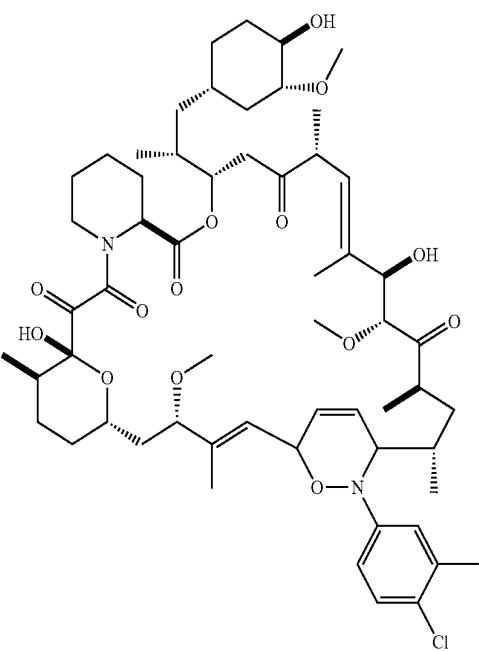
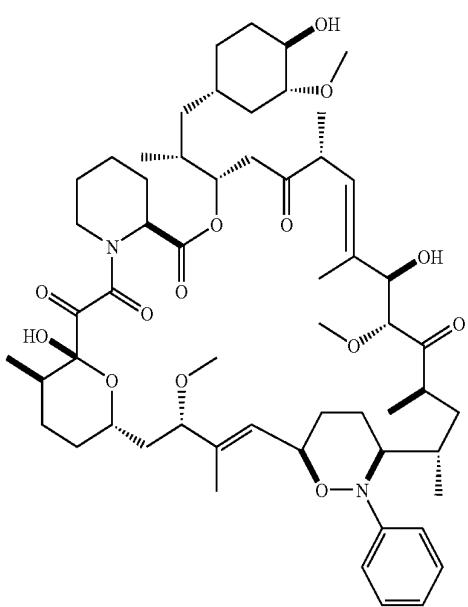
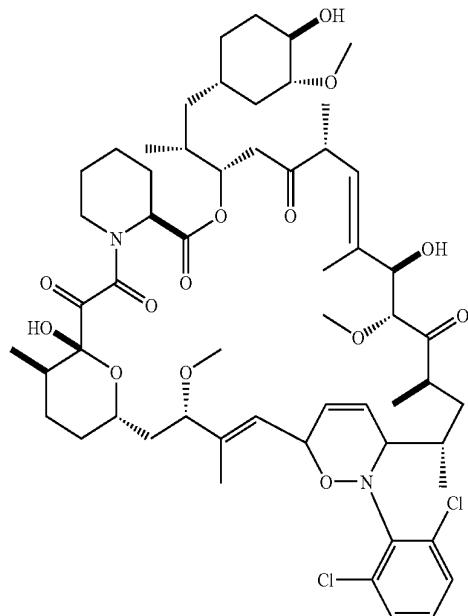
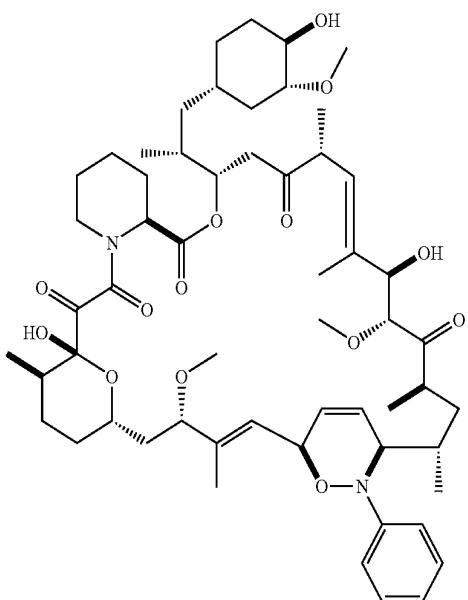
R₅, R₆, and R₇ are independently selected from the group consisting of H, OH, and OCH₃;

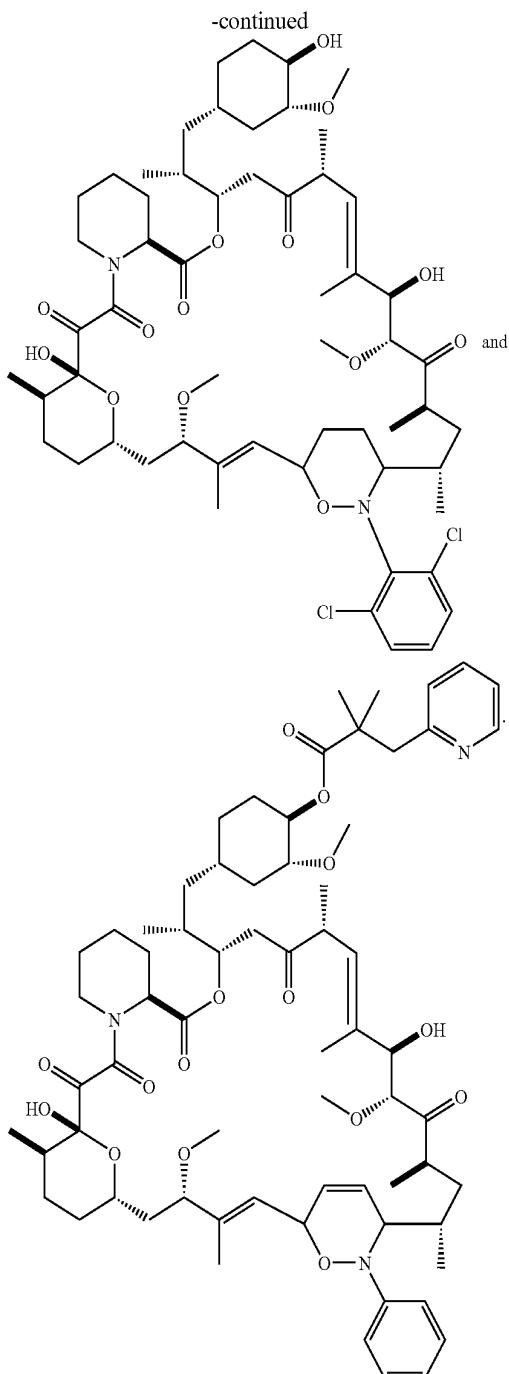
R₈ and R₉ are connected through a (i) single bond and are CH₂ or (ii) double bond and are CH;

R₁₅ is selected from the group consisting of C=O, CHOH, and CH₂; n is 1 or 2; or a pharmaceutically acceptable salt thereof.

45. The method of claim **44**, wherein the rapamycin analogue is selected from the group consisting of:

-continued





46. The method of claim **44**, wherein the immunophilin is FKBP52 or a functional fragment thereof having an amino acid sequence at least 95% identical, or identical, to 167 the amino acid sequence shown in FIGS. **12A-12D** (SEQ ID NO:11-14).

47. The method of claim **44**, wherein the calcium channel subunit is a $\beta 1$ subunit of the voltage gated L-type calcium channel, or a functional fragment thereof having an amino acid sequence at least 95% identical, or identical, to the amino acid sequence shown in FIGS. **11A-11J** (SEQ ID NO:1-10).

48. A method of stimulating neurite outgrowth of a neuronal cell, comprising contacting the neuronal cell with one or both of an antagonist of a $\beta 1$ subunit of a voltage gated L-type calcium channel, and/or an antagonist of FKBP52, under condition that reduce the activity or expression of the $\beta 1$ subunit or FKBP52.

49. The method of claim **48**, wherein the neuronal cell is selected from the group consisting of a dopaminergic, a cholinergic, a cortical, and a spinal cord cell.

50. The method of claim **48**, wherein the antagonist is an immunophilin ligand that forms a complex with the $\beta 1$ subunit and/or FKBP52.

51. The method of claim **48**, wherein the antagonist is an inhibitor of transcription of the calcium channel β subunit or FKBP52.

52. The method of claim **48**, wherein the antagonist is an antibody.

53. Use of an immunophilin ligand in the manufacture of a medicament for the prophylaxis or treatment of a condition associated with L-type calcium channel dysfunction.

54. The use according to claim **53**, wherein the immunophilin ligand is a rapamycin analogue having a heteroatom substituent at positions 1 and 4 of the rapamycin backbone.

55. Use of an immunophilin ligand in combination with an L-type calcium channel antagonist for the prophylaxis or treatment of a condition associated with L-type calcium channel dysfunction.

56. Use of a compound identified according to any of claims **13-17** in the manufacture of a medicament for the prophylaxis or treatment of a condition associated with L-type calcium channel dysfunction.

57. An immunophilin ligand for use in the prophylaxis or treatment of a condition associated with L-type calcium channel dysfunction.

58. A composition comprising an immunophilin ligand and an L-type calcium channel antagonist for use in the prophylaxis or treatment of a condition associated with L-type calcium channel dysfunction.

59. A compound identified according to any of claims **13-17** for use in the prophylaxis or treatment of a condition associated with L-type calcium channel dysfunction.

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