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Addendum

Small Molecule Enhancers of Rapamycin-Induced TOR Inhibition Promote Autophagy, Reduce Toxicity in Huntington's Disease Models and Enhance Killing of Mycobacteria by Macrophages

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Addendum to:

Small Molecules Enhance Autophagy and Reduce Toxicity in Huntington's Disease Models

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ABSTRACT

Upregulation of autophagy may have therapeutic benefit in a range of diseases that includes neurodegenerative conditions caused by intracytosolic aggregate-prone proteins, such as Huntington's disease, and certain infectious diseases, such as tuberculosis. The best-characterized drug that enhances autophagy is rapamycin, an inhibitor of the TOR (target of rapamycin) proteins, which are widely conserved from yeast to man. Unfortunately, the side effects of rapamycin, especially immunosuppression, preclude its use in treating certain diseases including tuberculosis, which accounts for approximately 2 million deaths worldwide each year, spurring interest in finding novel drugs that selectively enhance autophagy. We have recently reported a novel two-step screening process for the discovery of such compounds. We first identified compounds that enhance the growth-inhibitory effects of rapamycin in the budding yeast *Saccharomyces cerevisiae*, which we termed small molecule enhancers of rapamycin (SMERs). Next we showed that three SMERs induced autophagy independently, or downstream of mTOR, in mammalian cells, and furthermore enhanced the clearance of a mutant huntingtin fragment in Huntington's disease cell models. These SMERs also protected against mutant huntingtin fragment toxicity in *Drosophila*. We have subsequently tested two of the SMERs in models of tuberculosis and both enhance the killing of mycobacteria by primary human macrophages.

Macroautophagy (which we will call autophagy) is a critical process for cellular degradation of cytosolic contents, protein complexes and organelles. It involves the formation of double-membrane structures, called autophagosomes, which engulf a portion of cytoplasm. These autophagosomes ultimately fuse with lysosomes where their contents are degraded.[1]

Autophagy has numerous functions. We have shown that it is an important defense against aggregate-prone cytosolic proteins that accumulate in a range of neurodegenerative diseases.[2,3] These include polyglutamine-expanded proteins (in Huntington's disease and certain spinocerebellar ataxias), point mutations in α-synuclein (in certain forms of familial Parkinson's disease) and tau (in frontotemporal dementias).[4-10] Upregulation of autophagy enhances the clearance of these proteins and decreases toxicity in mouse and/or Drosophila models of many of these diseases.[2,3]

Another class of diseases that may benefit from strategies that enable autophagy upregulation are certain bacterial and viral infections, where the pathogens can be engulfed by autophagosomes and transferred to lysosomes for degradation. These include *Mycobacterium tuberculosis* (which causes tuberculosis), Group A *streptococcus* (which causes strep throat, toxic shock syndrome and necrotizing fasciitis), and viruses such as herpes simplex virus type I.[11-14]

The best characterized brain-accessible drug that upregulates autophagy is rapamycin.[3] Rapamycin induces autophagy by inhibiting the target of rapamycin (TOR) proteins, which are well conserved from yeast to man. The mammalian TOR is called mTOR. Although rapamycin is arguably the most specific kinase inhibitor known to date, its target also regulates numerous physiological processes that are independent of autophagy.[15] Indeed, rapamycin is in clinical use as an immunosuppressant for organ transplantation, but the immunosuppressive actions of rapamycin preclude its use in infectious diseases such as tuberculosis. Thus, there is a need for nonimmunosuppressive autophagy-inducing drugs. Such drugs may also be preferable for neurodegenerative diseases. One may argue that the potential benefits of rapamycin treatment in currently incurable conditions such as Huntington's disease may outweigh the side effects. However, as any autophagy-in-

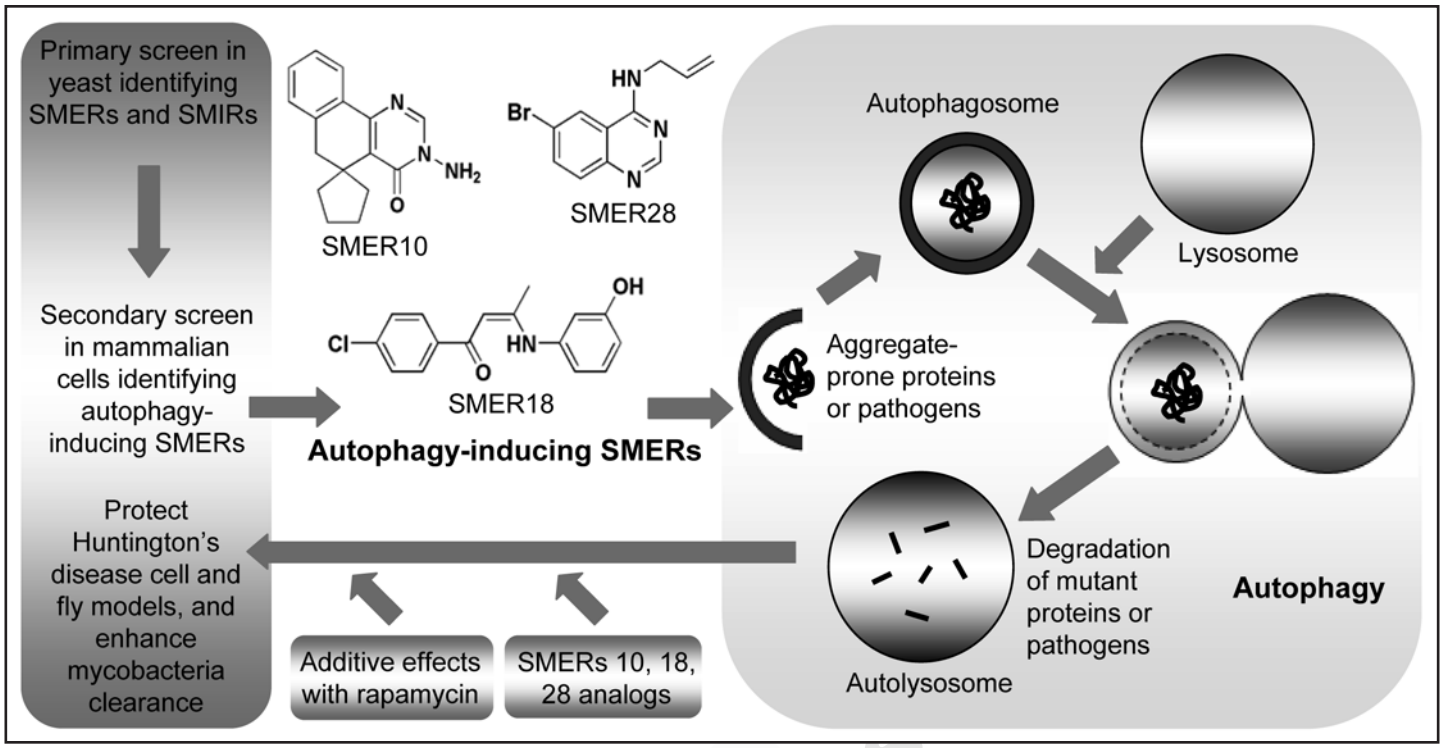

Figure 1. Schematic representation of identifying autophagy-inducing small molecules and their potential therapeutic application. A primary yeast screen identified small molecule enhancers of rapamycin (SMERs), on which a secondary screen was performed in mammalian cells to identify autophagy-inducing SMERs. Three SMERs (SMERs 10, 18 and 28) were identified that induce autophagy independently of rapamycin. These SMERs increased autophagic vesicles, thereby enhancing the clearance of mutant huntingtin and reducing its toxicity in cell and fly models of Huntington's disease. They also demonstrated additive protective effects with rapamycin. Further candidate drugs were identified by screening the structural analogs of these SMERs. The autophagy-inducing SMERs also enhanced the clearance of mycobacteria.

ducing treatment may need to occur over decades, a safer drug would be highly desirable.

We have recently described a novel pipe-line for the discovery of mTOR-independent compounds that induce autophagy (Fig. 1).[16] We screened 50,729 compounds for their abilities to enhance or suppress the growth-inhibitory effects of rapamycin in yeast (*Saccharomyces cerevisiae*).[17] As the mTOR pathway is conserved from yeast to man, our rationale was that the activities of some of these compounds would be conserved in mammalian systems, and that some may have an impact upon autophagy. From a structurally nonredundant set of 21 small-molecule inhibitors of rapamycin (SMIRs) and 12 small-molecule enhancers of rapamycin (SMERs), we identified 3 SMERs (SMER10, SMER18 and SMER28) that are nontoxic and that induce autophagy in mammalian cells.[16] Autophagy substrate clearance such as mutant huntingtin and A53T α-synuclein is SMER dose-dependent and only occurs in wild-type Atg5 (autophagy-competent) cells but not in Atg5 knockout (autophagy-incompetent) cells. SMER-mediated autophagy induction occurs at the stage of autophagosome formation.[16]

SMERs 10, 18 and 28 appear to act independently of, or downstream of, mTOR. They did not result in a decrease of mTOR activity as seen with the positive control rapamycin.[16] Furthermore, these SMERs in combination with rapamycin (at concentrations saturating for its pro-autophagic activity) resulted in greater rates of autophagy substrate clearance, compared to either the SMERs or rapamycin alone.[16] An analogue series for each of the SMERs was developed in order to assess preliminary structure-activity relationships. These analogues revealed a further 18 compounds that enhanced clearance of different autophagy substrates.[16]

Compatible with expectations, SMERs 10, 18 and 28 enhanced the clearance of mutant huntingtin fragments in cell models and protected against the toxicity of this polyglutamine-expanded protein in both cells and Drosophila.[16] One question that we have started to address since these compounds were published is whether they can also enhance the clearance of mycobacteria. To date, we have completed testing SMERs 18 and 28 (which had the highest activity with regard to clearance of the other autophagy substrates we examined). Both these SMERs reduced the numbers of viable intracellular mycobacteria in primary human macrophages (Fig. 2).

In conclusion, we have described a novel procedure for discovering autophagy-inducing drugs.[16] This is potentially scalable and may allow interrogation of larger small-molecule libraries. The compounds identified have potential utility for further development both for neurodegenerative diseases and for certain infectious diseases. These compounds may serve as probes for the identification of novel regulators of mammalian autophagy, a process that is still poorly understood.

METHODS

Primary human macrophage response to luminescent reporter mycobacteria. Human PBMC were isolated from peripheral blood of healthy consented volunteers using Lympholyte-H density gradients

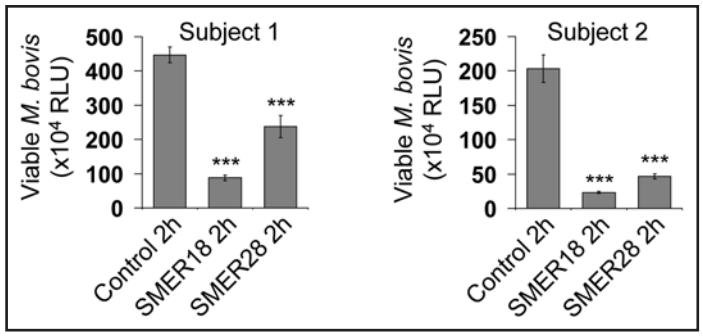

Figure 2. The effect of inducing mTOR-independent autophagy on intracellular mycobacterial replication. Macrophages from healthy volunteers were incubated for 1 h with *M. bovis* BCG-lux, washed to remove noninternalized mycobacteria, and incubated for 2 h with 43 μM SMER18 or 47 μM SMER28. Cell-associated luminescence (correlating with viable mycobacteria) was determined in sextuplicate and data are shown from samples from two different normal volunteers. Significance in subject 1: $p < 0.0001$ (SMER18), $p = 0.00039$ (SMER28); subject 2: $p < 0.0001$ (SMER18), $p < 0.0001$ (SMER28).

(Cedartree). These procedures comply with the local Institutional Review Board regulations for handling human subjects. Peripheral blood monocytes were enriched by negative selection utilizing a MACs based monocyte purification kit (Miltenyi Biotec, UK) and differentiated into macrophages by 7 d culture in RPMI media supplemented with L-Glutamine (2 mM), penicillin (100 U/ml), 20% heat-inactivated, filtered autologous serum and M-CSF (as previously described Ref. 18). Macrophages were incubated for 1 h, at a ratio of 1:10, with *M. bovis* BCG lux, a recombinant mycobacteria constitutively expressing *luxAB* genes from *Vibrio harveyi;* a well validated model system for examining mycobacterial killing.[19] Cells were washed to remove noninternalized mycobacteria. Cell-associated luminescence was measured, as previously described,[20] in sextuplicate samples 2 h after addition of the indicated compounds or vehicle alone.

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References

1. Klionsky DJ, Emr SD. Autophagy as a regulated pathway of cellular degradation. Science 2000; 290:1717-21.
2. Rubinsztein DC. The roles of intracellular protein-degradation pathways in neurodegeneration. Nature 2006; 443:780-6.
3. Rubinsztein DC, Gestwicki JE, Murphy LO, Klionsky DJ. Potential therapeutic applications of autophagy. Nat Rev Drug Discov 2007; 6:304-12.
4. Berger Z, Ravikumar B, Menzies FM, Oroz LG, Underwood BR, Pangalos MN, Schmitt I, Wullner U, Evert BO, O'Kane CJ, Rubinsztein DC. Rapamycin alleviates toxicity of different aggregate-prone proteins. Hum Mol Genet 2006; 15:433-42.
5. Ravikumar B, Duden R, Rubinsztein DC. Aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy. Hum Mol Genet 2002; 11:1107-17.
6. Ravikumar B, Vacher C, Berger Z, Davies JE, Luo S, Oroz, LG, Scaravilli F, Easton DF, Duden R, O'Kane CJ, Rubinsztein DC. Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. Nat Genet 2004; 36:585-95.
7. Sarkar S, Davies JE, Huang Z, Tunnacliffe A, Rubinsztein DC. Trehalose, a novel mTOR-independent autophagy enhancer,accelerates the clearance of mutant huntingtin and α-synuclein. J Biol Chem 2007; 282:5641-52.
8. Sarkar S, Floto RA, Berger Z, Imarisio S, Cordenier A, Pasco M, Cook LJ, Rubinsztein DC. Lithium induces autophagy by inhibiting inositol monophosphatase. J Cell Biol 2005; 170:1101-11.
9. Webb JL, Ravikumar B, Atkins J, Skepper JN, Rubinsztein DC. α-Synuclein is degraded by both autophagy and the proteasome. J Biol Chem 2003; 278:25009-13.
10. Shibata M, Lu T, Furuya T, Degterev A, Mizushima N, Yoshimori T, MacDonald M, Yankner B, Yuan J. Regulation of intracellular accumulation of mutant Huntingtin by *Beclin 1*. J Biol Chem 2006; 281:14474-85.
11. Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V. Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. Cell 2004; 119:753-66.
12. Nakagawa I, Amano A, Mizushima N, Yamamoto A, Yamaguchi H, Kamimoto T, Nara A, Funao J, Nakata M, Tsuda K, Hamada S, Yoshimori T. Autophagy defends cells against invading group A *Streptococcus*. Science 2004; 306:1037-40.
13. Ogawa M, Yoshimori T, Suzuki T, Sagara H, Mizushima N, Sasakawa C. Escape of intracellular *Shigella* from autophagy. Science 2005; 307:727-31.
14. Talloczy Z, Jiang W, Virgin IVth HW, Leib DA, Scheuner D, Kaufman RJ, Eskelinen E-L, Levine B. Regulation of starvation- and virus-induced autophagy by the eIF2α kinase signaling pathway. Proc Natl Acad Sci USA 2002; 99:190-5.
15. Sarbassov DD, Ali SM, Sabatini DM. Growing roles for the mTOR pathway. Curr Opin Cell Biol 2005; 17:596-603.
16. Sarkar S, Perlstein EO, Imarisio S, Pineau S, Cordenier A, Maglathlin RL, Webster JA, Lewis TA, O'Kane CJ, Schreiber SL, Rubinsztein DC. Small molecules enhance autophagy and reduce toxicity in Huntington's disease models. Nat Chem Biol 2007; 3:331-8.
17. Huang J, Zhu H, Haggarty SJ, Spring DR, Hwang H, Jin F, Snyder M, Schreiber SL. Finding new components of the target of rapamycin (TOR) signaling network through chemical genetics and proteome chips. Proc Natl Acad Sci USA 2004; 101:16594-9.
18. Floto RA, Clatworthy MR, Heilbronn KR, Rosner DR, MacAry PA, Rankin A, Lehner PJ, Ouwehand WH, Allen JM, Watkins NA, Smith KG. Loss of function of a lupus-associated FcγRIIb polymorphism through exclusion from lipid rafts. Nat Med 2005; 11:1056-8.
19. Kampmann B, Gaora PO, Snewin VA, Gares MP, Young DB, Levin M. Evaluation of human antimycobacterial immunity using recombinant reporter mycobacteria. J Infect Dis 2000; 182:895-901.
20. Floto RA, MacAry PA, Boname JM, Mien TS, Kampmann B, Hair JR, Huey OS, Houben EN, Pieters J, Day C, Oehlmann W, Singh M, Smith KG, Lehner PJ. Dendritic cell stimulation by mycobacterial Hsp70 is mediated through CCR5. Science 2006; 314:454-8.