BIO F366

LABORATORY PROJECT

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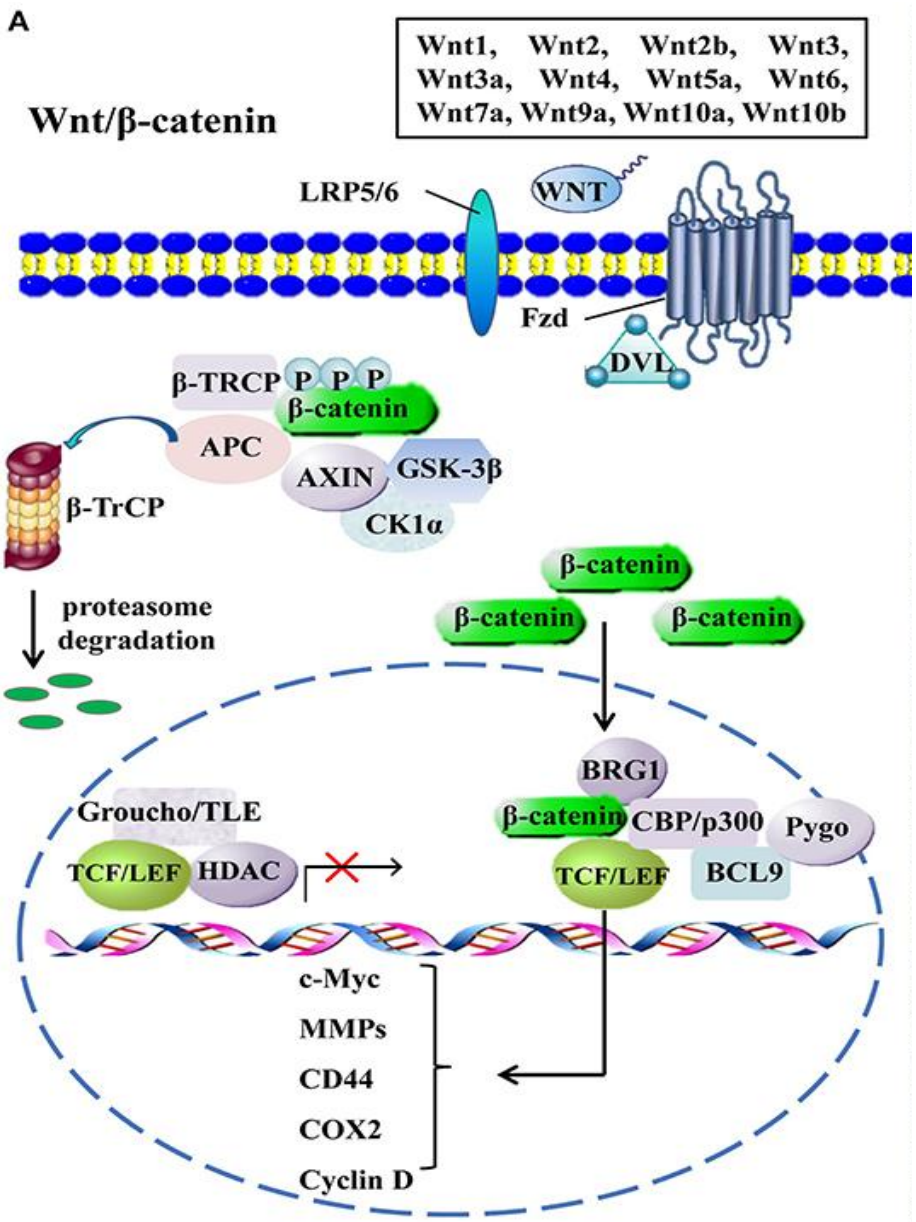
DRUG DISCOVERY USING THE PRINCIPLES OF PERTURBATION BIOLOGY AND TOOLS OF BIOMODELLING: Wnt pathway in colon cancer

Wnt PATHWAY


A
Wnt/β-catenin
Wnt1, Wnt2, Wnt2b, Wnt3,
Wnt3a, Wnt4, Wnt5a, Wnt6,
Wnt7a, Wnt9a, Wnt10a, Wnt10b
LRP5/6
WNT
Fzd
DVL
β-TRCP
P P P
β-catenin
APC
AXIN
GSK-3β
CK1α
β-TrCP
proteasome
degradation
β-catenin
β-catenin
β-catenin
BRG1
Groucho/TLE
β-catenin
CBP/p300
Pygo
TCF/LEF
HDAC
TCF/LEF
BCL9
c-Myc
MMPs
CD44
COX2
Cyclin D

- Mutational inactivation of the Adenomatous Polyposis Coli (*APC*) tumor suppressor is thought to be the initiating event in most sporadic and familial CRCs.
- Disruption of APC drives activation of the WNT signaling pathway, and a wealth of evidence suggests that WNT hyperactivation is the key oncogenic driver in most, if not all, CRCs.
- WNT ligands bind FRIZZLED (FZD) and LRP receptor complexes, initiating membrane recruitment of key scaffold proteins (AXIN, DVL), and disruption of the β-catenin destruction complex (minimally composed of AXIN, APC, CK1, GSK3β). In the absence of this complex, β-catenin accumulates in the cytosol, and through poorly understood mechanisms, translocates into the nucleus where it associates with TCF family transcription factors and a host of co-activators to drive transcription of target genes. The direct transcriptional output of Wnt activation is context dependent. For instance, in the mammalian colon, Wnt activation drives proliferation and stem-like phenotypes
- WNT-targeting approaches can be divided into three categories:
    - Targeting the WNT ligand-receptor interface

    Although direct WNT pathway agents are not yet in clinical practice, a number are in various stages of preclinical development, some are in early Phase clinical trials. Perhaps the most mature of all efforts to target hyperactive WNT signaling are small molecules and antibodies designed to block WNT-FZD ligand-receptor interaction at the cell surface. For example, Vantictumab (OMP-18R5) is an antibody targeting FZD receptors], and Ipafricept is a decoy-receptor, comprised of the FZD8 extracellular domain and the Fc domain of human immunoglobulin While both drugs have shown acceptable safety profiles and progressed to Phase 1b evaluation for breast, ovarian and pancreatic cancers, neither is under investigation for treatment of CRC. This is not surprising, as the majority of CRCs activate WNT signaling independent of actual WNT ligand. A similar paradigm is expected to hold true for strategies that aim to prevent the secretion of Wnt ligands (i.e., Porcupine inhibitors) or neutralize

Wnt ligand directly, thus reinforcing the challenge of targeting activated WNT signaling in CRC.

- Regulation of the endogenous destruction complex
  The destruction complex, composed of APC, AXIN, GSK3b, CK1, is the core network that regulates β-catenin degradation, and thereby serves as a cytosolic gatekeeper for β-catenin-mediated transcription. As the destruction complex enforces endogenous tumor suppression, it represents an attractive target to attenuate WNT signaling. In theory, there are numerous paths to stabilize the destruction complex, but the best explored is the regulation of AXIN function via Tankyrase activity.
- Direct interference with β-catenin-mediated transcription.
  The ultimate effector of WNT pathway activity is β-catenin mediated gene transcription. Hence, it follows that the most direct path to blocking WNT hyperactivation is by inhibiting this transcriptional response. However, it is clear from genetic studies that complete ablation of β-catenin is overtly toxic to normal intestinal epithelium

CETUXIMAB

Epidermal growth factor receptor (EGFR) inhibitors. Researchers have found that drugs that block EGFR may be effective for stopping or slowing the growth of colorectal cancer.

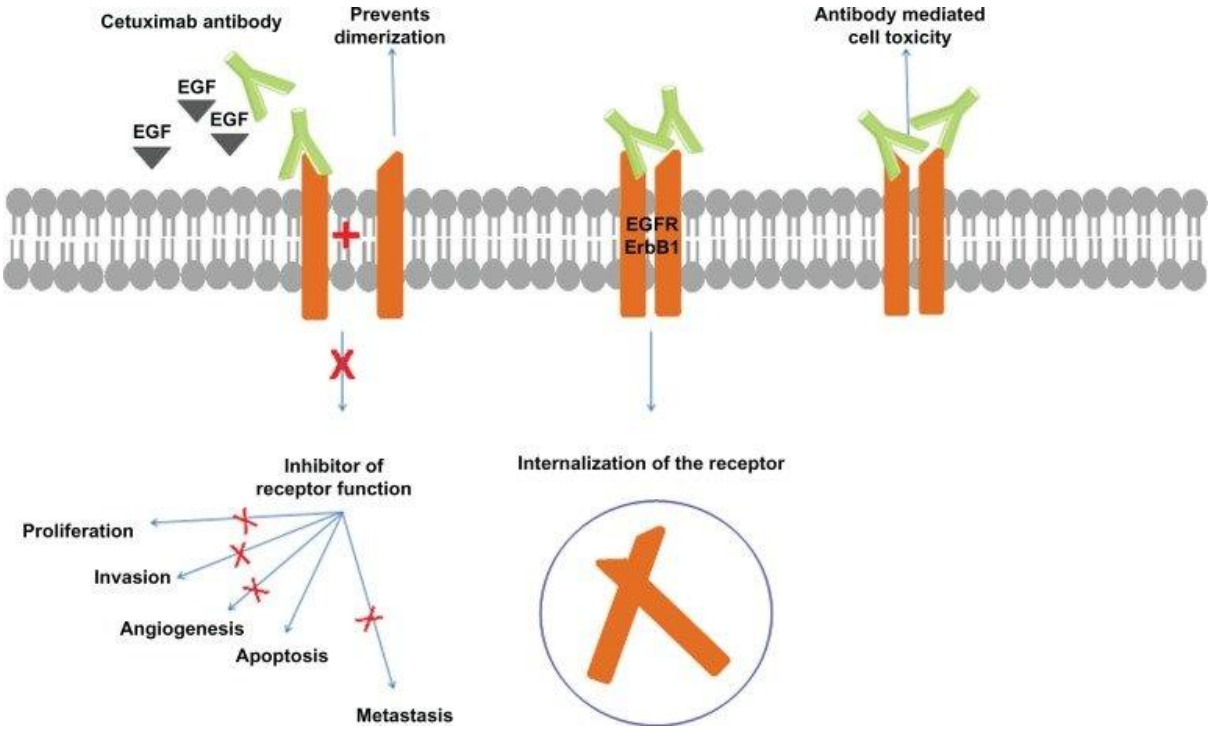
- **Cetuximab (Erbitux):** Cetuximab is an antibody made from mouse cells that still has some of the mouse structure,an IgG1 chimeric monoclonal antibody against epidermal growth factor receptor (EGFR), has activity against colorectal cancers that express EGFR.
- Recent studies show that cetuximab and panitumumab
  - Do not work as well for tumors that have specific mutations, or changes, to a gene called *RAS*.
  - ASCO recommends that all people with metastatic colorectal cancer who may receive an EGFR inhibitor have their tumors tested for *RAS* gene mutations.
  - If a tumor has a mutated form of the *RAS* gene, ASCO recommends that they do not receive EGFR inhibitors. Furthermore, the FDA now recommends that
  - Both cetuximab and panitumumab are only to be given to people with a tumor with non-mutated, sometimes called wild-type, *RAS* genes.

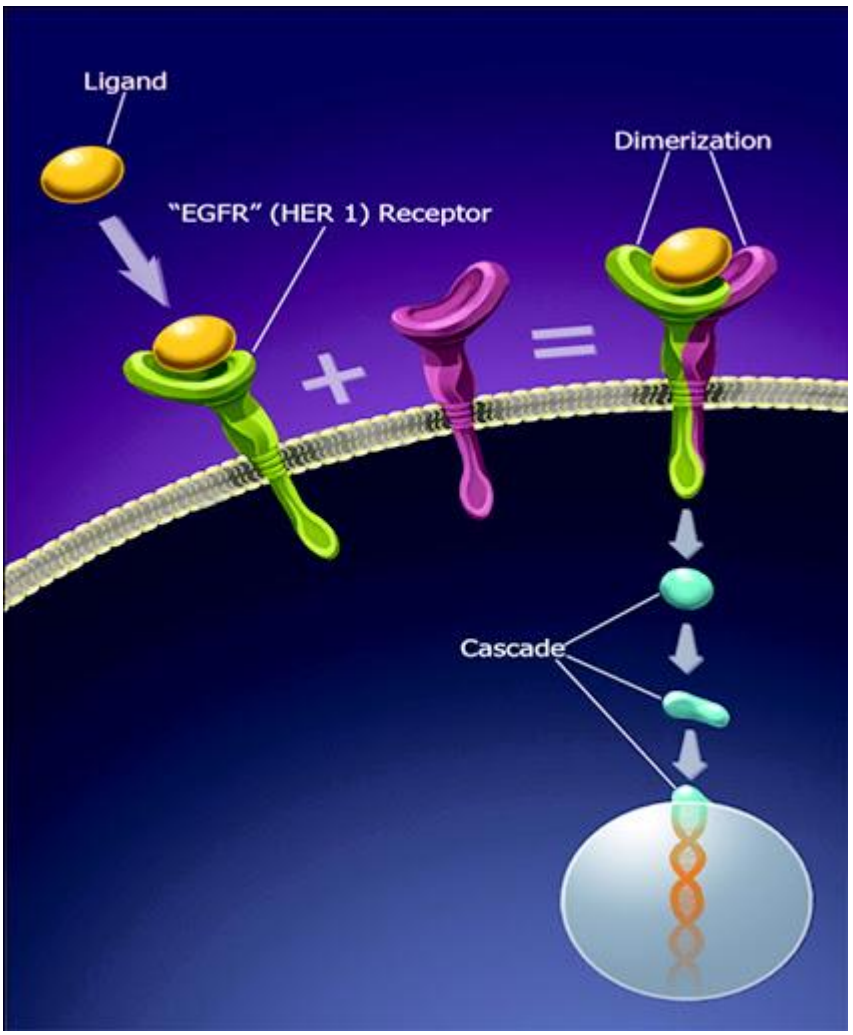
Agent	Target(s)	FDA-approved indication(s)
Cetuximab (Erbitux)	EGFR (HER1/ERBB1)	• Colorectal cancer (KRAS wild type) • Squamous cell cancer of the head and neck

- Cetuximab, an IgG1 chimeric monoclonal antibody against epidermal growth factor receptor (EGFR), has activity against colorectal cancers that express EGFR.
- Colorectal cancer, metastatic, KRAS wild-type (without mutation) - Cetuximab improves both overall survival and progression-free survival and preserves quality-of-life measures for patients with colorectal cancer where other treatments have failed.
- **Mechanism of action**
    - Cetuximab is a recombinant chimeric human/mouse IgG1 monoclonal antibody which binds to epidermal growth factor receptor (EGFR) and competitively inhibits the binding of epidermal growth factor (EGF) and other ligands
    - EGFR is a member of the ErbB family of receptors. When inactive, EGFR is a monomer, but when bound by epidermal growth factor or transforming growth factor-alpha (TGF-alpha), it forms homodimers or heterodimers with another member of the ErbB family of receptors.
    - Dimerization activates the intracellular tyrosine kinase region of EGFR, resulting in autophosphorylation and initiating a cascade of intracellular events.
    - The EGFR signaling pathway regulates cell differentiation, proliferation, migration, angiogenesis, and apoptosis, all of which become deregulated in cancer cells.
    - Cetuximab binds to EGFR with high specificity and with a higher affinity than either epidermal growth factor or TGF-alpha
    - blocking ligand-induced phosphorylation of EGFR.
    - Also, cetuximab enhances the effects of irinotecan and radiotherapy in experimental systems. *K-ras*, a small G-protein downstream of EGFR and a vital component of the EGFR signaling cascade, can acquire activating mutations in exon 2, thus isolating the pathway from the effect of EGFR and rendering EGFR inhibitors ineffective.
    - Cetuximab (CTX), a monoclonal antibody against epidermal growth factor receptor, is being widely used for colorectal cancer (CRC) with wild-type (WT) KRAS.
    - However, its responsiveness is still very limited and WT KRAS is not enough to indicate such responsiveness. Here,

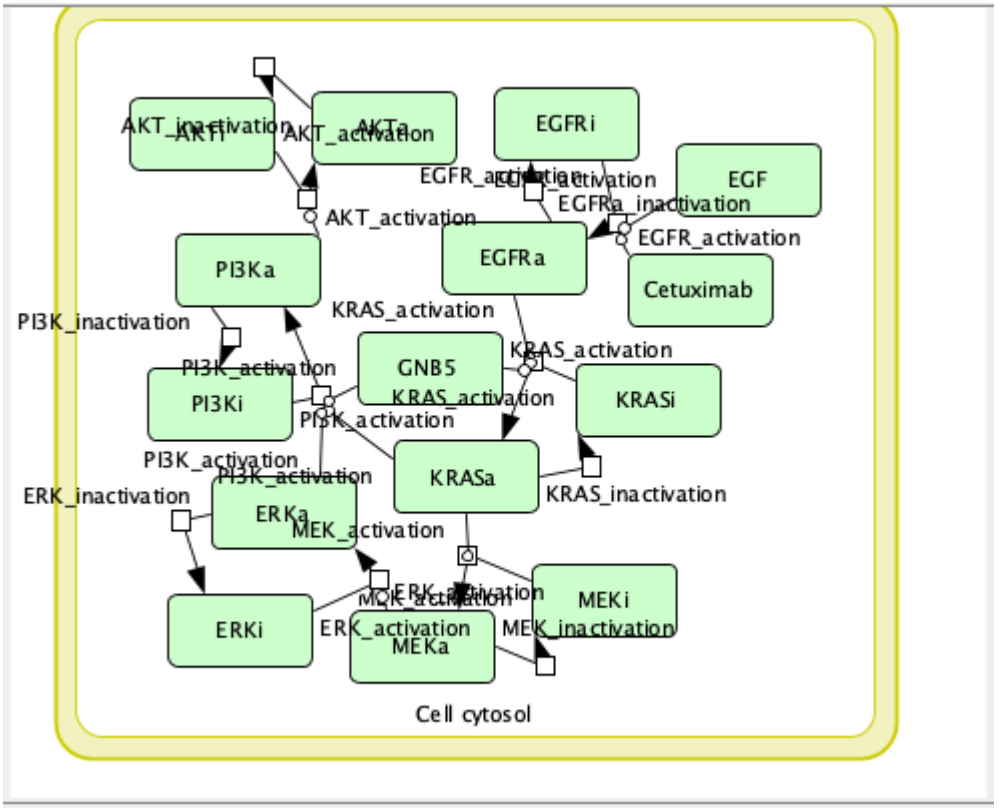
by analyzing the gene expression data of CRC patients treated with CTX monotherapy, we have identified DUSP4, ETV5, GNB5, NT5E, and PHLDA1 as potential targets to overcome CTX resistance.

- The knockdown of any of these five genes can increase CTX sensitivity in KRAS WT cells. Interestingly, we further found that GNB5 knockdown can increase CTX sensitivity even for KRAS mutant cells. We unraveled that GNB5 overexpression contributes to CTX resistance by modulating the Akt signaling pathway from experiments and mathematical simulation.
- Overall, these results indicate that GNB5 might be a promising target for combination therapy with CTX irrespective of KRAS mutation





SIMULATION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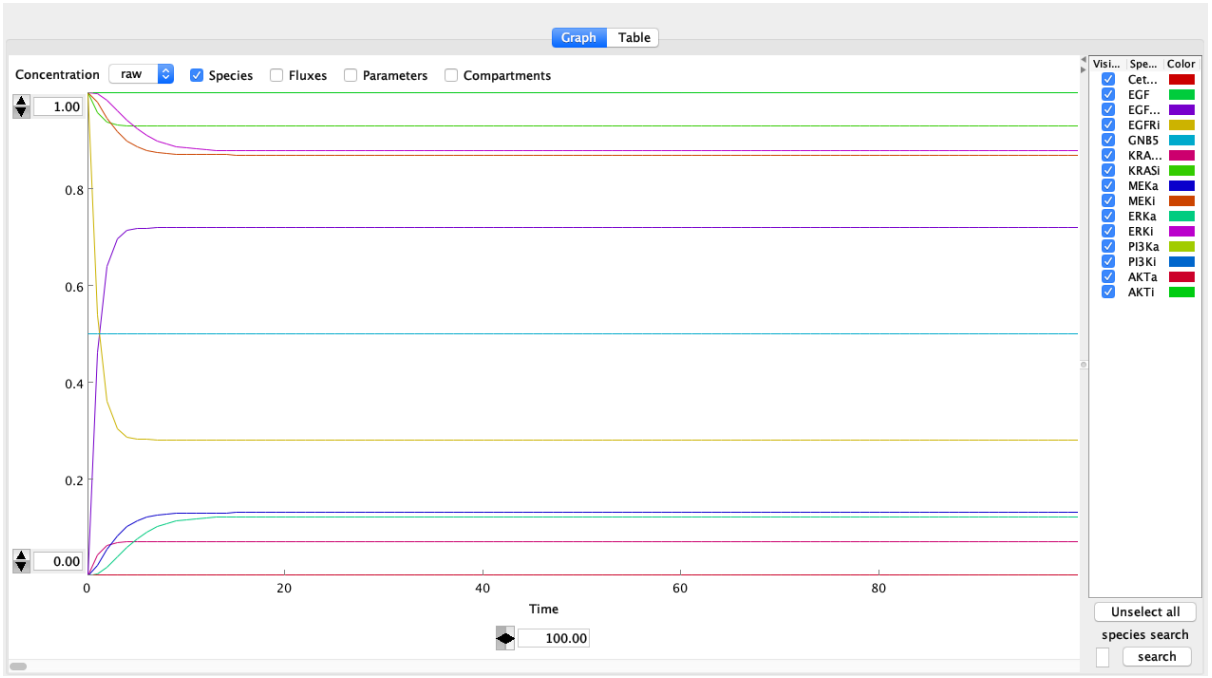
Cell cytosol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Visi...
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Cet...
EGF
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EGFRi
GNB5
KRA...
KRASi
MEKa
MEKi
ERKa
ERKi
PI3Ka
PI3Ki
AKTa
AKTi
Unselect all
species search
search

RESULTS AND CONCLUSIONS

- There is a limitation of use for EGFR inhibitors.
    - Patients with a colorectal tumor-bearing mutated K-ras did not benefit from cetuximab
    - Patients with a tumor-bearing wild-type K-ras did benefit from cetuximab.

DOXYCYCLINE

WNT/p21 circuit is driven by C-clamp target gene selection.

- The LEF/TCF family of transcription factors are downstream effectors of the WNT signaling pathway, which drives colon tumorigenesis.
- LEF/TCFs have a DNA sequence-specific HMG box that binds Wnt Response Elements (WREs).
- The “E tail” isoforms of TCFs are alternatively spliced to include a second DNA binding domain called the C-clamp.
- We show that induction of a dominant negative C-clamp version of TCF1 (dnTCF1E) induces a p21-dependent stall in the growth of DLD1 colon cancer cells
- Induction of a C-clamp mutant did not induce p21 or stall cell growth.
- Microarray analysis revealed that induction of p21 by dnTCF1EWT correlated with a decrease in expression of p21 suppressors that act at multiple levels from transcription (SP5, YAP1, RUNX1), to RNA stability (MSI2), and protein stability (CUL4A).
- We show that the C-clamp is a sequence specific DNA binding domain that can make contacts with 5’-RCCG-3’ elements upstream or downstream of WREs.
- The C-clamp-RCCG interaction was critical for TCF1E mediated transcriptional control of p21-connected target gene promoters.
- Our results indicate that a WNT/p21 circuit is driven by C-clamp target gene selection.
- Gene expression analysis of dnTCF and dnLEF induction in colon cancer cells.
- Dominant negative LEF/TCFs interferes with endogenous Wnt signaling by binding to Wnt Response Elements of target genes and displacing beta-catenin.
- Here we used induction of dnTCF-1 (wildtype and mutant forms of the C-clamp DNA binding domain) and dnLEF-1
- to identify genes that change expression at 8 hours and 23 hours post-induction.
- Three DLD-1 clonal stable cell lines created by tetracycline-regulated expression (T-REx) system were treated

with or without doxycycline for RNA extraction and hybridization on Affymetrix microarrays.

- One of the three cell lines is a mock cell line,a parental cell line that expresses only the Tet regulator and does not overexpress any protein when induced with doxycycline (Dox).
- The other three cell lines
    - can be induced by Doxycycline to overexpress wild-type dnTCF-1E or a mutant form of dnTCF-1E with five amino acid substitution mutations in the C-clamp of the E-tail, or dnLEF-1.
    - The mock cell line and dnLEF-1 cell lines were treated with or without Dox for 23 hr. The wild-type and mutant dnTCF-1E cell lines were treated with or without Dox for 8 and 23 hr, and as well, wild-type dnTCF-1E was induced with maximal amounts of Dox (hi: 1ug/ml) for maximum expression and gene expression regulation

HMG Box

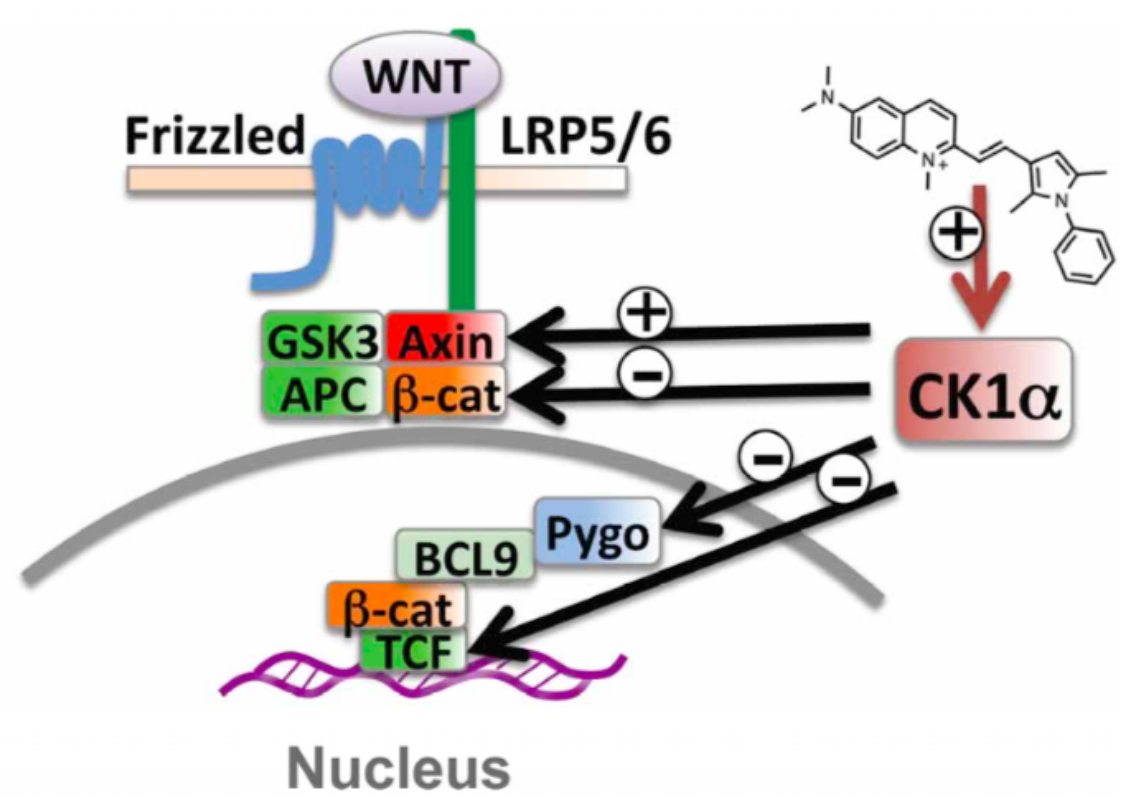
- High mobility group (HMG) box domains are involved in binding DNA, and may be involved in protein-protein interactions as well.
- The structure of the HMG-box domain consists of three helices in an irregular array.
- HMG-box domains are found in one or more copies in HMG-box proteins, which form a large, diverse family involved in the regulation of DNA-dependent processes such as transcription, replication, and strand repair, all of which require the bending and unwinding of chromatin.
- Many of these proteins are regulators of gene expression. HMG-box proteins are found in a variety of eukaryotic organisms, and can be broadly divided into two groups, based on sequence-dependent and sequence-independent DNA recognition; the former usually contain one HMG-box motif, while the latter can contain multiple HMG-box motifs.

HMG-box domains can be found in single or multiple copies in the following protein classes: HMG1 and HMG2 non-histone components of chromatin; SRY (sex determining region Y protein) involved in differential gonadogenesis; the SOX family of transcription factors [;

sequence-specific LEF1 (lymphoid enhancer binding factor 1) and TCF-1 (T-cell factor 1) involved in regulation of organogenesis and thymocyte differentiation]; structure-specific recognition protein SSRP involved in transcription and replication.

PYRVINIUM

Wnt/β-catenin signaling is critically involved in metazoan development, stem cell maintenance and human disease.
Inhibition of Wnt signaling by pyrvinium is due to allosteric activation of CK1α to regulate the stability of β-catenin and Axin in the cytoplasm and Pygopus and TCF/Lef1 in the nucleus.



Using Xenopus laevis egg extract to screen for compounds that both

- stabilize Axin2
- promote β-catenin turnover

pyrvinium, as a potent inhibitor of Wnt signaling (EC(50) of ~10 nM).

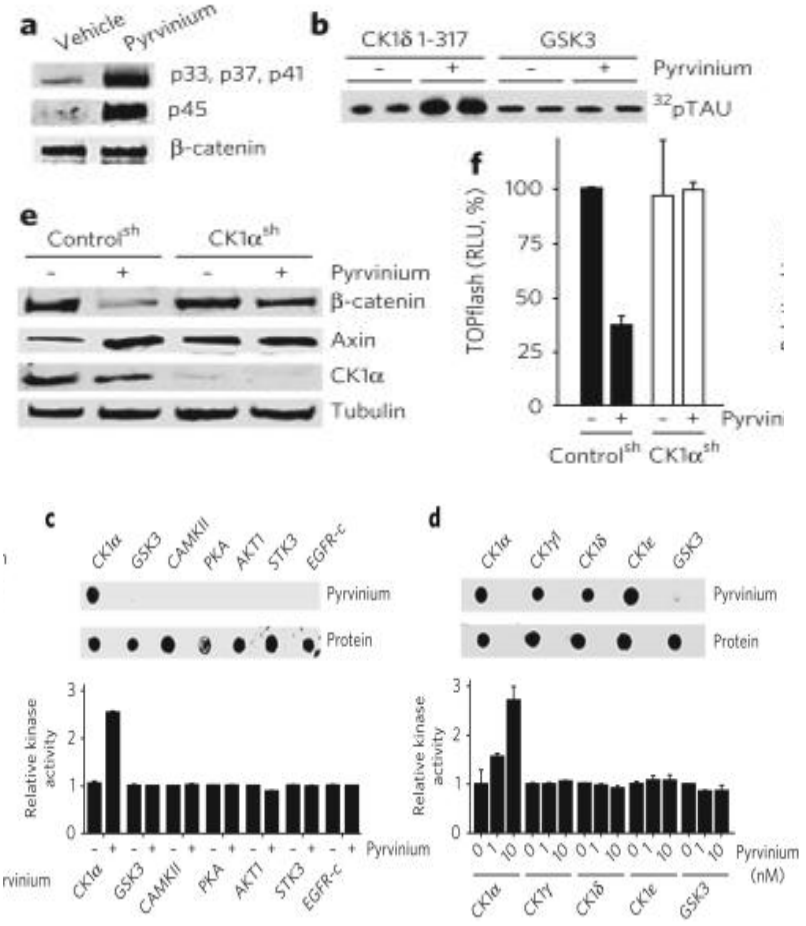
- We show pyrvinium binds all casein kinase 1 (CK1) family members in vitro at low nanomolar concentrations and pyrvinium selectively potentiates casein kinase 1α (CK1α) kinase activity.
- CK1α knockdown abrogates the effects of pyrvinium on the Wnt pathway.

CK1α is the critical target of pyrvinium

- Pyrvinium stimulates β-catenin phosphorylation *in vitro*. A kinase reaction was assembled *in vitro* with purified β-catenin, Axin, GSK3

and a constitutively active, truncated form of CK1δ (CK1δ1–317) (100 nM each) plus or minus pyrvinium (10 nM).

- Phosphorylation of β-catenin on GSK3 sites (p33, p37, p41) and the priming CK1α site (p45) was detected by immunoblotting.
- Pyrvinium stimulates CK1 activity *in vitro*. CK1δ1–317 (100 nM) was incubated with recombinant tau (100 nM) plus or minus pyrvinium pamoate (10 nM) in a kinase reaction containing [γ32P]ATP and underwent SDS-PAGE separation and autoradiography.
- Pyrvinium pamoate (10 nM) was incubated with purified recombinant kinases, and binding and kinase activities were assessed. Equivalent amounts (0.5 μg) were spotted for each protein.
- Pyrvinium binds and activates CK1α but not kinases representative of other major branches of the kinome.
- Pyrvinium binds all full-length CK1 isoforms tested but only activates CK1α. Graphs for c, d show mean ± s.e.m., performed in triplicate. (e, f) Downregulating CK1α blocks the biochemical and transcriptional responses to pyrvinium.
- A Jurkat cell line expressing inducible shRNA for CK1α (CK1αsh) was incubated with pyrvinium pamoate (30 nM) for 24 h. Lysates were immunoblotted for β-catenin, Axin and tubulin (loading control) or assayed for TOPflash to assess Wnt signaling.
- For the TOPflash assays, cells were treated with Wnt3a. Graph shows mean ± s.e.m., normalized to cell number and performed in triplicate.

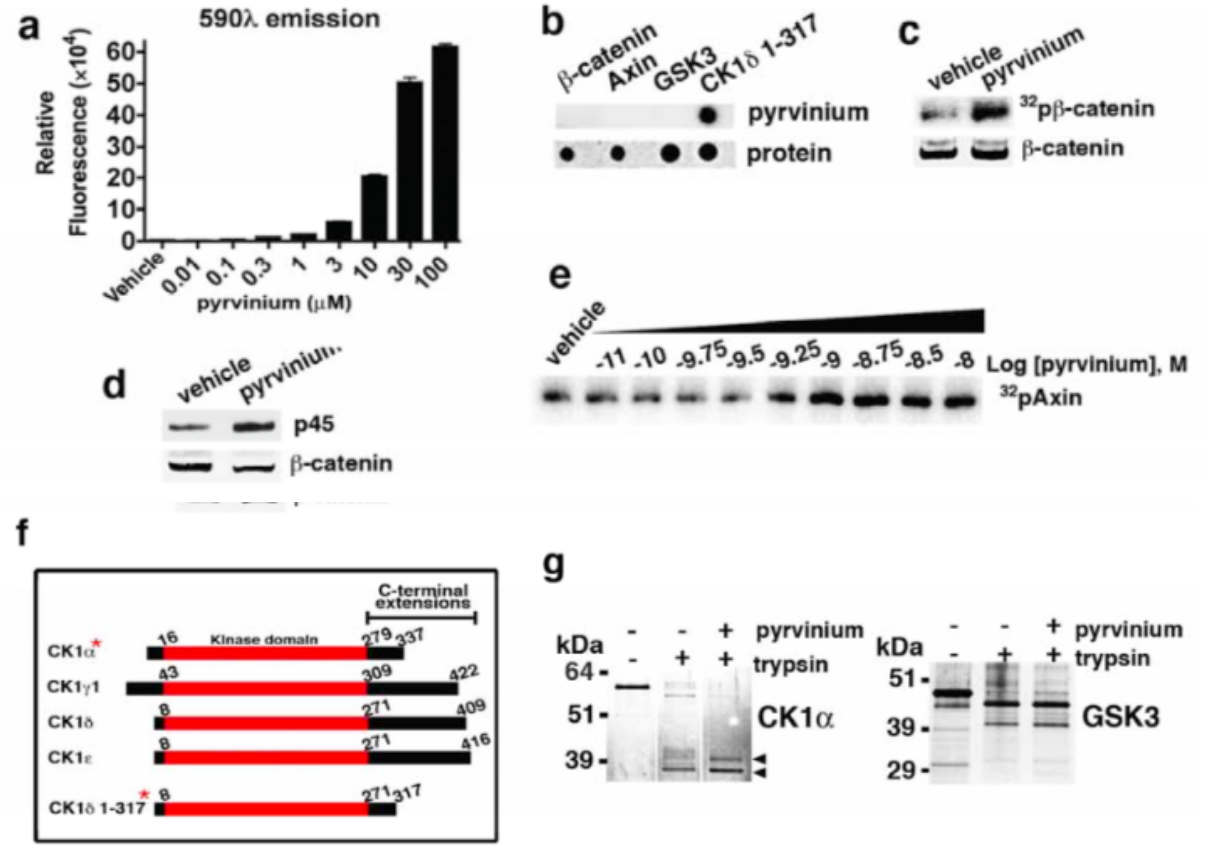


- In addition to its effects on Axin and β-catenin levels,
- pyrvinium promotes degradation of Pygopus, a Wnt transcriptional component. Pyrvinium treatment of colon cancer cells with mutation of the gene for adenomatous polyposis coli (APC) or β-catenin inhibits both Wnt signaling and proliferation. Our findings reveal allosteric activation of CK1α as an effective mechanism to inhibit Wnt signaling and highlight a new strategy for targeted therapeutics directed against the Wnt pathway.

- Pyrvinium binds CK1 *in vitro*. β-catenin, Axin, GSK3, and CK1δ1-317 (0.5 μg each) were spotted on nitrocellulose, incubated with pyrvinium pamoate (10 nM), and fluorescence detected (**c**,**d**).

- Pyrvinium stimulates phosphorylation of β-catenin by CK1 *in vitro*

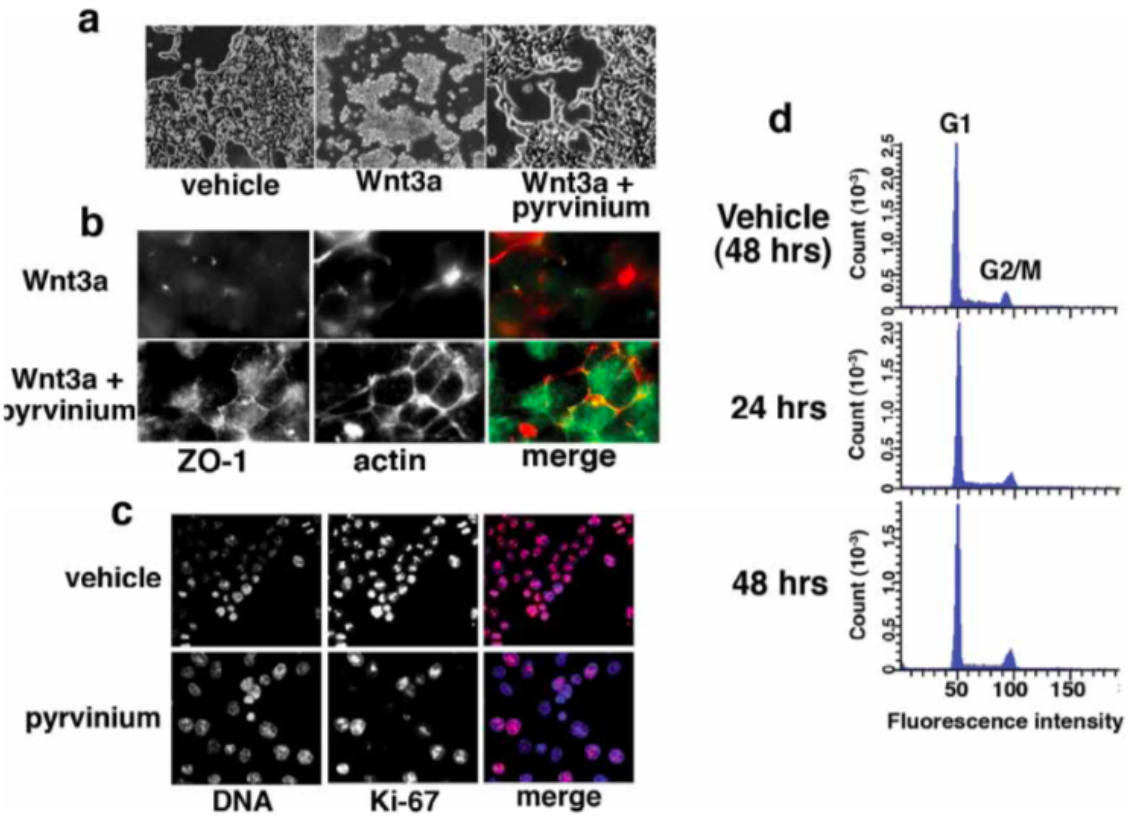
- Purified β- catenin and CK1 (100 nM each) were incubated in a kinase reaction containing [γ32P]ATP in the absence or presence of pyrvinium pamoate (1 nM).
- Samples were analyzed by SDS-PAGE/autoradiography. (**d**) CK1 and β-catenin (100 nM each) were incubated in a kinase reaction and phospho-Ser45 β-catenin (p45) detected by immunoblotting. (**e**) Pyrvinium stimulates *in vitro* phosphorylation of Axin by CK1 in a dose-dependent manner. Purified Axin and CK1 (100 nM each) were incubated in a kinase reaction containing [γ32P]ATP in the absence or presence of varying concentrations of pyrvinium pamoate. Samples were analyzed by SDS-PAGE/autoradiography.
- Recombinant CK1δ1-317 was used as the source of CK1.
- Autophosphorylation of the C-terminal extensions of CK1 isoforms has been shown to inhibit kinase activity.
- Of particular note is that CK1α contains a shorter C-terminal extension compared to the other CK1 isoforms. CK1δ1- 317 is a truncated form that lacks a portion of the C-terminal extension of CK1δ. Asterisks mark isoforms activated by pyrvinium (α and δ1-317).
- Pyrvinium affects the conformation of CK1α *in vitro* as assayed by limited trypsin proteolysis. Sliver stained gel indicate prominent CK1α fragments (arrowheads) that only appear when purified, recombinant CK1α is trypsinized in the presence of pyrvinium pamoate (100 nM). No observable difference in the digest pattern is detected when GSK3 is trypsinized in the absence or presence of pyrvinium pamoate.



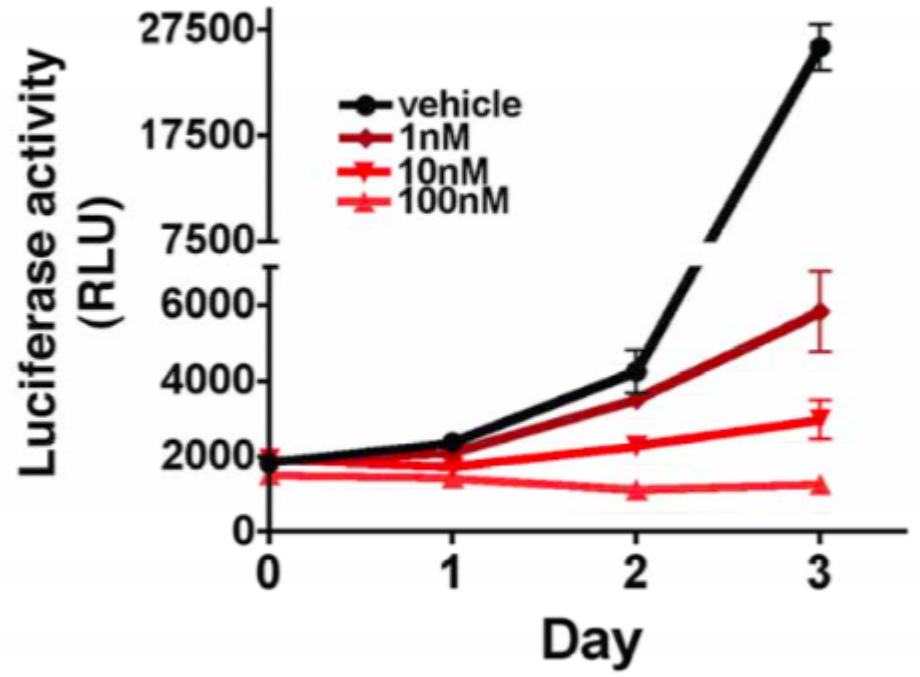
Pyrvinium inhibits Wnt3a-induced changes in cell morphology and repression of the junction marker, ZO-1. HEK 293 cells were treated with Wnt3a in the absence or presence of pyrvinium pamoate (10 nM) for 7 days.

- Phase-contrast micrographs (20X magnification) show reversion of Wnt3a-induced changes in morphology by pyrvinium pamoate.
- Fluorescent micrographs (40X magnification) show that pyrvinium pamoate enhances ZO-1 staining in Wnt3a-treated cells. Phalloidin staining of actin marks the cell cortex.
- Pyrvinium decreases proliferation of HCT116 WTKO cells without affecting cell- cycle phasing.
- Fluorescent micrographs (100X magnification) of cells treated with or without pyrvinium pamoate (100 nM) for 48 hours, fixed, and stained for Ki-67, a proliferation marker.

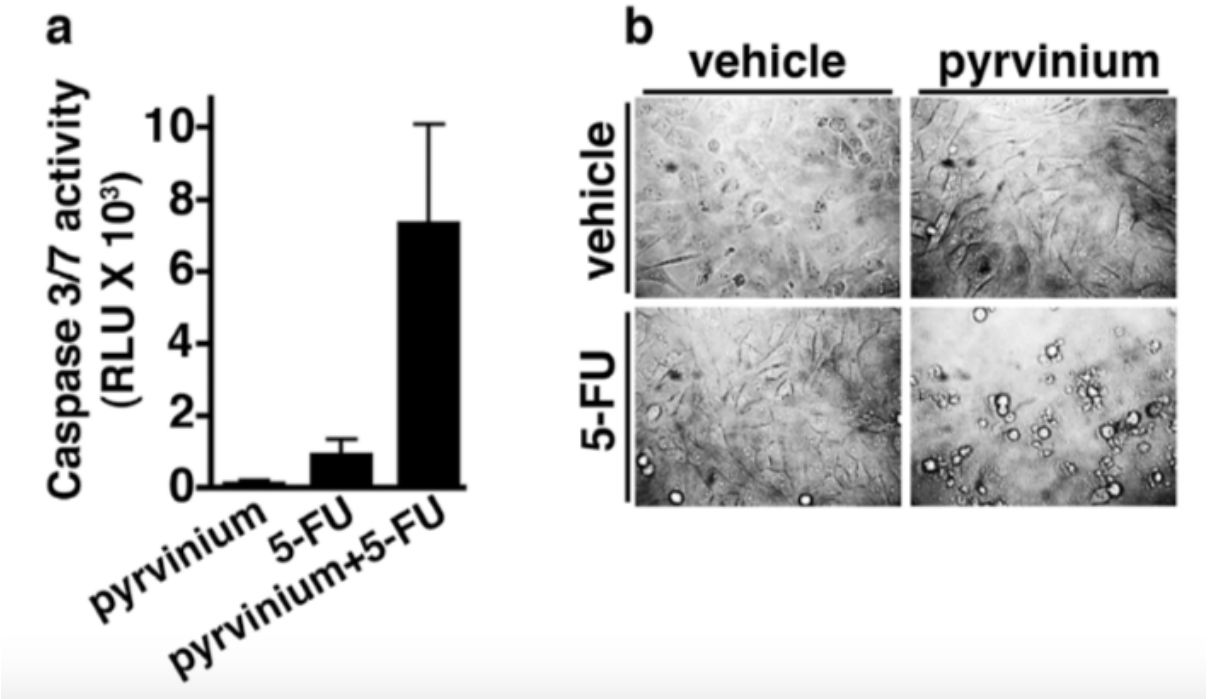
- Cells were treated with or without pyrvinium pamoate (100 nM) for 24 or 48 hours, fixed, stained with DAPI, and analyzed by FACS.



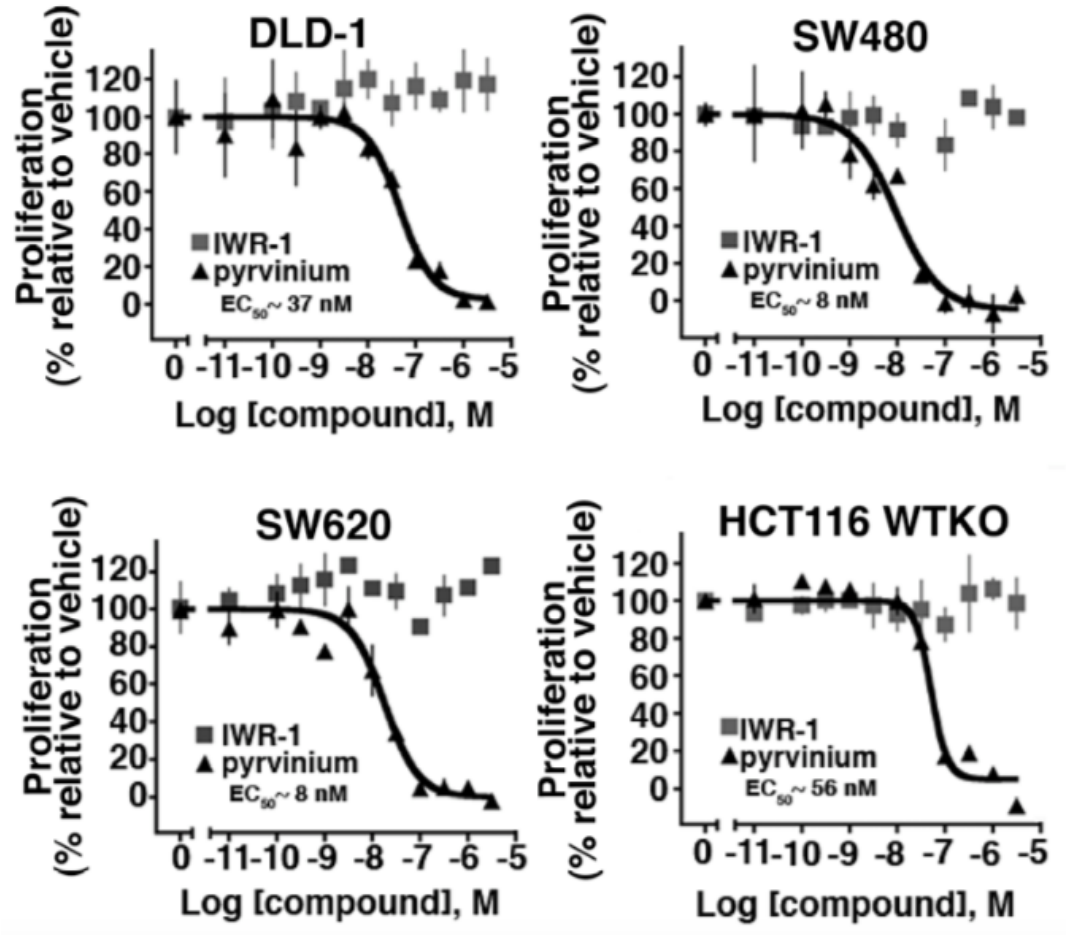
- **Pyrvinium inhibits the growth/proliferation of cultured cancer cells in a dose-dependent manner.** HCT116 cells were incubated with varying concentrations of pyrvinium pamoate and viability assessed at the indicated times using CellTiter-Fluor (Promega).



- **Pyrvinium potentiates the apoptotic effects of 5-FU on colon cancer cells.**
    - The highly metastatic colon cancer line, SW620, was treated with combinations of pyrvinium pamoate (100 nM) and 5-FU (5 μM).
    - (**a**) Mean ± s.e.m. of caspase 3/7 activity normalized to vehicle-treated cells is graphed (performed in triplicate).
    - (**b**) Phase contrast micrographs (20X magnification) show enhanced blebbing (indicative of apoptosis) of cells treated with both compounds.



- **Pyrvinium but not IWR-1 decreases viability of colon cancer cells under normal serum conditions.** Colon cancer lines (SW480, SW620, HCT116 WTKO, and DLD-1) were treated for 72 hours with the indicated concentrations of pyrvinium pamoate or IWR-1 in normal growth media (10% FBS) and cell viability determined.



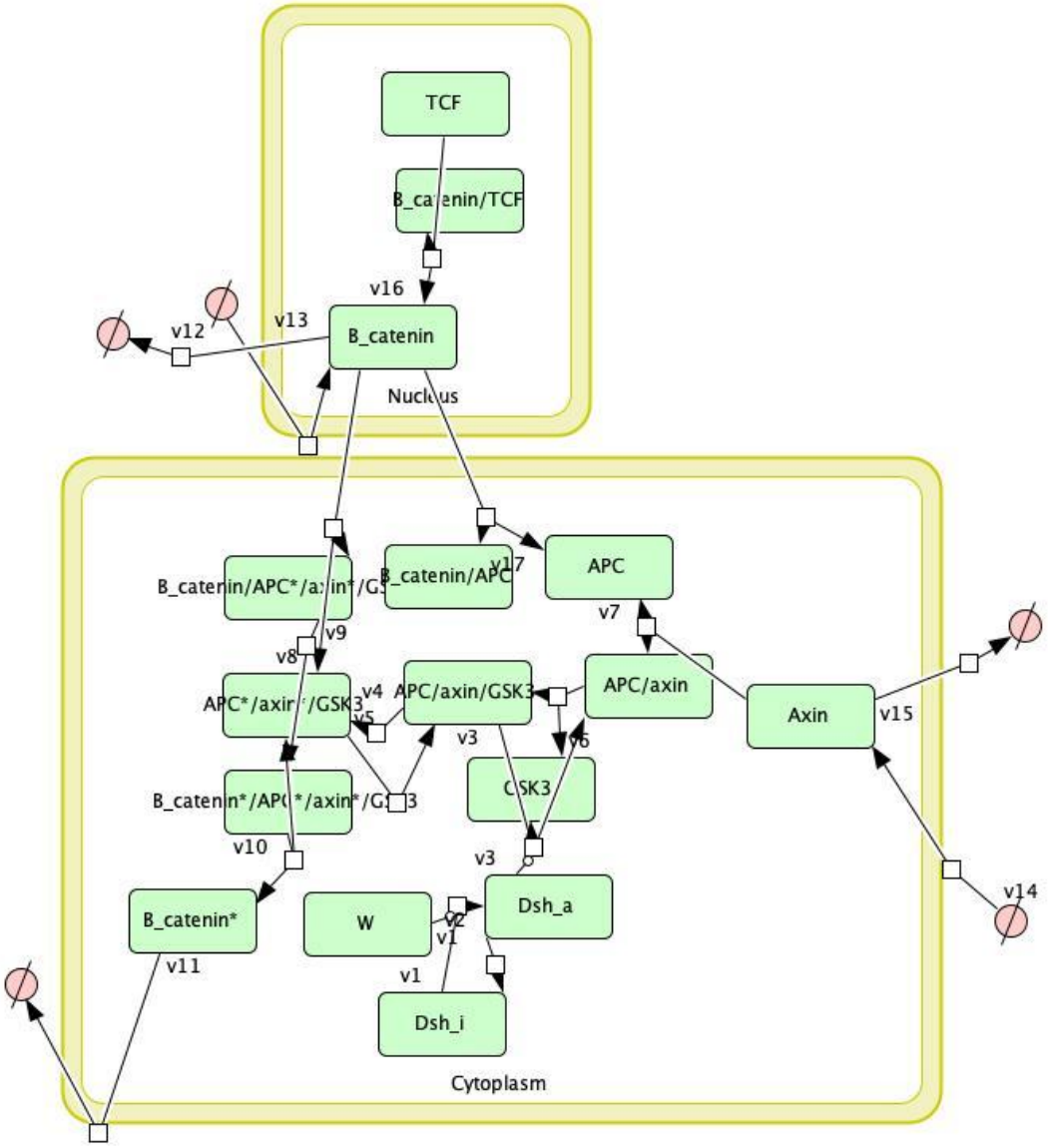
- **Effects of Pyrvinium over 4 days**

Name	nxid6748	nxid8670	nxid10541	nxid10349
Axin2	236	109	72	37
Ccnb1	119	35	39	22
Cdk6	210	144	160	71
Cdkn1a	29	12	10	13
Cdkn1b	72	41	17	48
Ctnnb1	747	433	476	432
Kras	50	30	36	31
Lgr5	20	9	7	7
Myc	36	27	23	6
Wnt3	5	3	2	0

- **CK1 effected APC and Axin in Wnt Pathway**
    - Wnt signaling plays an important role in both oncogenesis and development.
    - Activation of the Wnt pathway results in stabilization of the transcriptional coactivator beta-catenin.
    - Recent studies have demonstrated that axin, which coordinates beta-catenin degradation, is itself degraded.
    - Although the key molecules required for transducing a Wnt signal have been identified, a quantitative understanding of this pathway has been lacking.
    - We have developed a mathematical model for the canonical Wnt pathway that describes the interactions among the core components: Wnt, Frizzled, Dishevelled, GSK3beta, APC, axin, beta-catenin, and TCF.
    - Using a system of differential equations, the model incorporates the kinetics of protein-protein interactions, protein synthesis/degradation, and phosphorylation/dephosphorylation.
    - We initially defined a reference state of kinetic, thermodynamic, and flux data from experiments using Xenopus extracts. Predictions based on the analysis of the reference state were used iteratively to develop a more refined model from which we analyzed the effects of prolonged and transient Wnt stimulation on beta-catenin and axin turnover.
    - We predict several unusual features of the Wnt pathway, some of which we tested experimentally. An insight from our model, which we confirmed experimentally, is that the two

scaffold proteins axin and APC promote the formation of degradation complexes in very different ways.

- We can also explain the importance of axin degradation in amplifying and sharpening the Wnt signal, and we show that the dependence of axin degradation on APC is an essential part of an unappreciated regulatory loop that prevents the accumulation of beta-catenin at decreased APC concentrations.
- By applying control analysis to our mathematical model, we demonstrate the modular design, sensitivity, and robustness of the Wnt pathway and derive an explicit expression for tumor suppression and oncogenicity.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APC
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v4
v5
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Axin
v15
v3
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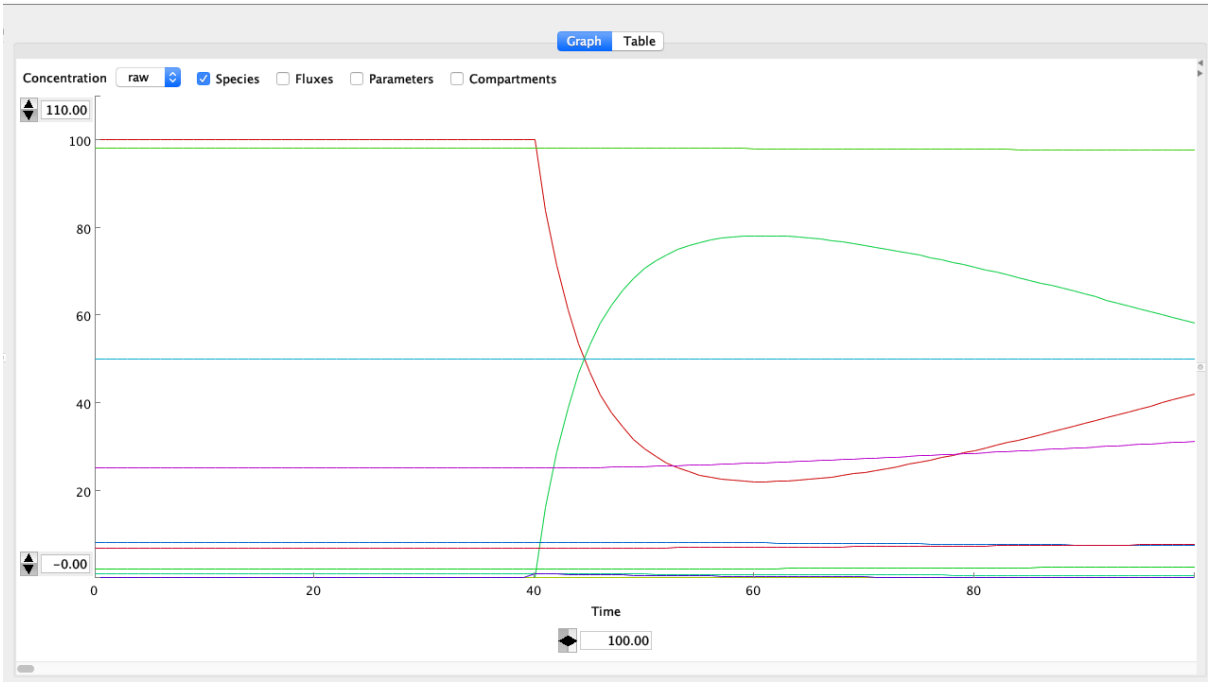
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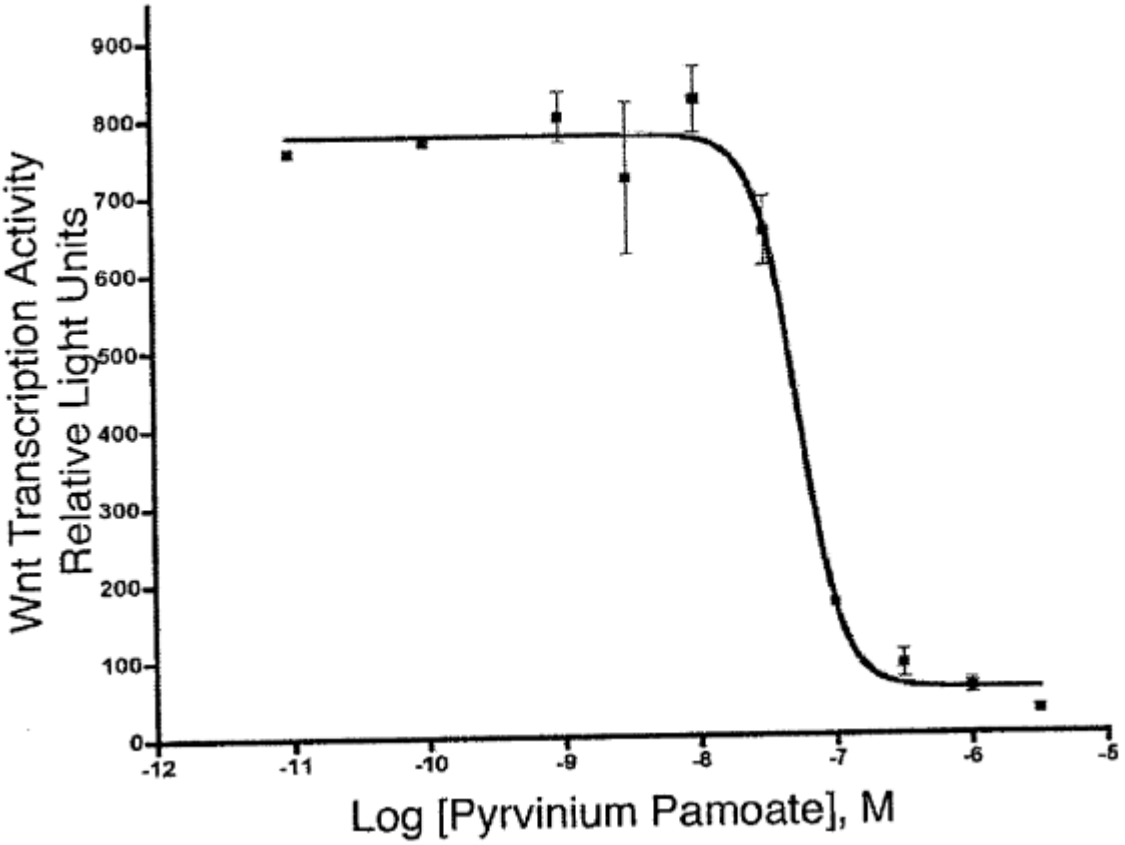
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400
300
200
100
0
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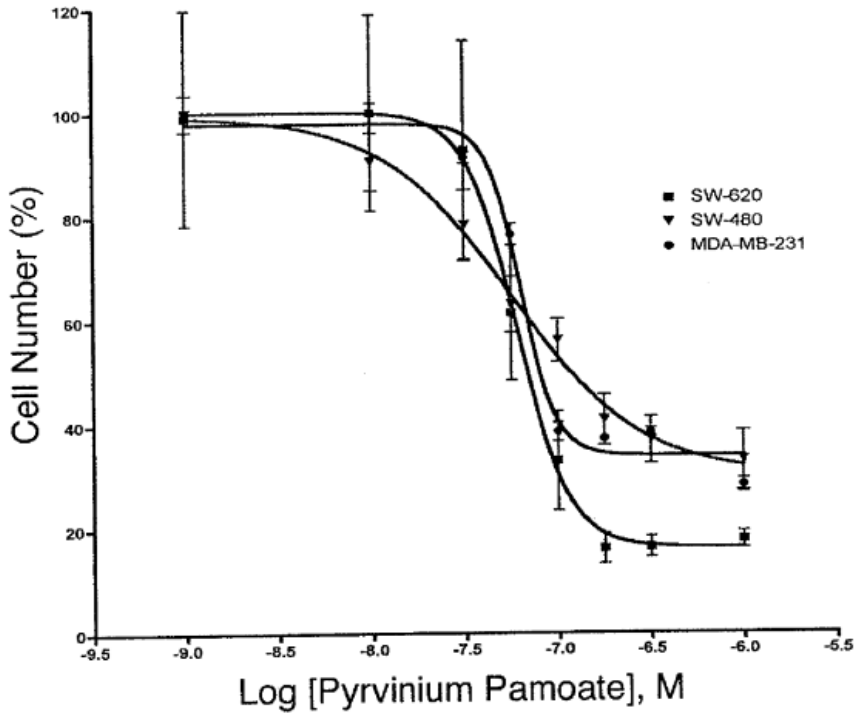
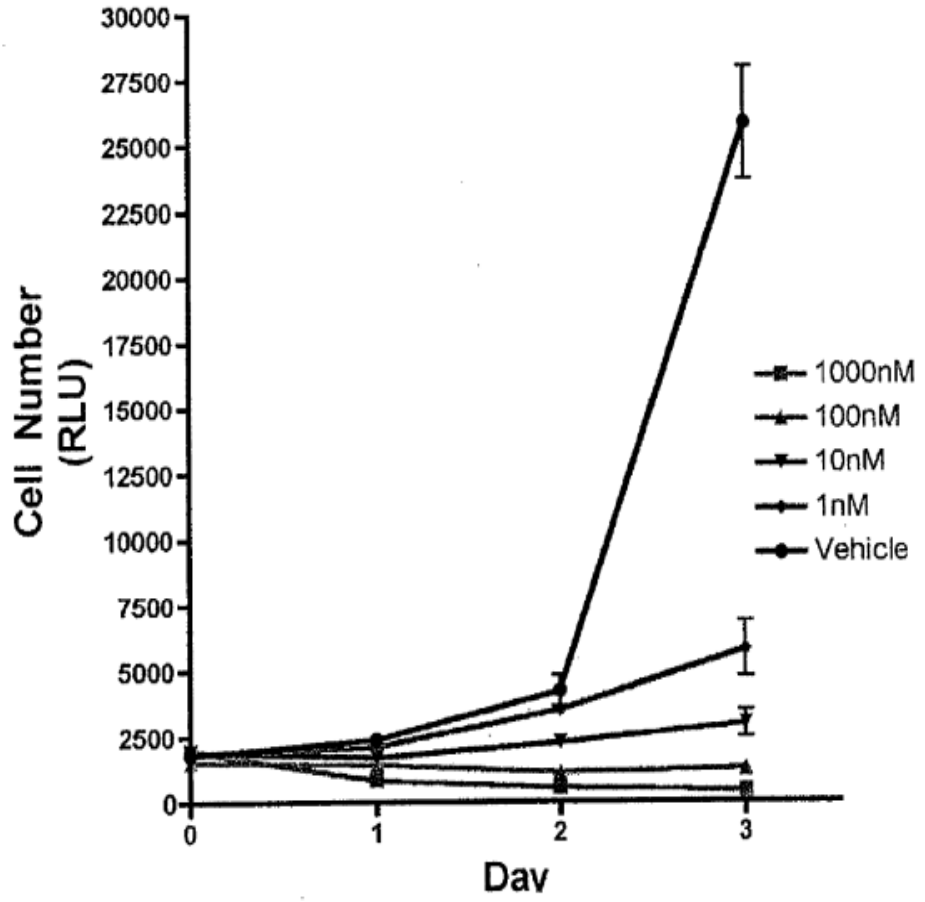
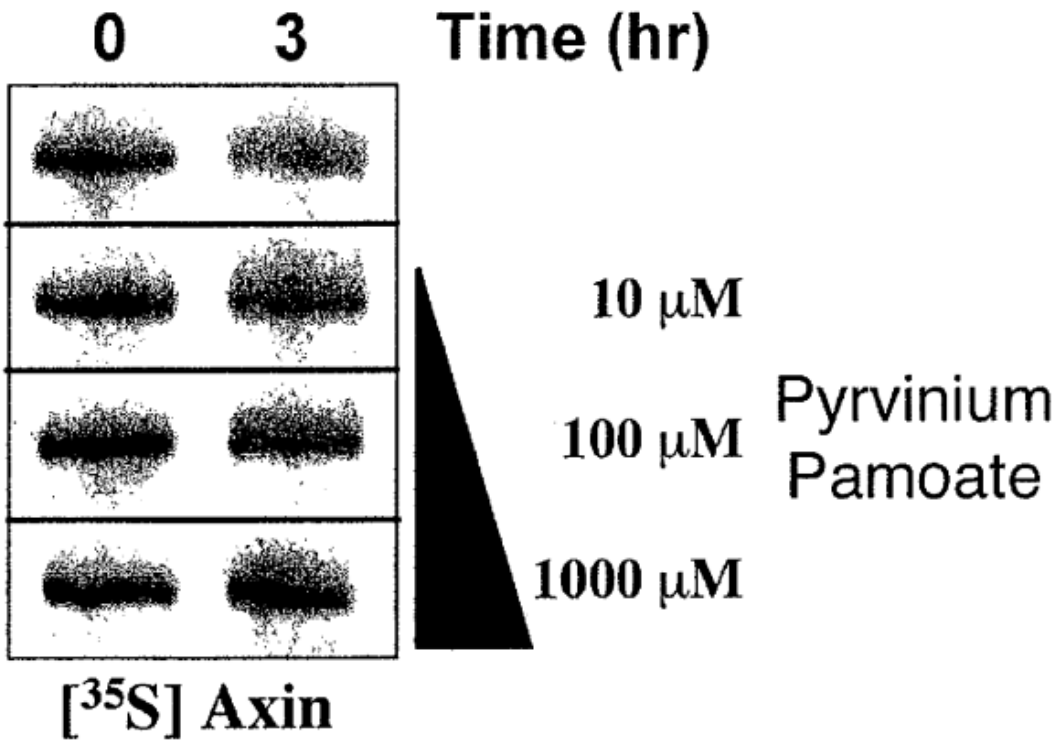
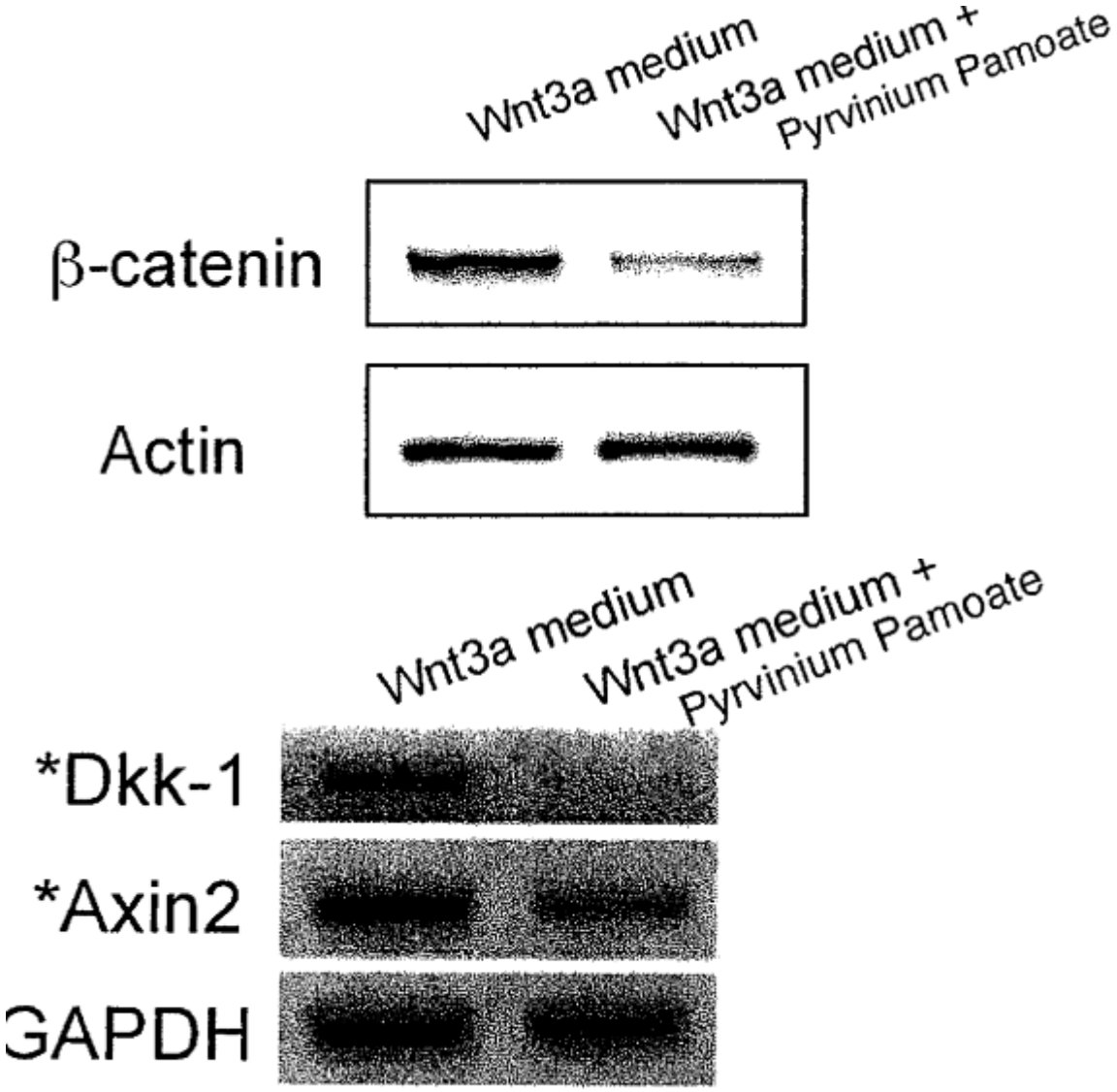
-11
-10
-9
-8
-7
-6
-5
Log [Pyrvinium Pamoate], M


Cell Number (%)
120
100
80
60
40
20
0
-9.5
-9.0
-8.5
-8.0
-7.5
-7.0
-6.5
-6.0
-5.5
SW-620
SW-480
MDA-MB-231
Log [Pyrvinium Pamoate], M

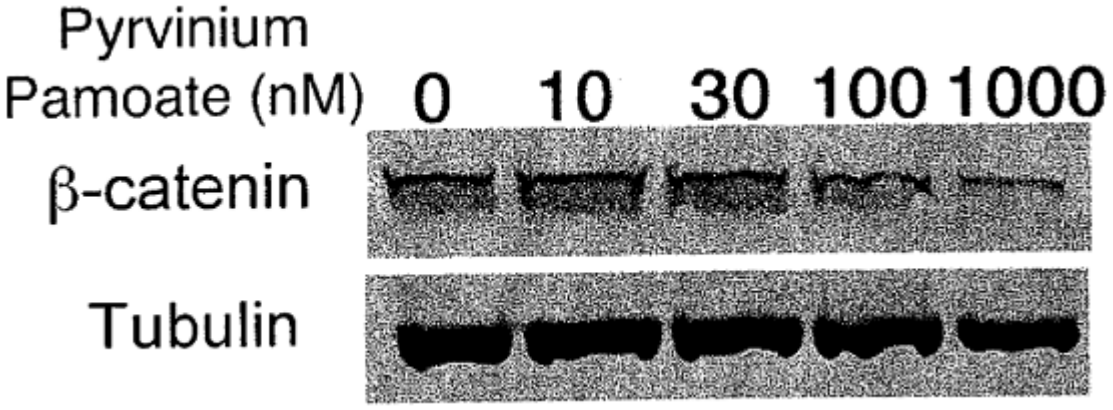
HCT 116

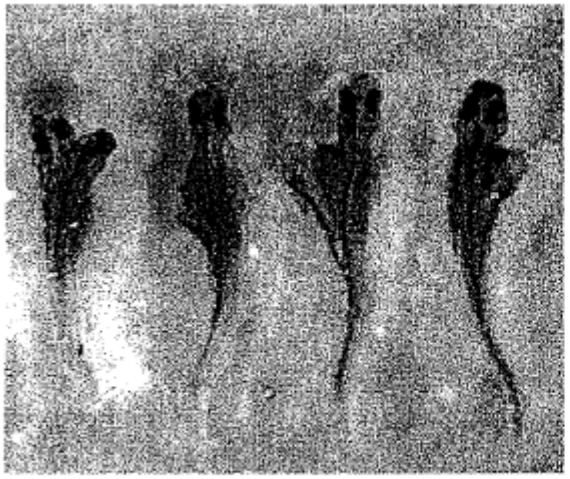

Cell Number (RLU)
30000
27500
25000
22500
20000
17500
15000
12500
10000
7500
5000
2500
0
0
1
2
3
Day
1000nM
100nM
10nM
1nM
Vehicle


0
3
Time (hr)
10 μM
100 μM
1000 μM
Pyrvinium
Pamoate
[35S] Axin

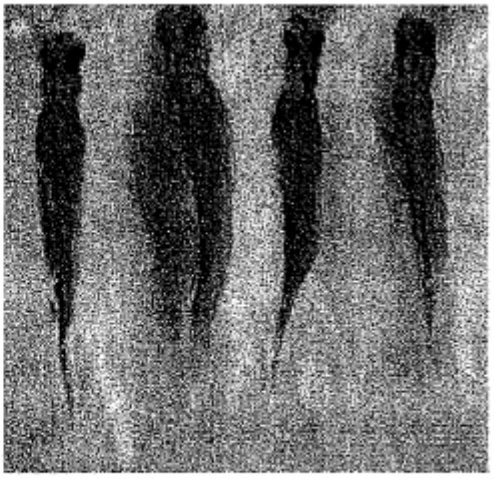


* Wnt target genes





Wnt 8



Wnt 8 +
Pyrvinium pamoate

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