# Identification and characterization of novel small molecule inhibitors of ubiquitin specific peptidase 1 (USP1)

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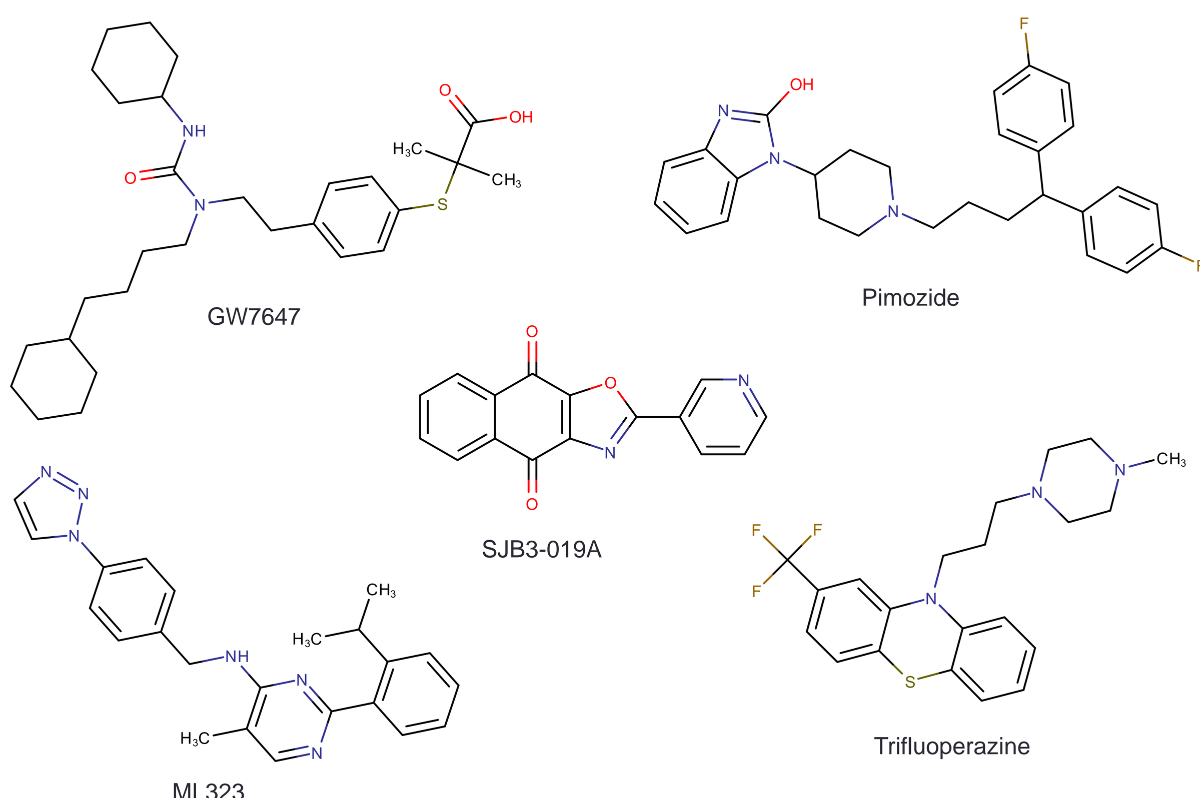
## BACKGROUND

- Deubiquitinating enzymes (DUBs) are an important group of regulators in the ubiquitin-proteasome system, responsible for cleaving ubiquitin from substrate proteins.[1]
- Ubiquitin specific peptidase 1 (USP1), in complex with USP1-associated factor 1 (UAF1), deubiquitinates substrates involved in key oncogenic pathways.[2]
- USP1 regulates the DNA damage response by deubiquitinating FANCD2, FANCI, and PCNA, which are major modulators of interstrand crosslink repair and translesion synthesis.[3, 4, 5]
- In addition, USP1 sustains cancer cell stemness by increasing the stability of ID proteins through deubiquitination.[6]
- Previously, inhibition of USP1 has been suggested to be used to increase the effect of DNA damaging drugs on cell viability.[2, 7, 8]
- As part of Medivir's DUB drug discovery efforts, we have evaluated the tractability and the feasibility of the USP1/UAF1 complex as a drug target using the Medivir DUB platform, and initiated in house hit and lead finding activities to identify novel chemical entities for inhibition of this target.

## CHARACTERIZATION OF PUBLISHED USP1 INHIBITORS

- Initially, we characterized a set of published USP1 inhibitors in order to identify tool compounds for biological assays, and to evaluate their suitability as starting points for a hit and lead finding activity.[8, 9, 10]
- Reported USP1 inhibitors such as pimozide, GW7647 and trifluoperazine are shown to be inactive in the K48-diubiquitin cleavage assay.
- While displaying good permeability, ML323 and SJB3-019A are either non-selective, or characterized by low solubility or poor metabolic stability.

### Evaluated literature compounds

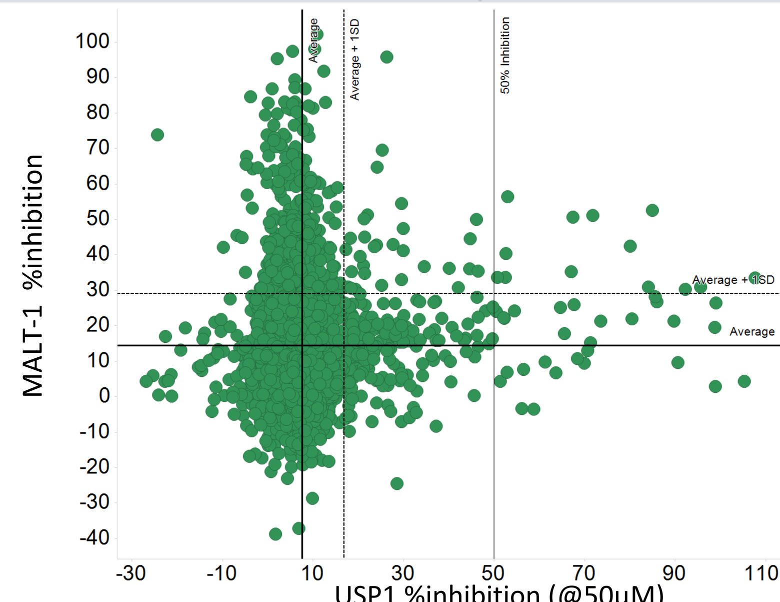



| Compound | ML323 | Pimozide | GW7647 | SJB3-019A | Trifluoperazine |
|---|---|---|---|---|---|
| Parent MW | 384.4 | 461.5 | 502.7 | 276.2 | 407.5 |
| USP1 $IC_{50}$ (µM) | 6.3 | >100 | >100 | 6.1 | >100 |
| USP7 $IC_{50}$ (µM) | >100 | >100 | 17 | 14 | 79 |
| USP14 $IC_{50}$ (µM) | >100 | >100 | N.T | 5.4 | N.T |
| USP47 $IC_{50}$ (µM) | >100 | >100 | 43 | 14 | >100 |
| Kinetic Solubility in PBS (µM) | 5 | <1 | 80 | 12 | 78 |
| Caco-2 Papp ($x10^{-6}$ cm/sec) | 25 | 12 | 5 | 15 | Very low recovery |
| HLM CLint (µl/min*mg) | 250 | 66 | >300 | <6 | 79 |

## SCREENING OF A DUB-TARGETED LIBRARY

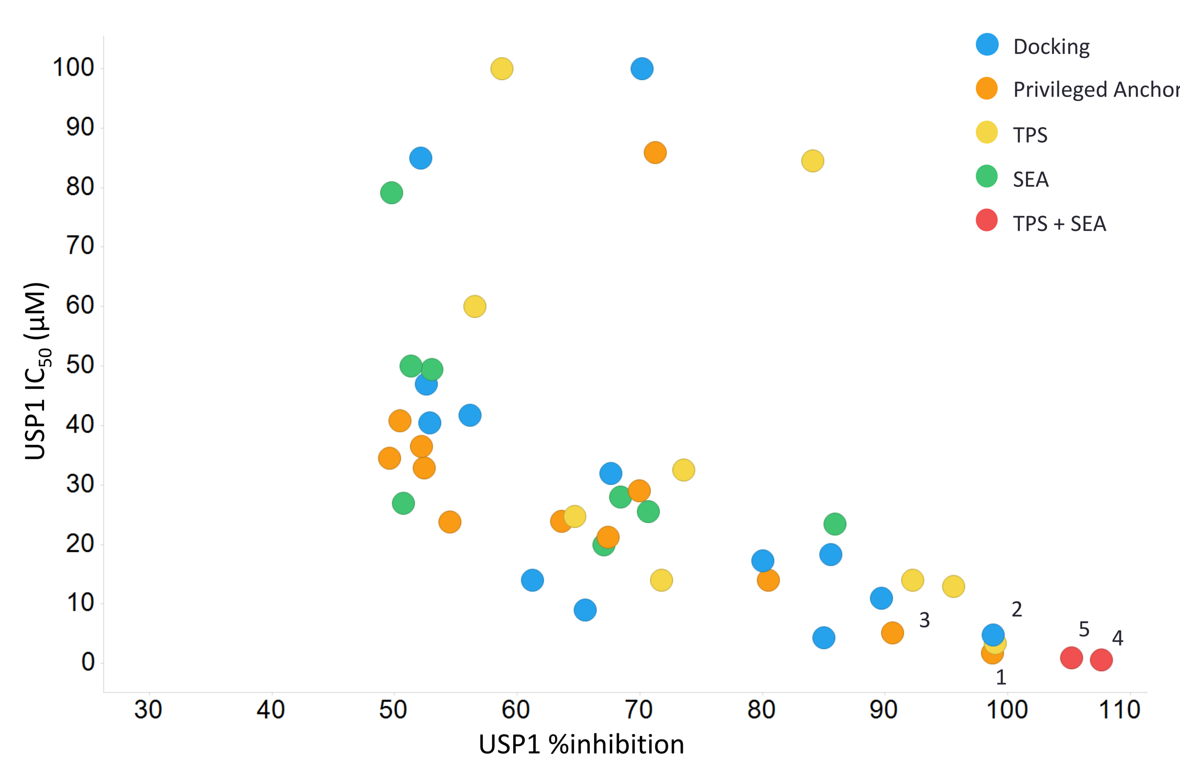
- In order to identify a starting point for a chemistry program, we constructed a target-focused library using the BioAscent Compound Cloud.[11]
- Compounds with putative reactive motifs, undesirable functional groups or low predicted solubility were filtered out.
- The selection of compounds for inclusion was subsequently based on three different approaches:
  - In silico docking to published DUB structures with the PDB entry 3NHE for USP2 and 2AYO for USP14.[12]
  - Structural similarity to known DUB inhibitors using the Similarity Ensemble Approach (SEA)[13] or a Topological Pharmacophore Search (TPS).[14]
  - Privileged anchor fragments (PAFs),[15] a target independent chemo-informatic approach to enrich bioactive motifs.
- The library of 4500 compounds was screened at 50µM, against a number of in house DUB targets as well as against MALT-1, a non-DUB cysteine protease.
- 43 preliminary hits (inhibition >50%) were identified in the K48-diubiquitin cleavage assay used for USP1/UAF1.
- The preliminary hits displayed good selectivity over the unrelated cysteine protease MALT-1.

### Screening results




### Hit confirmation

- The preliminary hits were evaluated with a concentration response curve. The majority of the hits could be confirmed and the USP1 $IC_{50}$ values were in good agreement with the results of the single point screen.
- From the distribution, we could see that the majority of hits were from the Docking or Privileged Anchor inclusion criteria. The most active compounds though, were included based on both the TPS and SEA.
- The most interesting compounds were counter-screened in other USP assays available internally, which showed that the tested compounds were non-selective inhibitors.




| Compound | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Parent MW | 334.4 | 377.5 | 248.4 | 236.3 | 323.4 |
| USP1 $IC_{50}$ (µM) | 1.7 | 4.7 | 5.2 | 0.6 | 0.9 |
| USP7 $IC_{50}$ (µM) | 10 | >100 | >100 | >100 | 63 |
| USP9x $IC_{50}$ (µM) | >100 | 4.8 | 13 | 0.9 | 1.2 |
| USP14 $IC_{50}$ (µM) | NT | 7.1 | NT | 1.5 | 1.2 |
| USP47 $IC_{50}$ (µM) | 5.7 | >100 | >100 | >100 | 88 |
| Kinetic Solubility in PBS (µM) | 3 | >100 | >100 | 10 | 2 |

## IDENTIFICATION OF IMPROVED USP1 INHIBITORS

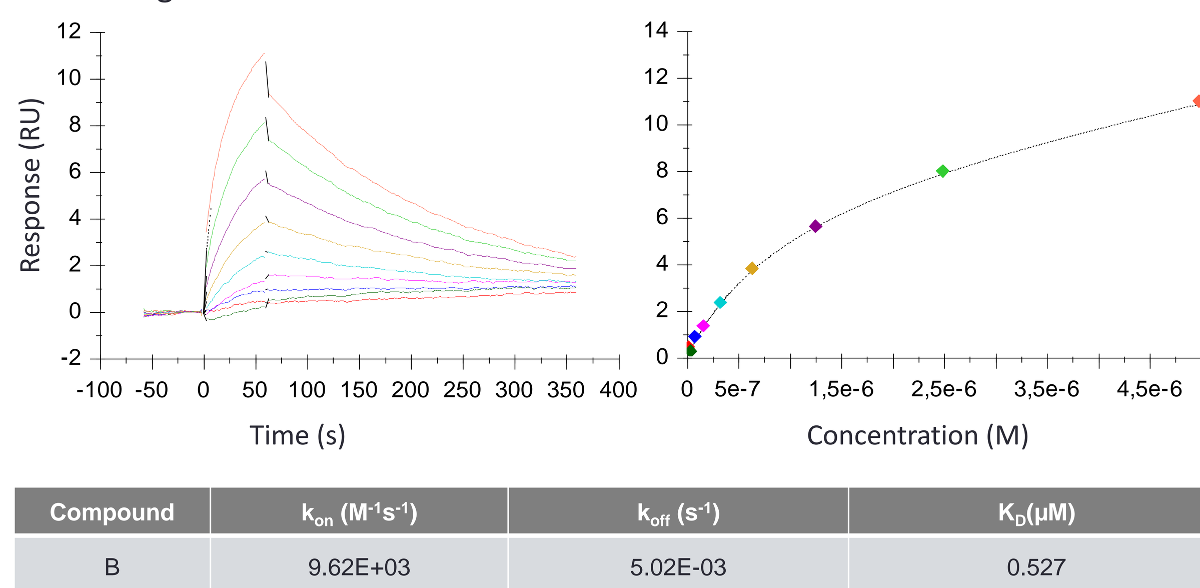
- In house elaboration led to the identification of multiple compounds with substantially improved biological and physicochemical properties.
- The representative compounds A, B and C showed very good selectivity over a set of established DUBs.
- To further characterize the compounds, we subdued them a selectivity profiling (Ubiquigent DUB*profiler*™). The compounds were active against USP1/UAF1 but showed no significant inhibition in a single point screen against 26 different DUBs (data not shown).
- Furthermore, the compounds have high permeability and good solubility in a kinetic solubility assay in phosphate buffered saline (PBS) at pH 7.4.
- Intrinsic clearance in human liver microsomes (HLM) is high and is the current focus of ongoing compound optimization.

| Compound | A | B | C |
|---|---|---|---|
| Parent MW | 476.0 | 505.5 | 509.5 |
| USP1 $IC_{50}$ (µM) | 0.11 | 0.052 | 0.072 |
| USP7 $IC_{50}$ (µM) | >100 | >100 | >100 |
| USP14 $IC_{50}$ (µM) | >100 | >100 | >100 |
| USP47 $IC_{50}$ (µM) | >100 | >100 | >100 |
| Kinetic Solubility in PBS (µM) | 15 | 74 | 9.5 |
| Caco-2 Papp ($x10^{-6}$ cm/sec) | 16 | 7.4 | 17 |
| HLM CLint (µl/min*mg) | 180 | 72 | >300 |

## BINDING VALIDATION

### Surface Plasmon Resonance (SPR)

- To further validate our in house compounds, we established an SPR binding assay using a truncated USP1, complexed with UAF1, and established immobilization protocols.
- The catalytic activity of this USP1/UAF1 complex was similar to the full length USP1/UAF1 complex, and its inhibition by compound B was demonstrated.
- The results below show a saturable binding of compound B with a $K_D$ value in the sub-µM range.
- The binding model fits to a 1:1 binding model and kinetic analysis of the binding event indicates a slow on-rate and a slow off-rate.

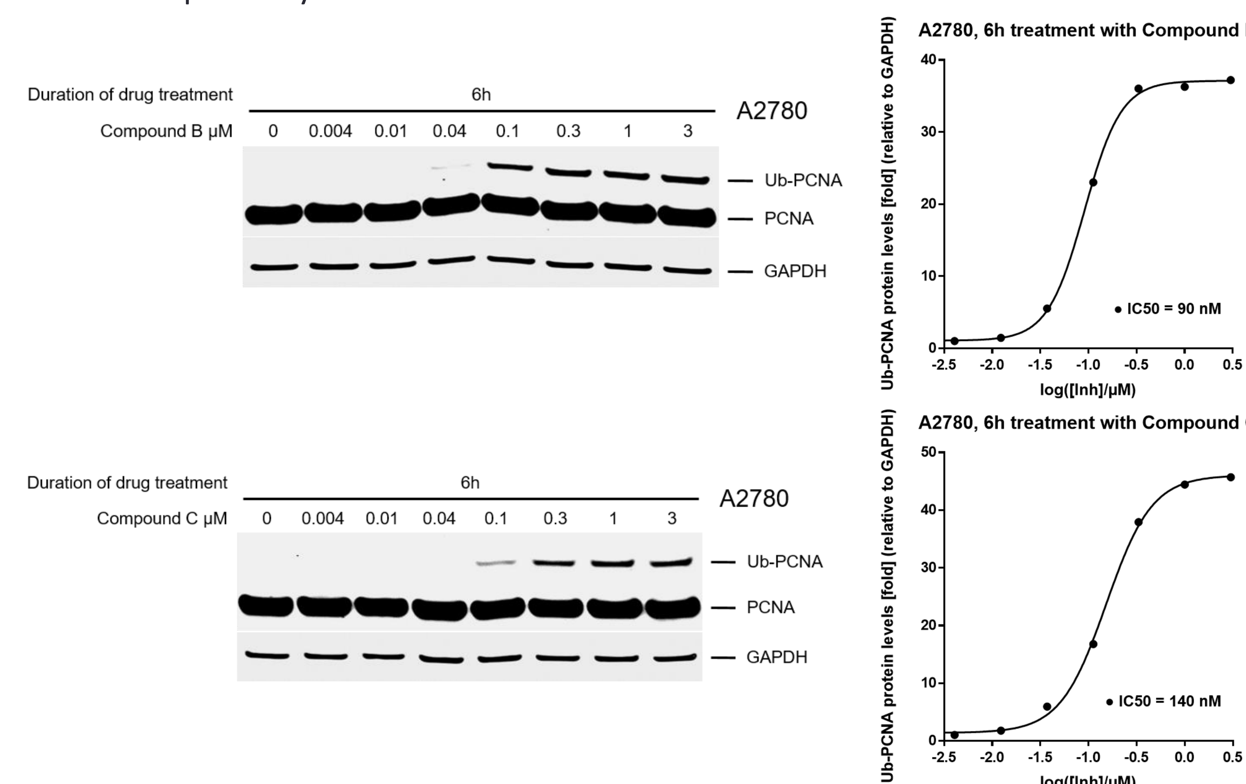



| Compound | $k_{on}$ ($M^{-1}s^{-1}$) | $k_{off}$ ($s^{-1}$) | $K_D$(µM) |
|---|---|---|---|
| B | 9.62E+03 | 5.02E-03 | 0.527 |

## EFFECT OF USP1 INHIBITION IN CELLS

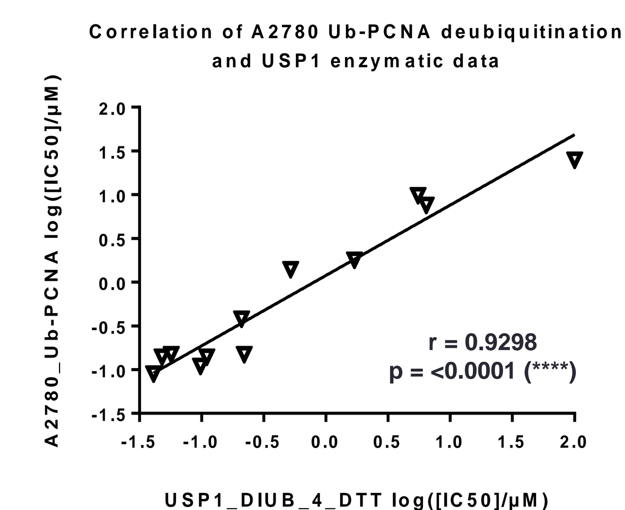
### Effect on the USP1 substrate Ub-PCNA

- Compounds B and C demonstrated robust activity in a cellular assay assessing Ub-PCNA deubiquitination, with $IC_{50}$ values of 90 and 140 nM respectively in the ovarian cancer cell line A2780.



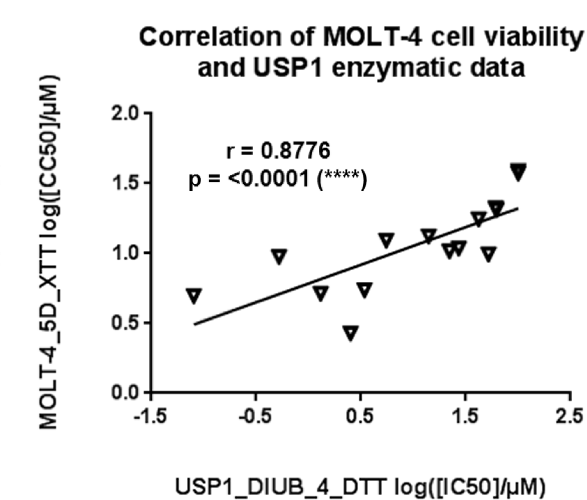

### Correlation between enzymatic assay and cell efficacy assay

- The cellular Ub-PCNA de-ubiquitination assay and the enzymatic USP1 K48-diubiquitin cleavage assay show an excellent correlation for the compounds tested.

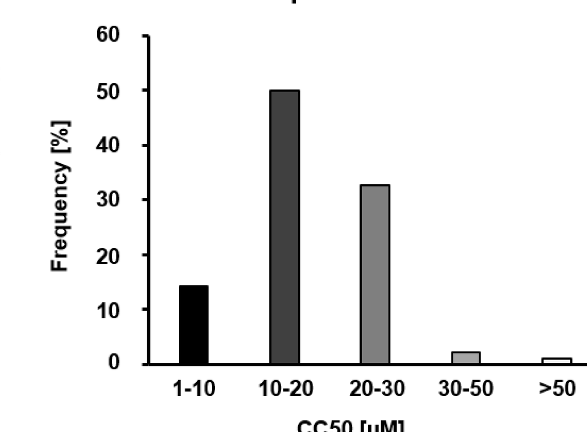



### Cell viability data

- The cell viability assay, using MOLT-4 cells, shows a very good correlation with the enzymatic USP1 K48-diubiquitin cleavage assay.




- A representative USP1-specific compound decreased cell viability with $CC_{50}$ values below 30 µM in most of the 92 cell lines, which have been tested in a cancer cell line profiling panel (Oncolead GmbH).




## SUMMARY

- We have described the design and evaluation of a DUB targeted library that rendered µM-potency hits that were suitable for further elaboration.
- Optimization resulted in potent inhibitors of USP1 with excellent selectivity profiles and promising *in vitro* pharmacokinetic properties.
- SPR were used to confirm specific binding to a catalytically active USP1 construct and allowed evaluation of compound binding kinetics.
- The compounds are active in cell-based assays of USP1 function, with potencies correlating with enzyme data, as well as in a viability screen against a panel of cancer cell lines.
- These potent and selective inhibitors are currently undergoing *in vivo* evaluation in cancer disease models.

## METHODS

- USP1/UAF1 (Boston Biochem) was assayed using DiUb48-4 FRET substrate (Life Sensors). Ub-VME-proteasome activated USP14 (produced in house) and USP9x (Boston Biochem) were assayed using ubiquitin rhodamine 110 (Life Sensors) as substrate. USP7 (produced in house) and USP47 (Life Sensors) were assayed using DiUb48-1 FRET substrate (Life Sensors).
- A2780 cells were treated with compound B or C for 6h. The resulting lysates were analysed by Western Blot using antibodies against PCNA (Santa Cruz) and GAPDH (Cell Signalling).
- After treating MOLT-4 cells with compounds for 5 days, cell viability was measured by XTT.

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