ESTABLISHMENT OF A METHODOLOGICAL PLATFORM FOR THE EXPLORATION OF MCL1 INHIBITORS

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Introduction

Many cancer cells succeed to survive by neutralizing apoptotic triggers. A frequently found evasive mechanism is to keep proapoptotic BCL2 family proteins (e.g. BAK; BIM) in check. To avoid apoptosis, cancer cells overexpress anti-apoptotic BCL2 family proteins (e.g. BCL2, BCLXL or MCL1). Latter proteins block pro-apoptotic effectors via binding to the so called BH3 binding domain and thus act as oncogenic survival factors. Therapeutic strategies aim at the disruption of this protein-protein interaction to reactivate apoptosis. Small molecular weight compounds targeting BCL2 and BCLXL such as ABT-263 (Navitoclax®) have been successfully promoted into clinics. However, there is still an urgent need for inhibitors to MCL1, which would be especially useful for MCL1-addicted cancers as well as for cancers that show resistance to ABT-263.

For discovery and optimization of compounds targeting MCL-1, our first aim was to have a reliable biochemical binding assay that would allow to detect the disruption of binding of MCL1 or BCLXL to the BH3 binding domain of its proapoptotic counterpart such as BAK.

Secondly, we needed a test system to reflect the cellular activity, so we went to establish an apoptosis- and a proliferation-assay for the multiple myeloma cell line NCI-H929, which is known to be addicted to MCL1.

Thirdly, we were interested in an assay system that would substantiate the mechanism of action. To that end, we established a combinatorial assay that would reflect the two-sided survival strategies of cells using MCL1 as well as BCLXL/BCL2 as cooperating survival mediators.

Application of this combinatorial approach to 100 tumor and 2 normal cell lines led to observation of different synergistic phenotypes.

Methods

MCL1/BCLXL-BAK BINDING ASSAY

C-terminally His-tagged proteins MCL1(172-327) or BCLXL (1-212) were incubated with indicated compound concentrations for 15 min before N-terminally biotinylated BAK peptide GQVGRQLAIIGDDINR was added for 30 min. Upon addition of Streptavidin-labeled Europium and APC-labeled anti-His antibody (Perkin Elmer) for 30 min at 30°C and 30 min at 4°C, time-resolved FRET fluorescence was detected.

APOPTOSIS ASSAY

Cells were seeded the day before compounds were added at indicated concentrations. Compounds were added by nanodrop-dispenser. 0.1% DMSO (solvent) and Staurosporine (1.0E-05 M) served as Low control (0% apoptosis) and High control (100% apoptosis), respectively. After incubation for 6 h, plates were developed with Caspase-Glo 3/7 reagent (Promega) and luminescence was measured. The IC50 values were determined by sigmoidal slope regression using the software GraphPad Prism 5.

PROLIFERATION ASSAY

Cells were seeded the day before compounds were added at indicated concentrations. Compounds were added by nanodrop-dispenser. 0.1% DMSO (solvent) and Staurosporine (1.0E-05 M) served as High control (100% viability) and Low control (0% viability), respectively. For combination studies, Compounds were added to cells in a checkerboard pattern.

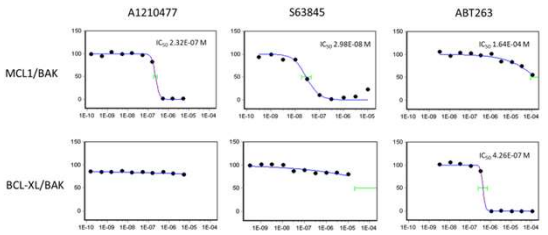
After incubation for 72 h, plates were developed with CellTiterGlo reagent (Promega) and luminescence was measured. The IC50 values were determined by sigmoidal slope regression using the software GraphPad Prism 5.

FOR ANALYSIS OF COMBINATORIAL EFFECTS, TWO METHODS WERE APPLIED:

Bliss-Factor analysis: Taking the effects observed at the different concentrations of the compounds alone, a Bliss-Factor matrix was used [E1+E2=E1+E2-E1xE2] to calculate the expected effects for a merely additive situation. This was compared with the actual data obtained. The difference of both numbers is given in the Bliss factor analysis, showing positive numbers for synergistic and negative numbers for antagonistic effects. These calculations are shown as three-dimensional plot: a hill on the plot indicates synergism, a valley antagonism.

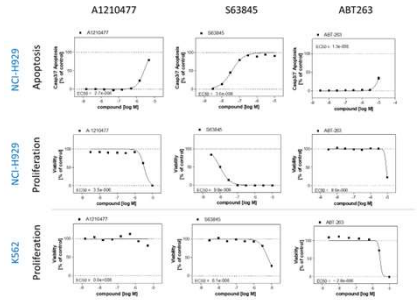
Comparison of dose-response curves of the respective first compound with and without the different concentrations of the second compound. Counts obtained for the second compound alone were set to a new High value (100%) and data obtained at different concentrations of the first compound were set in relation to that High value. An additive combination results in overlapping dose-response curves as compared to the curve obtained with the first compound alone. A synergistic combination would improve the potency of the first compound with increasing amounts of the second compound and vice versa an antagonistic combination would reduce the potency.

BINDING ANALYSIS OF MCL1 AND BCL XL INHIBITORS



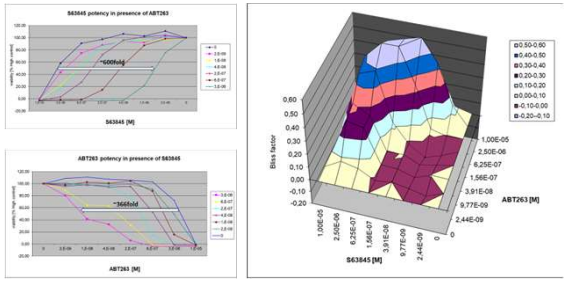
MCL1 inhibitors (A1210477; S63845) and BCL-XL inhibitor ABT263 show distinct, mutually exclusive inhibition on MCL1 or BCLXL binding to BAK

IMPACT OF MCL1 AND BCL XL INHIBITORS ON THE VIABILITY OF LEUKEMIA CELLS



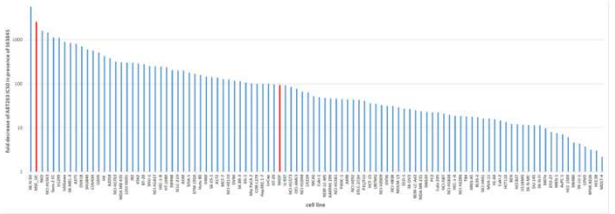
MCL1- but not BCLXL-inhibitors inhibit proliferation and induce apoptosis in MCL1 overexpressing MM cell line NCI-H929, while CML cell line K562 shows resistance.

IMPACT OF S63845/ABT263 COMBINATION ON K562 CELL PROLIFERATION



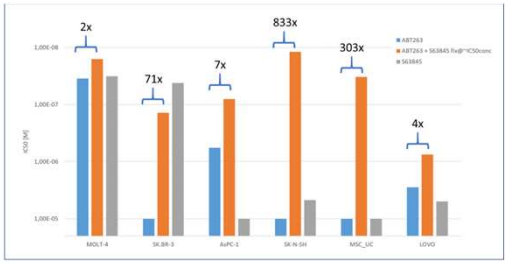
K562 cells turn sensitive to BCLXL inhibitor ABT263 when combined with MCL1-inhibitor S63845 and vice versa, resulting in up to 600 fold decreased IC50 and significant hill formation in Bliss factor analysis.

ANALYSIS OF SYNERGISM OF S63845 AND ABT263 ON 102 CELL LINES



MCL1 and BCL-XL inhibitors acted synergistically on essentially all cell lines tested. However, different types of responder cells were observed. Critically for therapeutic considerations, also compounds also strongly synergized on normal cell types (e.g. MSC and NHDF, red bars). Synergy is shown as fold decrease of ABT263 IC50 in the presence of S63845 (at fixed conc of ~ IC50) over the IC50 of ABT263 alone.

DIFFERENT SYNERGISTIC PHENOTYPES



ABT263 sensitivity	High	Low	Medium	Low	Low	Low
S63845 sensitivity	High	High	Low	Low	Low	Low
Synergism	Low	Medium	Low	High	High	Low

Different types of synergistic responder cells are shown, depending on their sensitivity towards the two agents alone as well as on their synergistic response. Differences may reflect the dependence of the cell lines on the various BCL2 family members that contribute to survival. In extremes, in MOLT4 cells either single target inhibition is sufficient for almost maximal effect, while in LOVO cells even the combined inhibition of both targets hardly shows an effect, suggesting the involvement of other survival mechanisms. Even apparent inactivity of both agents alone does not guarantee no effect – in contrast – here the highest synergistic effects and absolute activities of compound combinations were observed. Interestingly, this was observed also for primary MSC, pointing at a risk of applying this combination clinically.

Summary and Conclusion

- We have successfully established a biochemical binding assay for MCL1/BAK, as well as apoptosis and proliferation assays for MCL1-addicted NCI-H929 cells.
- These systems were successfully validated with MCL1 inhibitors A1210477 and S63845, the latter being ~10fold more potent in binding assays and ~100fold more potent in cellular assays. In contrast, BCL-XL inhibitor ABT-263 proved inactive.
- We have established a combinatorial setup for synergy analysis of MCL1-inhibitors with ABT-263.
- Combinatorial studies on 100 tumor cell lines reveals wide-spread synergistic effects, however, the degree varies and different synergistic phenotypes are observed.
- Strong synergism is observed also for two primary cell types pointing at the risk of applying this combination clinically.