

**DEVELOPMENT OF BIOCONJUGATES AND THEIR
MODUL CONSTRUCTS FOR TARGETED THERAPY
OF CANCERS WITH HIGH MORTALITY**

Excerpt from the results obtained in frame of the grant

NVKP_16-1-2016-0036

supported by the National Research, Development and Innovation Office

ISBN 978-963-489-286-1

Budapest, 2020

Design and synthesis of novel apoptosis-inducing cytotoxic drug leads for conjugation with carrier molecules and development of Tumor Targeting Drug Conjugates

Béla Bertók¹, György Dormán¹, László Kőhidai², Orsolya Láng², Csaba Magyar³

¹ComInnex Inc. Budapest, Hungary

²Semmelweis University, Budapest, Hungary

³Institute of Enzymology, Research Centre for Natural Sciences, Hungarian Academy of Sciences, Budapest, Hungary

Introduction

During the last 10 years, ComInnex specialized on developing high-quality drug-like molecules in order to cover novel chemical space of potential biological targets. ComInnex's original strategy focuses on non-flat 3-dimensional templates. The new compounds are prepared *via* hydrogenation of diverse heterocycles resulting in screening libraries with more favorable physicochemical properties and various functionalities. That approach, which was combined with up to date, *in silico* methods to generate focused Target Oriented Libraries *via* a complex platform (Figure 1), was an excellent starting point to synthesize novel compounds that might be applied as original drug leads alone or in combination with carrier compounds to develop novel Small Molecule Targeting Drug Conjugates (SMTDCs).

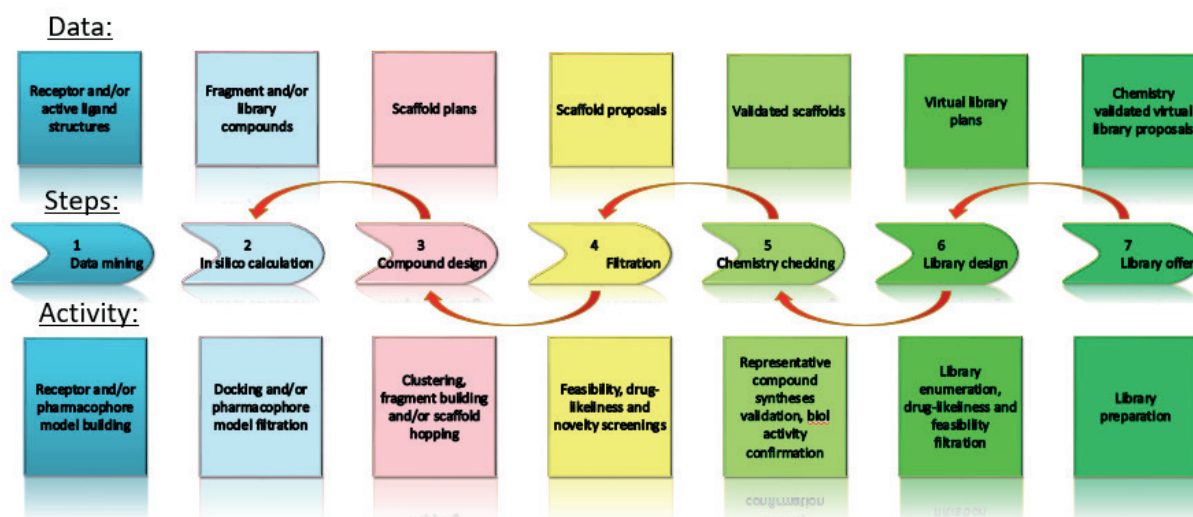


Figure 1. Target Oriented Library platform (TOL) general workflow and steps

Cancer is still one of the most challenging lethal diseases in medicinal chemistry with no or reduced surviving rate. Albeit some types of tumors already have potential treatment, there are still many tumor types without effective therapy. Our focus turned to pancreatic cancer, since it has a particularly high mortality rate (the general 5-year survival rate for

people with pancreatic cancer is 9%). The applied chemotherapies have several problems such as high resistance factor for anticancer drugs and low tolerability. The cytotoxic compounds applied in tumor treatment typically induce necrosis with toxic side-effects and inflammation, which is in contrast with the natural control mechanism of elimination and utilization of the malfunctioning cells regulated by programmed cell death (apoptosis) (Figure 2).¹

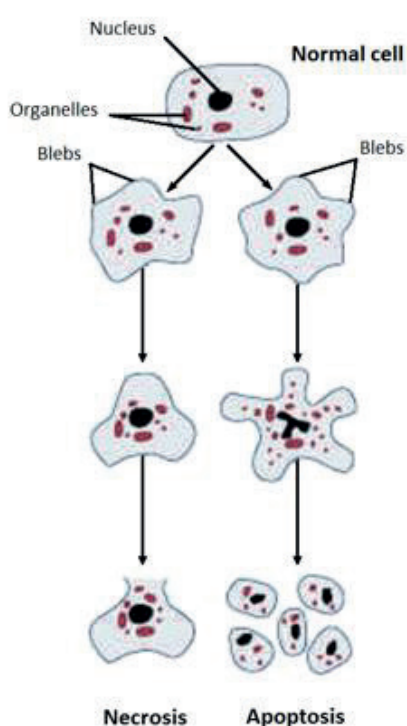


Figure 2. Main differences in necrotic and apoptotic elimination of cells

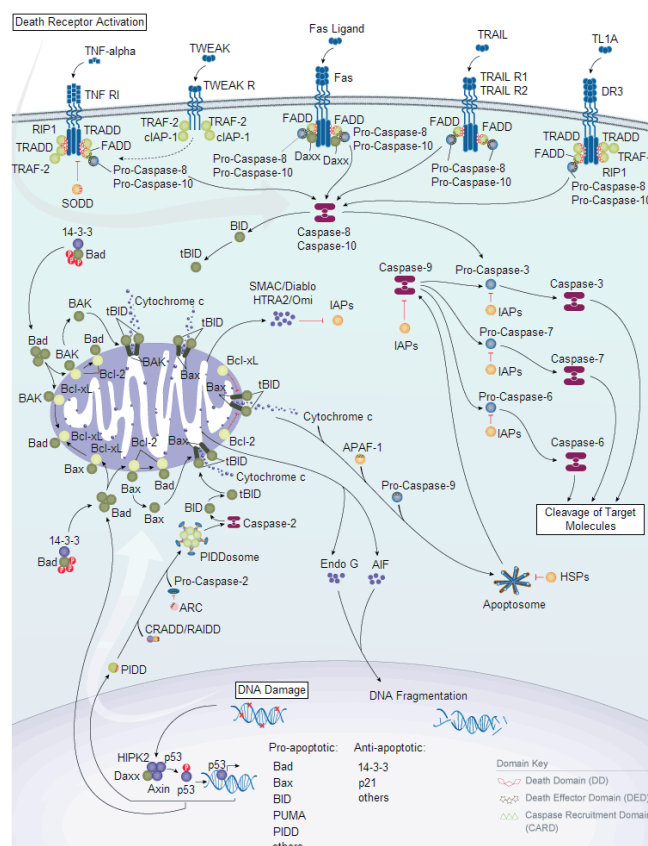


Figure 3. Apoptosis signalling pathways

Cancer is associated with the damage of the programmed cell death regulation, resulting in blocked elimination of the tumor cells, together with the resistance developed to chemotherapy. In cancer, the apoptotic signaling is de-regulated, particularly by the activation of an anti-apoptotic system, which allows cancer cells to escape this program leading to uncontrolled proliferation and tumor survival.²

Apoptosis has a complex regulation mechanism (Figure 3), since on one hand, it has a vital role in removing malignant cells, on the other hand, the uncontrolled or released apoptosis might result finally in killing the patient. The goal we targeted was, therefore, to develop novel apoptosis-inducing or regulating cytotoxic lead compounds, which might also be conjugated with specific carrier molecules to ensure selectivity only on cancer cells. Our strategy can be summarized as Triple Action Magic Bullet approach (Figure 4).

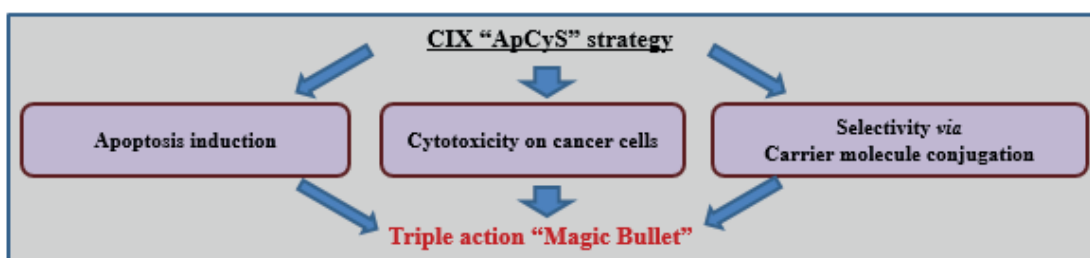


Figure 4. Triple Action Magic Bullet strategy

Design, synthesis and filtration of a diversified focused library

Several hundred diverse compounds were designed applying the ComInnex TOL platform. The compounds were filtered *via* chemical feasibility and physicochemical properties. The parameters were set according to the ADME properties characteristic to the existing small molecule drug developments:

MW	<350
clogP	<3.5
HBD	<5
HBA	<9

Finally, more than 200 compounds of different heterocycles were delivered and screened for cytotoxic activity on various cancer cell lines in the focus of two pancreatic cancer cell lines, PANC-1, and MiaPaCa-2. Six *structures* having cytotoxic activity higher than 10^{-4} were found from which 5 skeletons are illustrated in Figure 5. Two molecule cores which had more hits were picked for further development as starting points.

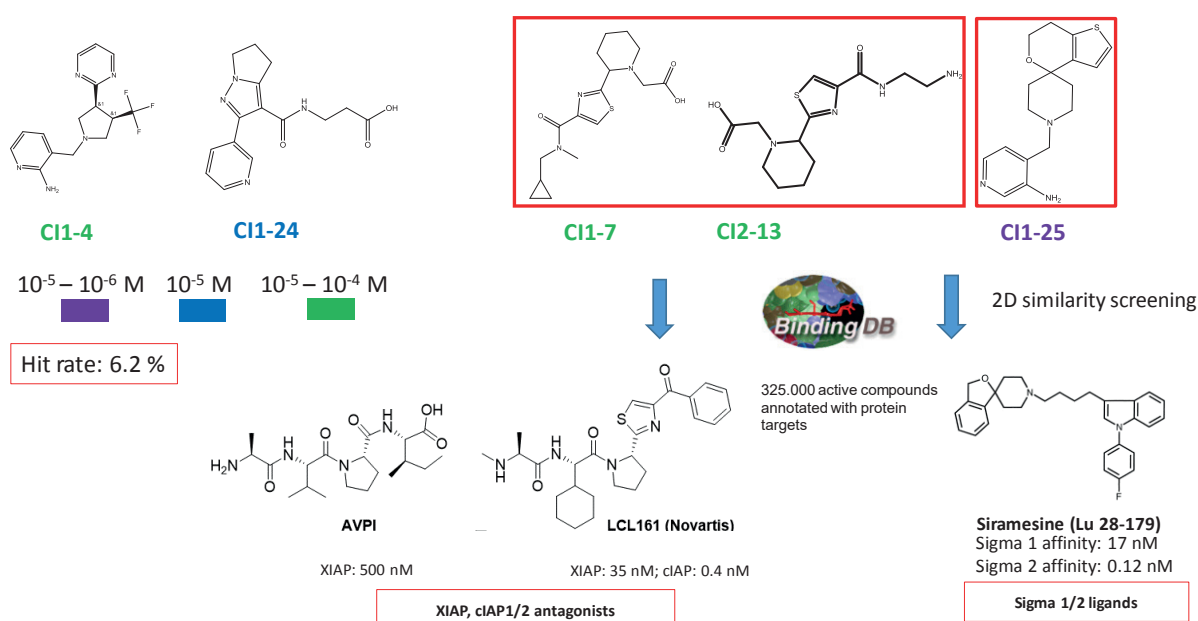


Figure 5. 1st round *in vitro* cytotoxicity screening: best hits/ *in silico* protein target prediction

2D similarity search based on the structure of active compounds applying annotated databases (*e.g.* BindingDB) confirmed the biological potency and revealed the potential proteins targets. Compound group of CI1-7/CI2-13 and CI1-25 showed similarity to existing bioactive compounds in development as XIAP antagonists (member of the Inhibitor of Apoptosis Protein family) and sigma-1,2 ligands, respectively.

The physiological function of Inhibitor of Apoptosis Protein family (IAP) and their antagonists^{3,4}

Natural anti-apoptotic proteins play an important role in the regulation of the programmed cell death (apoptosis). Those proteins regulate (inhibit) the activation of caspases. One of the most important protein groups in that regulation pathway is the Inhibitors of Apoptosis Proteins, comprising various proteins with high similarity (XIAP: X-linked IAP, cIAP1/2: cellular IAP1/2). XIAP inhibits initiator (Caspase-9) and effector caspases (Caspase-3 and -7), through the interaction of its BIR domains (BIR3 and BIR2) with the N-terminal tetrapeptide (AVPI) of the SMAC (Second Mitochondria-derived Activator of Caspases) or in other term, DIABLO (direct IAP-binding protein with low pI) protein. (Figure 6)

The IAP or XIAP antagonists such as SMAC/DIABLO and their mimetics temporarily suspend the inhibition of caspases. Intensive research activity is going on the field. Several SMAC mimetics were developed in the last couple of years,^{5,6} and 7 of them reached clinical phases. One of the first compounds, the Novartis' LCL161 (Figure 6) is particularly promising and has recently entered Phase II. clinical trials. That compound has not only hung up the inhibition of apoptosis but in parallel directly induces apoptosis by activating TNF α . The compound mimics the AVPI tetrapeptide, which is the protein binding motif of the natural SMAC peptide.

SMAC/AVPI is able to bind to the BIR3 domain of cIAPs as well and this interaction induces auto-degradation of the protein through E3 ligase mediated ubiquitination by the UBA/RING domain. However, if SMAC mimetics (compounds having AVPI-like structural motifs) are linked to specific binders of a POI (Protein of Interest) the conjugate could induce ubiquitination and degradation of this protein. Auto-ubiquitination and targeted ubiquitination can be balanced by achieving binding selectivity towards XIAP *vs.* cIAPs.

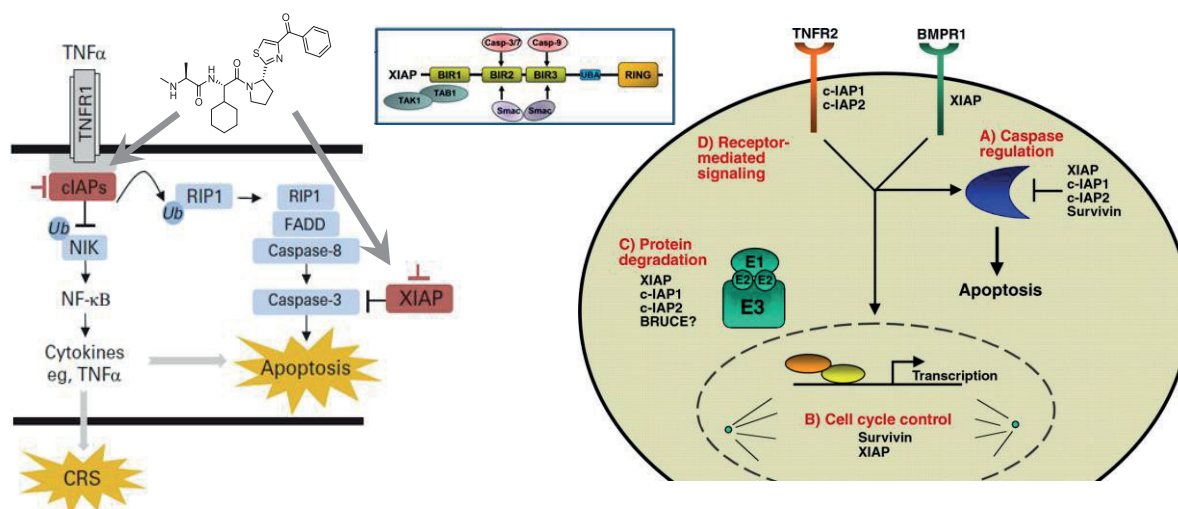


Figure 6. The role of IAP ligands in apoptotic signaling pathways

Major physiological roles of sigma receptor 1 and 2 (1R, 2R) and their ligands

Sigma receptors play important roles in various diseases such as CNS-linked neurodegenerative diseases (e.g. Alzheimer Disease) and neuropathic pain; malfunction of ion-channels; the abnormal regulation of the cell proliferation and apoptosis induction (Figure 7). Based on their role in the latter regulation pathways, sigma receptors have become potential targets in anti-cancer therapy.⁷ Sigma receptors exist in two major forms (sigma-1 and sigma-2). While the crystal structure of the sigma-1 receptor has been recently elucidated, the 3D structure of the sigma-2 receptor is not known yet. Recent studies (cloning and sequencing) revealed that sigma-2 is an endoplasmic reticulum (ER)-resident membrane protein (TMEM97).⁸ It is a four-transmembrane protein which regulates the cholesterol transport and increases the rate of internalization of LDL.⁹ The downstream effect of the sigma-2 receptor binding results in the activation of the caspase-3 enzymes. Up to now sigma-1 and -2 receptors lack natural ligands identified.¹⁰ In addition, the agonist or antagonist nature of the ligands is rather ambiguous, therefore the compounds that have affinities towards the receptors are simply referred to as Sigma receptor ligands.

According to numerous studies, the sigma-2 receptor is overexpressed in solid tumors, therefore it could serve as a tumor marker and could be considered as a potential anticancer drug target. The activity pattern, the binding mode and the nature of the ligands are particularly important for designing such agents, since while S2R agonist and S1R antagonist induce apoptosis, Pentazocine, a sigma-1 agonist, promotes cell proliferation.¹¹

Sigma ligands could also serve as targeting elements in small molecule drug conjugates (SMDCs) (as an analogy to the peptide drug conjugates (PDCs). Interestingly,

sigma ligands conjugated to various IAP antagonists showed increased cytotoxic activities in several cell-lines compared to the parent, separate small molecules.¹² While in these small-molecule drug conjugates the IAP antagonist serves as a cytotoxic “warhead”, attachment of the targeting sigma ligand has multiple actions:

- a.) increases the internalization of the conjugate into the cell,
- b.) contributes with its intrinsic antiproliferative effects.

On the other hand, recent TMEM97 knock-out studies revealed that the transmembrane receptor is not needed for the cytotoxic effects of sigma-2 ligands,¹³ thus, further studies are required to elucidate the molecular mechanism of the exact mode of action. The synergistic effect could also be attributed to the IAP antagonist’s protein degradation capability, therefore such conjugates were defined as SNIPERs (Specific and Non-genetic Inhibitor of apoptosis protein (IAP)-dependent Protein Erasers).¹⁴

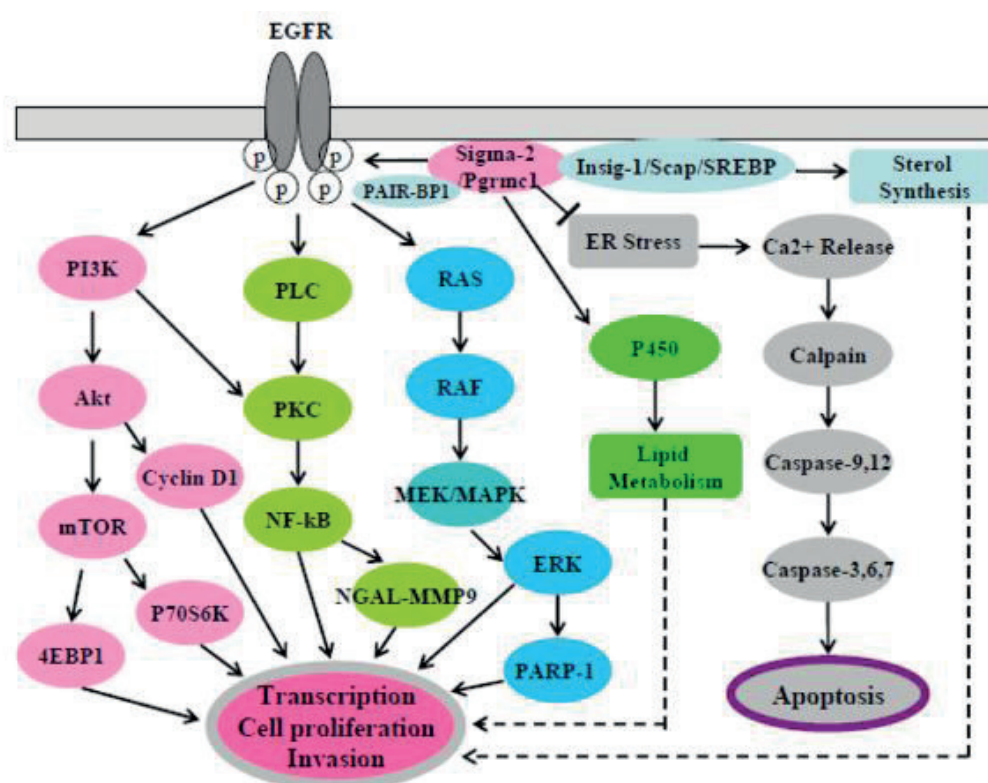


Figure 7. A hypothetical scheme of sigma-2 receptor/PGRMC1 signaling pathways in cancer cells¹⁵

Rational analog design of XIAP antagonist compounds

During the last couple of years, several structure – activity caveats were revealed. Looking at the structure of the natural ligand AVPI (alanine-valine-proline-isoleucine) tetrapeptide, 4 structural motifs (amino acids) were (P1, P2, P3, P4) determined.¹⁶ The relevance and the optional structural replacement for each motif is summarized in Figure 8.

As it was also reported, the P1, P2 dipeptide plays a critical role in binding to BIR3, thus, it acts as an “anchor”. The ring-embedded structure of the P2, P3 interface is similarly important and only isosteric replacements are allowed. Thus, P4 module offers the highest variability for novel compound design. During our rational design process, we identified the common or similar structural elements with LCL161 and applied the above modular approach for successively change the P3 and P4 motifs. In summary, we preferred changing the thiazole ring at 2,4 and 5 position. (Figure 9)

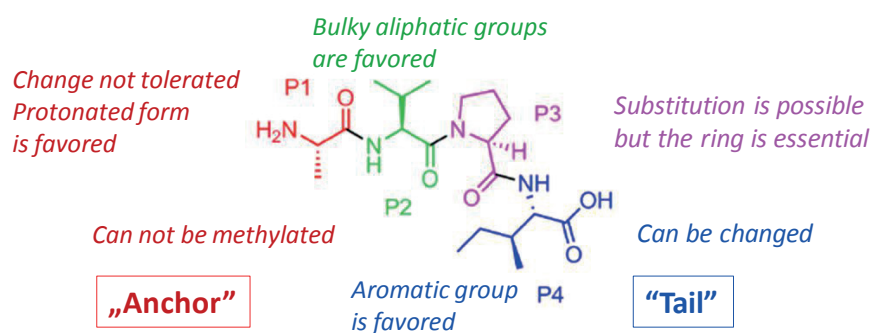


Figure 8. AVPI-based structure – activity relationships

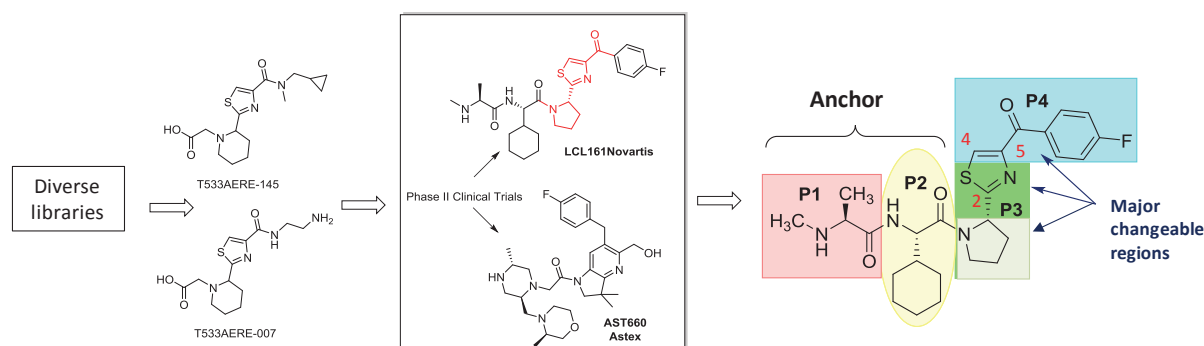


Figure 9. Rational design of novel XIAP antagonists

2D/3D chemoinformatics approaches. Virtual screening of the thiazole library

Based on the above rational design approach, a substituted thiazole library was generated (Figure 10).

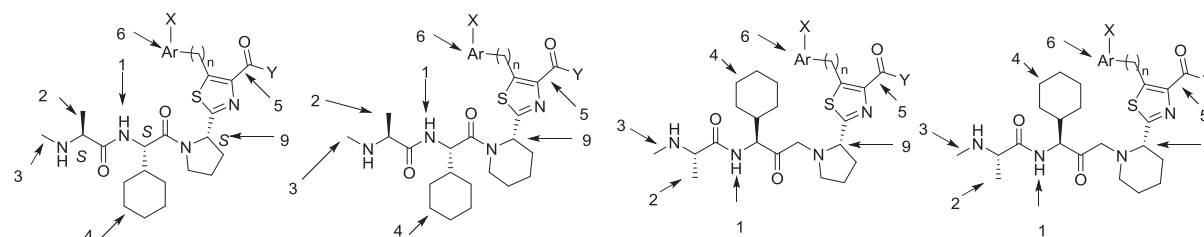


Figure 10. Major structural variations of the thiazole virtual library

The virtual library was first filtered for drug-likeness using the standard physicochemical parameter ranges followed by docking to the 3D structure of XIAP BIR3 domain. The available crystal structures of XIAP BIR3 (3HL5, 3CM2, 2OPY, 2JK7, 4HY0) allowed to generate 3D models in order to identify the major interactions of the known hit compounds and allowing to select the most effective XIAP antagonists.

For 3D modelling Schrödinger "Small Molecule Drug Discovery Suite" software package was used. Based on the results of cross docking calculations, the 4HY0 crystal structure was selected leading to the best performing model of XIAP BIR3 domain. To incorporate flexibility of the receptor, the Induced Fit Docking module was applied, followed by MM-GBSA binding free energy calculations.

The ΔG free energy values correlated with the biological activities of the reference compounds (XIAP-BIR3 K_d values: AVPI: 580 nM; AVPF (AVPI Ile \rightarrow Phe) 290 nM; LCL161: 52.7 nM). Some of the library members (e.g. Lead 1 (S)) showed lower free energy values (Figure 11).

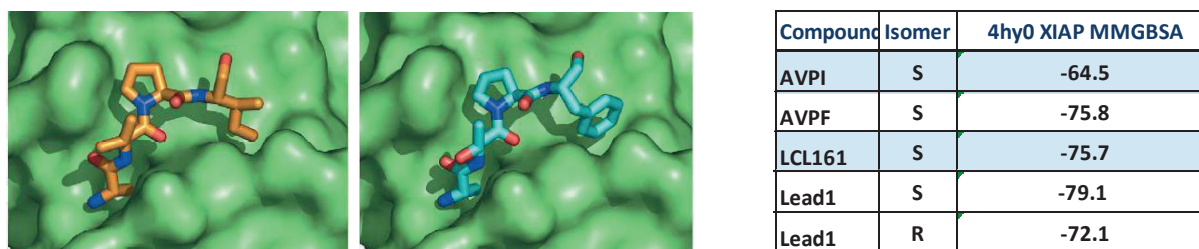


Figure 11. a.) 3D modelling for virtual screening (AVPI- and AVPF-XIAP complex – 4hy0) b.) ΔG free energy values (kcal/mol) of the reference and CIX lead compounds. (Note: Lead 1 = CI6_7 see later)

In vitro screening later confirmed that even the R/S diastereomeric mixture of Lead1 (CI6-7) showed increased cytotoxicity compared to LCL161. Additional cheminformatics methods were applied to improve the performance of the virtual screening. The possible binding features of the small molecules can be assessed by their 3D conformational flexibility and shape (ChemAxon MarvinSketch Screen3D).¹⁷ Applying flexible alignment analysis and molecular dynamics, the statistically average conformations generated allows to rapidly compare the 3D similarity between two compounds. The 3D similarity measures are expressed in 3D Tanimoto coefficient (T3D). 3D similarity helps the analog design revealing close 3D shape between the active compounds and the candidates in rational design (3D similarity of LCL161 and AVPF, T3D= 0.7) as it is illustrated in Figure 12.

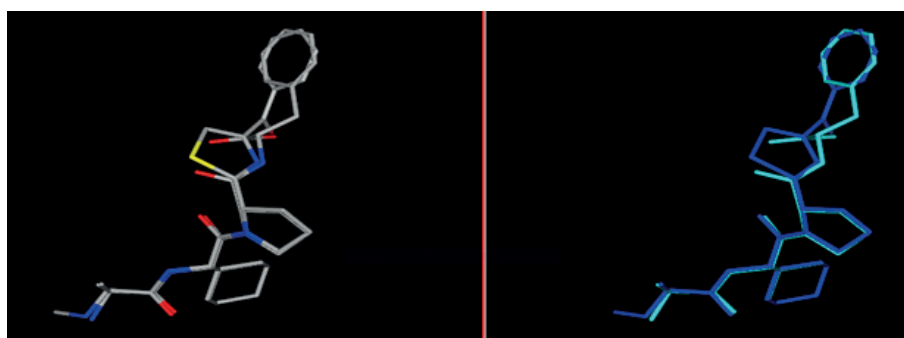


Figure 12. 3D similarity of LCL161 vs. AVPF 3D similarity (T3D = 0.7)

In addition, based on the structure of available XIAP-BIR3 binding ligands (20) a consensus pharmacophore model was developed. For model validation, LCL161 was used as a “required match”. The ADHHR_4 model was selected as the best performing consensus pharmacophore arrangement (BEDROC α 160.9: 0.98) (Figure 13). This model has sufficient capacity for *in silico* screening of an extended virtual thiazole library (72k).

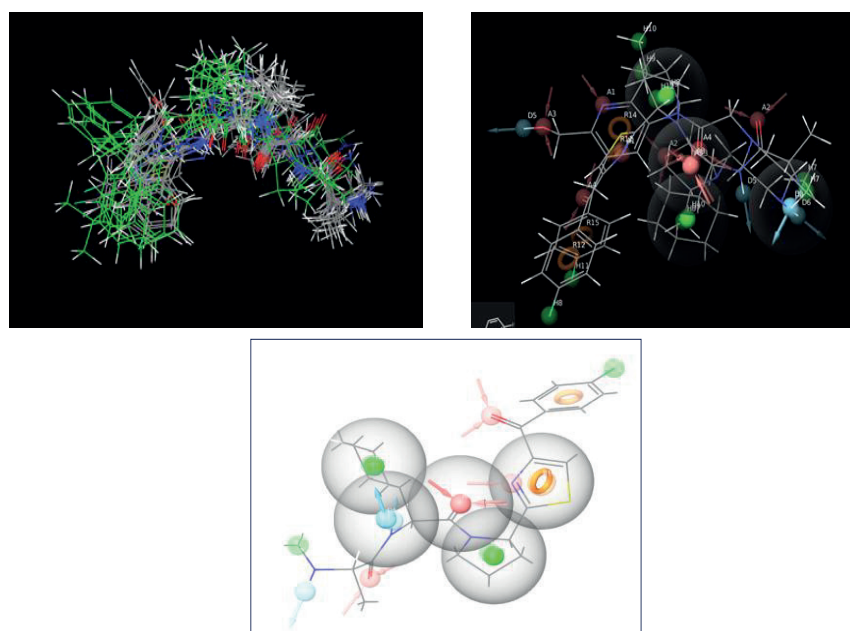


Figure 13. Generation of pharmacophore models for XIAP BIR3 binding ligands: Matching 20 compounds; identification of the major pharmacophoric features; ADHHR_4 as best performing model (LCL161 is shown as the best match).

Scaffold hopping

Using our established pharmacophore model and 3D similarity comparisons of known IAP ligands applying MarvinSketch Screen 3D, novel compounds were designed (Figure 14).

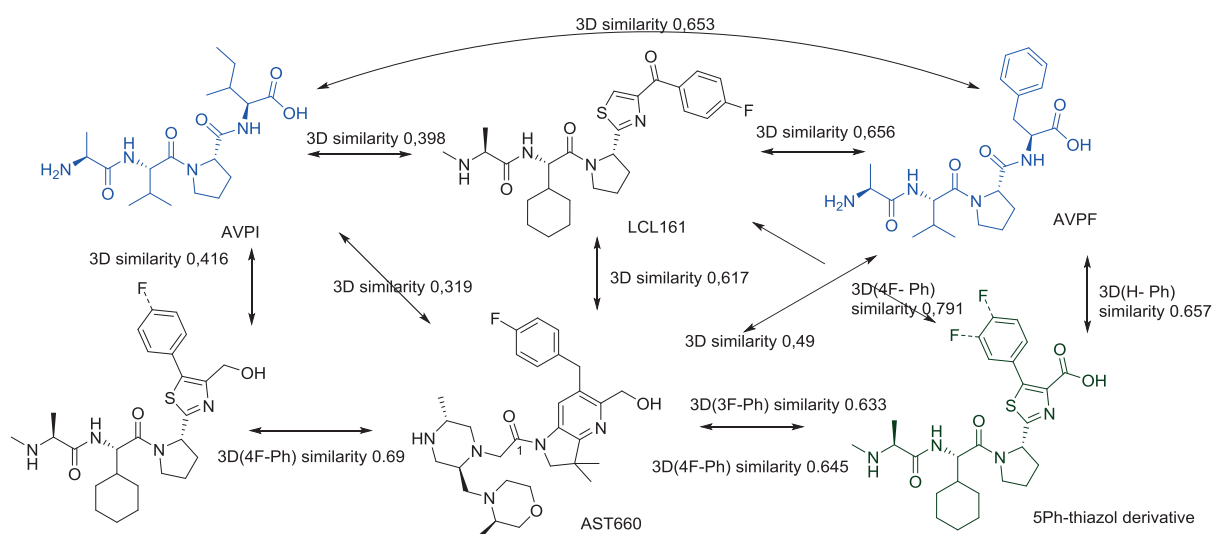


Figure 14. 3D similarity correlations of IAP ligands

Based on the similarity results, the substitution and the modification of the position of the phenyl group in the thiazole ring showed further possibility. Increasing the freedom on the phenyl ring by inserting a methylene group had even better fitting and potential, as shown by the respective *Tanimoto* coefficients: T3D=0.86 vs 0.78 as illustrated in Figure 15.

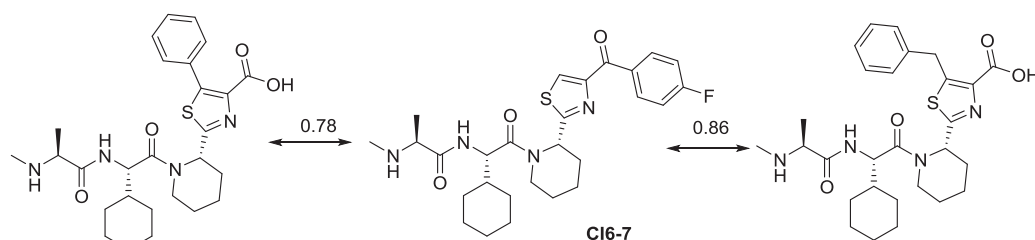


Figure 15. Scaffold hopping via 3D similarity of phenyl and benzyl substituted thiazole

The possibility of the modifications was confirmed by the biological results (Tables 1 and 7). The introduction of a new functional group in the molecule allowed us to develop new derivatives and a new possibility to conjugate the molecule with linkers and carrier compounds.

Cytotoxicity of novel XIAP antagonists

The best-fitting compounds were prepared and tested. The results confirmed the expected binding and cytotoxicity. The results are summarized in Table 1.

The following conclusions have been drawn from the biological data:

1. There is a long-lasting dogma in the publications that replacement of the pyrrolidine ring to piperidine decreases the XIAP antagonist activity. „At the proline position (*of AVPI*),

replacement with a variety of amino acids leads to a dramatic loss in binding affinity. ...piperidine, bearing a six-membered ring, exhibits ...10-fold decreases in the K_d value...” (Cong *et al.*).⁶ We found that such replacement (LCL161 → CI6_7) led to 2-4-fold cytotoxic activity increase and with this “irrational” modification we entered a patent-free area. Our modification was supported by the 3D modelling studies.

2. We identified a novel substitution pattern on the thiazole ring (CI12_3 and CI12_7) that showed improved or equal cytotoxic activities compared with LCL161. These novel analogs opened new opportunities for further analog design.

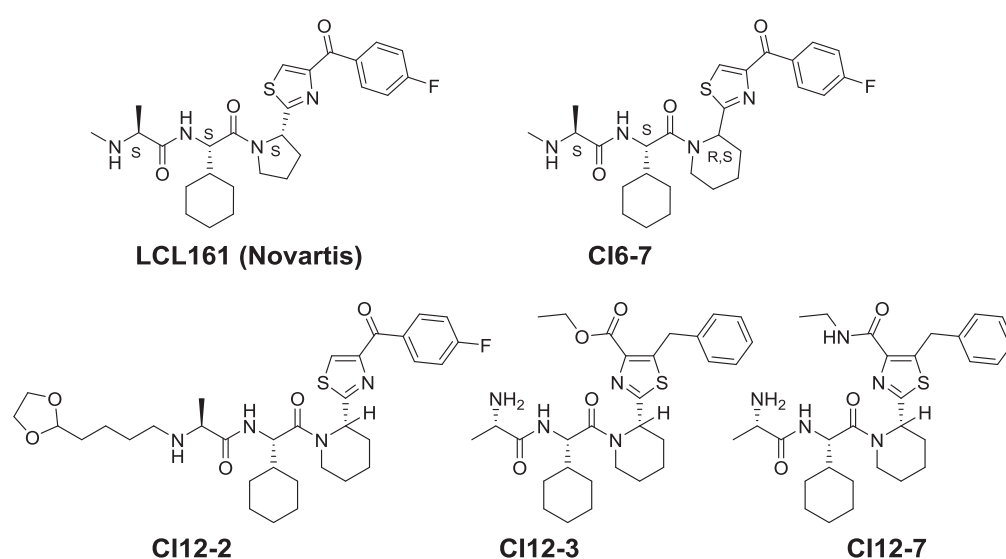


Table 1 shows that the different forms of the CIX Lead1 (CI6-7) are 2-4-fold more active than LCL161, particularly on pancreatic tumor cells, the IC₅₀ value of CI6-7 2xTFA (SSS) on PANC-1 cell line is 11 μM – 72 h, while LCL161 base is cytotoxic at 58 μM concentration on the same cell line and 72 h incubation period.

Table 1. The IC₅₀ values are shown in μM concentration

Compounds (form/isomer)	Test ID	PANC-1 – xCELLigence SP (Pancreas)			A2058 (Melanoma)		
		24 h	48 h	72 h	24 h	48 h	72 h
LCL161 base (SSS)	CI4-49	54.9	79.5	58.5			
LCL161 xHCl(SSS)	CI6-6	100	100	67.5	99	55	52
CIX CI6-7 xHCl (RS,SS)	CI6-7	32	37	31	18	21	14
CIX CI6-7 base (RS,SS)	CI9-4						9
CIX CI6-7 2xTFA (RS,SS)	CI10-2			14.3			13.9
CIX CI6-7 2xTFA (SSS)	CI10-3			11.7			14.6
CIX CI6-7 2xTFA (RSS)	CI10-4			23.2			11.8
CIX CI12-2, base (SSS)	CI12-2	18.2	17.6	19.9	22	46.4	12.8
CIX CI12-3 base (SSS)	CI12-3	26.4	32.7	36.4	21	13.6	8.19
CIX CI12-7 base (SSS)	CI12-7	93	54.6	60	50.4	27.3	35.8

Compounds (form/isomer)	Test ID	EBC1 (Lung)			Colo205	MiaPaca (Pancreas)	MdaMb231 (Breast)
		24 h	48 h	72 h	72 h	72 h	72 h
LCL161 base (SSS)	CI4-49				39.4	16.8	
LCL161 xHCl(SSS)	CI6-6	> 100	84	54			
CIX CI6-7 xHCl (RS,SS)	CI6-7	29.5	25	25			
CIX CI6-7 base (RS,SS)	CI9-4			7.7	7.4	8.8	1.5
CIX CI6-7 2xTFA (RS,SS)	CI10-2				14.7	6.5	
CIX CI6-7 2xTFA (SSS)	CI10-3				13.3	6.2	
CIX CI6-7 2xTFA (RSS)	CI10-4				11.9	10.3	
CIX CI12-2, base (SSS)	CI12-2	30.3	34.7	41.1	15		
CIX CI12-3 base (SSS)	CI12-3	32.1	18.8	13.2	11		
CIX CI12-7 base (SSS)	CI12-7	70.5	38.2	28.2	24.6		

Property forecast and drug likeliness of the lead compound¹⁸

The measured and calculated physicochemical parameters listed in Table 2 were similar to the reference compounds, thus the predicted character of the lead compound is conformed with the drug-likeliness criteria needed for further development.

Table 2. Drug likeliness of Lead 1 compared with relevant drug in clinical trials

Parameter	LCL161	Lead 1 (CI6-7)	AST660
Mw	500.63	514.66	539.70
HBD	2	2	2
HBA	5	5	7
TPSA	91.4	91.4	81.17
CLogP (calculated)	3.78	4.22	2.62
ChromLogD (measured)	3.0	3.1	3.1*
Property Forecast Index ¹⁹	5.0	5.1	5.1

*Literature data²⁰

Development of further XIAP antagonist hit compounds

The new molecules have potential modification possibilities in the P3 and P4 positions.

The key elements as the chirality and the size of the P3 ring, as well as the substitution possibilities of the thiazole ring were systematically checked. The potential development directions are shown in Figure 16.

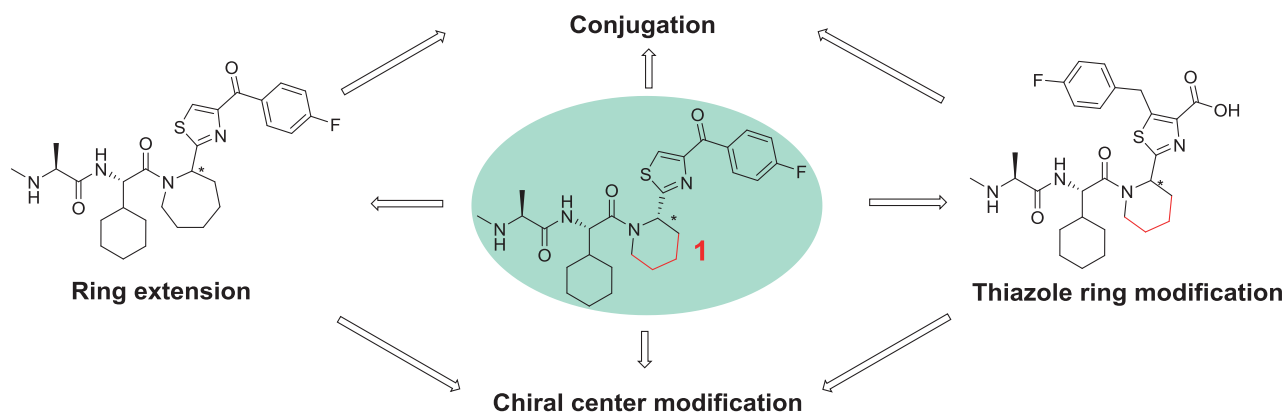
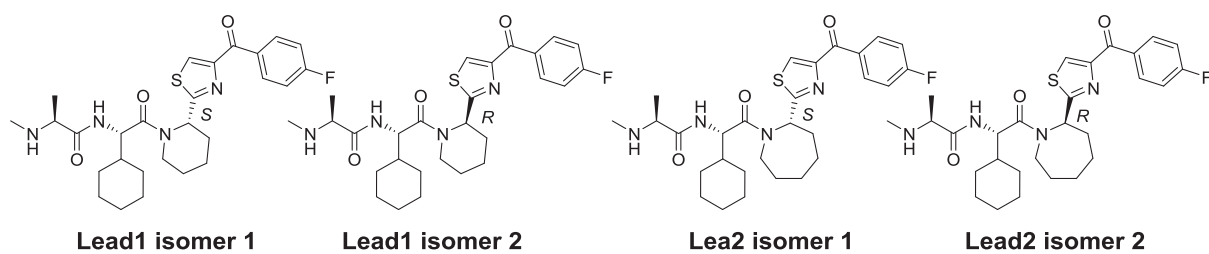


Figure 16. Development possibilities of the Lead 1 compound

Effect of the chirality and extension of the P3 ring

The piperidine ring was enlarged to azepane and the epimers of the stereoisomers of the molecules were docked in our *in silico* model. Both compound pairs fit in the receptor with high binding energy.



#	Compound	Isomer	XIAP BIR3 (4HY0) MMGBSA (Kcal/mol)
1	AVPI	1	-64.5
2	AVPI	2	-63.3
3	AVPF	1	-75.8
4	AVPF	2	-58.8
5	LCL161	1	-75.7
6	LCL161	2	-77.0
7	Lead1	1	-79.1
8	Lead1	2	-72.1
9	Lead2	1	-80.6
10	Lead2	2	-72.8

Figure 17. *In silico* binding values of the epimers and the ring extension

The results listed in Figure 17 showed that the new compounds and epimers were fitting well in the model; therefore the molecules were prepared and tested. The results summarized in Table 7 confirmed that the modification is possible, serving a novel set of compounds.

Rational analog design and development of Sigma 1/2 ligands

Rational analog design of Sigma-1 or Sigma-2 receptor ligands started from the initial hit compound CI1-25.

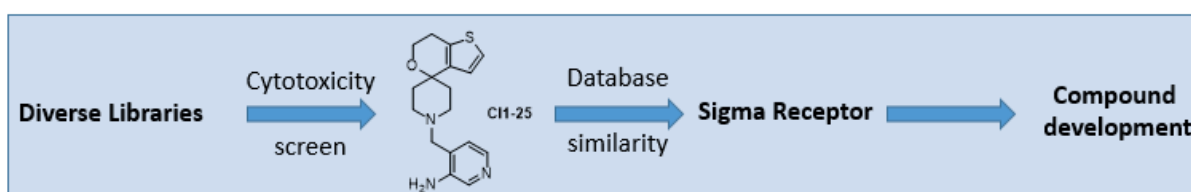


Figure 18. Development scheme of novel Sigma Receptor ligands

Retaining the spiro ring system, R_1 (alkyl, aryl) and R_2 substituents were varied and systemically designed applying the TOL platform.

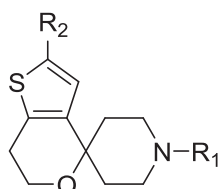


Figure 19. Rational design of dihydrospiro[piperidine-4,4'-thieno[3,2-c]pyran] library

A two-dimensional compound library was generated applying *in silico* methods and compound development based on the cytotoxicity screen results.

Cheminformatics methods to support the rational design of the sigma-1,2 ligands

The 3D modelling is very challenging, since crystal structure is only available for sigma-1 proteins, furthermore, similar structures could act as agonists or antagonists and often allosteric interactions could also be predominant. Therefore, 3D and homology methods were only used for fragment screening or confirmation of the biological activities. The 5HK1 sigma-1 receptor crystal structure was selected for the 3D model building based on the results of cross-docking experiments of sigma-1 ligands with various receptors. The binding site was optimized by using Induced Fit Docking calculation with selected ligands. The fragment library was docked to the optimized binding site using the Schrödinger Glide SP program.

The best-performing fragments were selected and used in analog design. The novel virtual structures were first filtered for drug-like properties and synthetic feasibility, and followed by virtual screening using pharmacophore models (Figure 20). After ranking the compounds, the promising virtual hits were docked to 5HK1 (Figure 21) and ranked again based on the free energy values.

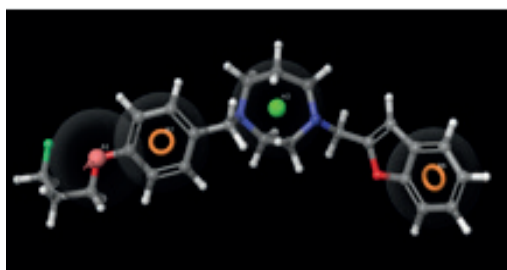


Figure 20. Pharmacophore model based on the structure of Haloperidol

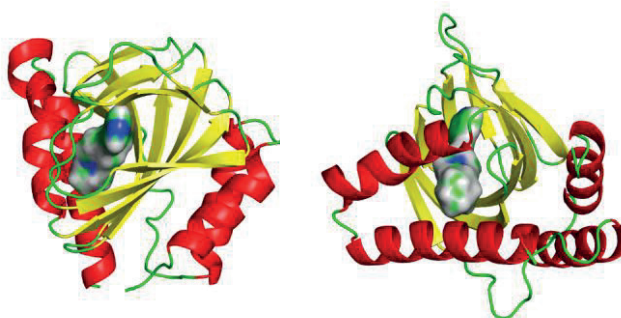


Figure 21. Binding model of CI7-8 lead compound to 5HK1 S1 receptor

Development of Sigma receptor ligands

Based on the rational analog design strategy and virtual screening, more than 50 compounds were synthesized and pre-screened for cytotoxicity (Figure 22).

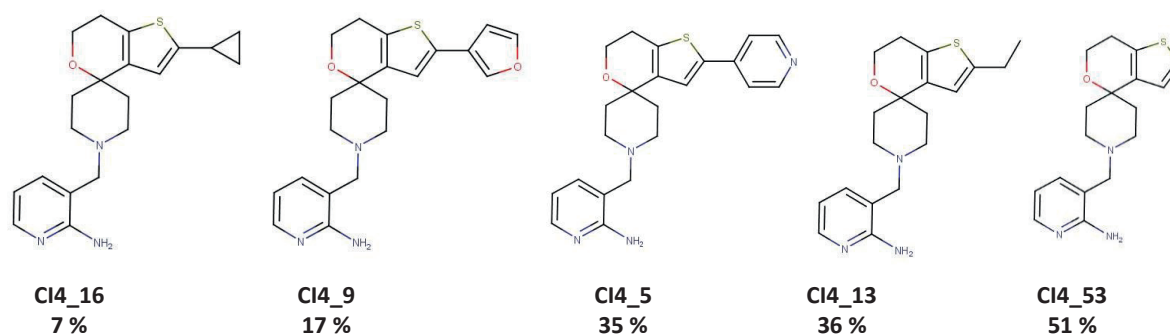
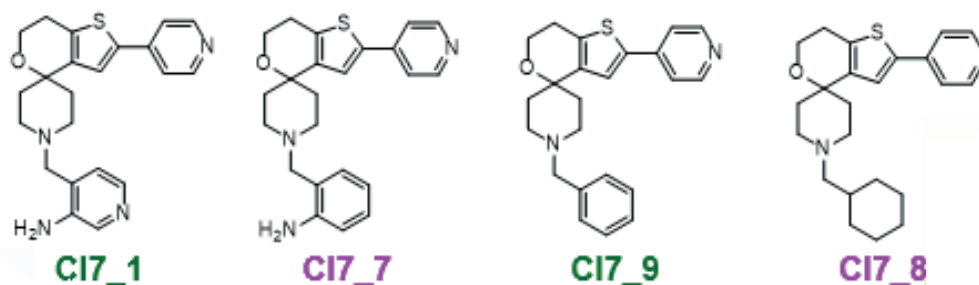


Figure 22. Cytotoxicity results of the first rounds of sigma-1,2 ligands (PANC1 cell line; cell viability %, 72 h, at 10^{-4} M concentration)

Based on the results, a second analog set was prepared according to the original rational design plan. The pyridine-substituted dihydrospiro[piperidine-4,4'-thieno[3,2-c]pyran] series, by removing the *N* atom and the amino group from the pyridinyl-methyl side chain; plus saturating the aromatic ring, led to the identification CI7_8 as a potential lead compound.



The cytotoxic effect of the new compounds was tested on different cell lines (Table 3). The modifications showed a robust correlation with the *in silico* model (Table 4). For hit validation, a new batch of CI7_8 was synthesized (ID: CI9_1); the concentration dependency was measured and the IC_{50} values were determined. (Table 5)

Table 3. Cytotoxicity results of selected sigma 1-2 ligands (various cell lines; cell viability %, at 24, 48, 72 h, in 10^{-4} M concentration).

ID	PANC1			A2058 - Melanoma			EBC1 - Lung			Colo205- Colon		
	24	48	72	24	48	72	24	48	72	24	48	72
CI7_1	53	62	80	68	67	67	78	44	26	72	39	23
CI7_7	17	16	16	23	11	9	62	23	14	39	18	14
CI7_8	11	10	9	27	15	13	48	22	14	37	18	14
CI7_9	69	76	41	30	35	37	55	31	34	88	53	64

Table 4. The free energy values and the physicochemical parameters of sigma lead compounds (PDB: 5HK1)

Compound	MM-GBSA ΔG (kcal/mol)	MW	HBA	HBD	LogP	Fsp3	TPSA	Rotable Bonds
CI7-7	-81.8	391.5	4	1	3.07	0.35	51.4	3
CI7-8	-89.0	382.6	3	0	4.29	0.61	25.4	3
Haloperidol	-84.4	375.9	3	1	3.66	0.38	40.5	6

Table 5. IC₅₀ values of CI9_1 lead compound using various cancer cell lines measured at 24, 48 and 72 hrs (values are in μM).

ID	PANC1			A2058 - Melanoma			EBC1 - Lung			Colo205- Colon		
	24	48	72	24	48	72	24	48	72	24	48	72
CI9_1 (CI7_8)	76.6	71.2	53.1	55	48.1	3.37	66.2		8.3	66.2	24	8.7

CI9_1 was particularly efficient in melanoma, lung cancer and colon cancer cell lines, showing low micromolar cytotoxicity. Although the scaffold was the same, these compounds were significantly different from the Esteve compounds, which were reported earlier as sigma ligands.^{21,22} Depending on the substituent pattern around the above core the compounds show affinities to both Sigma-1 and Sigma-2 receptors with varying ratio. The synthesized novel dihydrospiro[piperidine-4,4'-thieno[3,2-c]pyran] derivatives (CI7_8 or CI9_1) showed equal cytotoxicity to the reference XIAP antagonist LCL161.

Generating Small Molecule Targeting Drug Conjugates by linking novel XIAP antagonists and novel sigma-1,2 ligands

Since one of the major objectives of the project was to connect the apoptosis inducer “warhead” to targeting molecules, the CI6-7 compound was extended with a linker at the P1 alanine site (CI12-2).

Hawkins and his coworkers previously reported sigma-2 conjugates with XIAP antagonists. Since sigma-2 receptors are overexpressed in many proliferating tumor cells and the complexed ligands were internalized in the cancer cells, they assumed that sigma-2 receptor was an attractive target for drug delivery.¹² They attempted to conjugate apoptosis-inducing compounds (“warheads”) such as XIAP antagonists with sigma-2 ligands as a targeting or delivering tool. They constructed a typical Small Molecule Targeting Drug Conjugate compound.

Similarly, novel conjugates were rational to design and test since we identified both novel XIAP antagonist and sigma receptor ligands. Based on the above analogy we assume that our sigma ligands act also as at least partially sigma-2 binding ligands. (Figure 22)

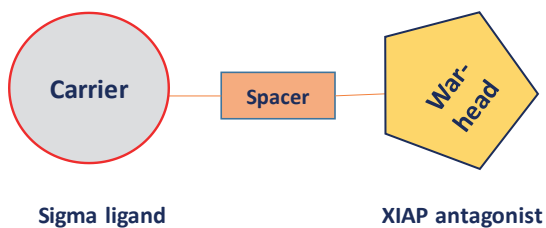


Figure 23. General structure of the XIAP antagonist sigma-2 ligand conjugates

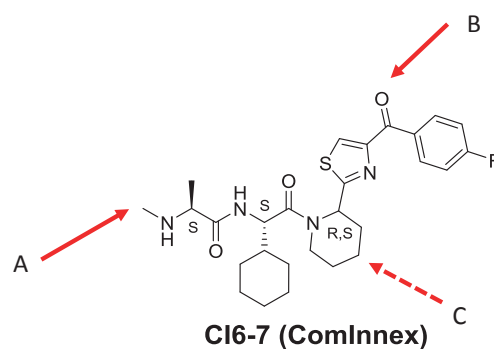


Figure 24. Possible conjugation sites in CI6-7

it has been established that the XIAP antagonist sigma-2 ligand conjugate exhibited higher cytotoxic activities than either of the parent (monomeric) compounds. More significant cytotoxicity increase was observed with the XIAP antagonist portion, while the cytotoxic activity was doubled for the sigma-2 ligand. In order to design a XIAP antagonist sigma-2 ligand conjugate, the optimal linker position should be identified. For the XIAP antagonist portion there were several options based on literature analogies:

Since previously we have already synthesized CI12-2 for possible multipurpose conjugation and the compound showed reasonable activity, we have the A-variation in hand. For the sigma-2 ligand portion, only limited connection site was feasible through the spiro-piperidine substructure. These two linkers, containing compounds with the dioxolane masked aldehyde functionality, are shown in Figure 25. Surprisingly, CI11_4 had poor cytotoxic activity, which accounted for some uncertainty for the expected activity of the conjugate. The linker length was predicted by *in silico* modelling studies as 5 carbons. (Figure 26)

The biological activity of the SMDT conjugate showed excellent cytotoxic activities on several cell lines, interestingly, with little time dependency. The conjugate doubled the biological activity towards the XIAP antagonist and showed more than one order of magnitude cytotoxicity increase compared with the sigma-2 ligands (Table 6).

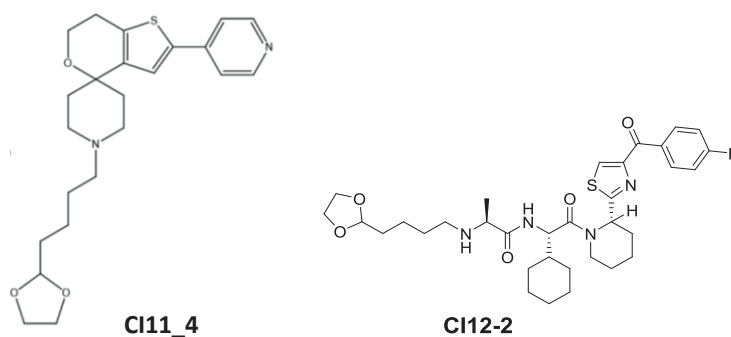


Figure 25. Two halves of the linker-containing portion as potential compounds for assembly

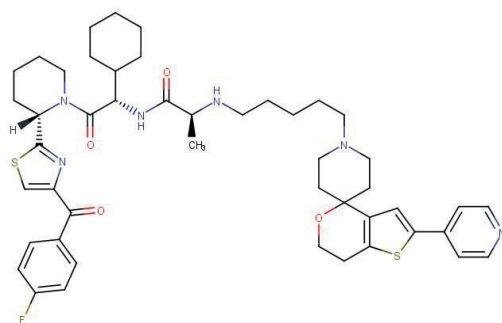


Figure 26. The XIAP antagonist sigma-2 ligand conjugate (CI12_1)

Table 6. Comparative IC₅₀ (μM) of the Chimera compound on tumor cells

	ID	PANC1			Melanoma			EBC1 - Lung			Colo205- Colon			
		Time	24	48	72	24	48	72	24	48	72	24	48	72
XIAP antagonist	CI10-3			11.7			14.6							13.3
XIAP antagonist with linker	CI12-2	18.2	17.6	19.9	22	46.4	12.8	30.3	34.7	41.1	15			15
Sigma ligand	CI9_1 (CI7_8)	76.6	71.2	53.1	55	48.1	3.37	66.2		8.3	66.2	24		8.7
Sigma - XIAP ant. Conjugate	CI12_1	6.5	6.71	6.56	5.18	5.54	3.44	6.85	6.97	6.58	6.73	5.93		3.17

Cytotoxicity screening of XIAP antagonists and sigma-1,2 ligands

Impedimetric assay

Biological tests were performed on different tumor cell lines, PANC1 (pancreatic adenocarcinoma), COLO-205 (colon cancer), A2058 (melanoma), EBC1 (lung cancer), Miapaca (pancreatic ductal adenocarcinoma) and MDA MB231 (breast cancer). Cytotoxicity was measured by impedance-based technique²³ in an xCELLigence SP (ACEA) system.

Colorimetric assay

In the case of COLO-205, A2058 and EBC-1 cell lines, a colorimetric assay (alamarBlue- or MTT-test) was used to determine the antiproliferative/cytotoxic effects of the ONC201 derivatives as references. These colorimetric assays were chosen because for these cell lines there was no stable plateau phase (A2058) or there was only a weak/negligible adhesion (COLO-205 and EBC-1), therefore detection with the xCELLigence System was not feasible.

Summary of the cytotoxicity results

The results of the representative compounds are listed in Table 7.

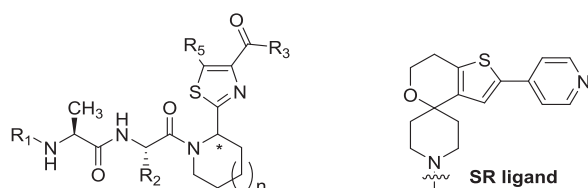


Table 7. The IC₅₀ results of selected IAP binders indicating the sensitive cells among the 6 tested cancer lines (Pancreas, lung, breast, colon and melanoma)

Code	R ₁	R ₂	R ₃	R ₅	n	* isomer	Salt	IC ₅₀ μM (72h)	Cell line
CI6-7	Me	cHex	pFPh	H	1	RS	HCl	14	Melanoma
CI9-4	Me	cHex	pFPh	H	1	RS	Base	2	Brest
CI10-2	Me	cHex	pFPh	H	1	RS	TFA	7	MiaPaca
CI10-3	Me	cHex	pFPh	H	1	S	TFA	6	MiaPaca
CI10-4	Me	cHex	pFPh	H	1	R	TFA	10	MiaPaca
CI11-1	H	cHex	pFPh	H	1	S	HCl	40	Panc1
CI11-2	H	cHex	pFPh	H	1	RS	HCl	33	Panc1
CI12-5	Me	cHex	NHEt	Ph	0	RS	Base	35	Panc1
CI12-1	-(CH₂)₅-SR ligand	cHex	pFPh	H	1	S	Base	0.8	Melanoma
CI12-2	-(CH ₂) ₅ -2-dioxolane	cHex	pFPh	H	1	S	Base	20	Panc1
CI12-3	H	cHex	NHEt	Bn	1	RS	Base	25	Colo205
CI12-4	H	cHex	OH	Bn	1	RS	Base	>100	
CI12-7	H	cHex	OEt	Bn	1	RS	Base	8	Melanoma
CI12-6	H	BuNH ₂	pFPh	H	1	S	Base	54	Melanoma
CI14-1	-CH ₂ (CH ₂) ₂ CO ₂ CH ₃	cHex	pFPh	H	1	S	Base	7	Brest
CI14-2	-CH ₂ (CH ₂) ₄ CH ₂ OH	cHex	pFPh	H	1	S	Base	6	Brest
CI14-3	-(CH₂)₅-SR ligand	cHex	pFPh	H	1	R	Base	0.8	Melanoma
CI15-3	-(CH ₂) ₃ -CO ₂ -(CH ₂) ₆ -SR ligand	cHex	pFPh	H	1	S	Base	25	Colo205
CI15-4	Me	cHex	O ⁻ -(CH ₂) ₅ -SR ligand	Bn	1	S	Base	2	Colo205
CI15-6	Me	CH ₂ (CH ₂) ₂ CO ₂ CH ₃	pFPh	H	1	S	Base	>100	
CI15-5	Me	CH ₂ CH ₂ SCH ₃	pFPh	H	1	S	Base	34	Colo205
CI15-7	Me	cHex	pFPh	H	2	RS	Base	14	Colo205

Collateral sensitivity determination of XIAP antagonists and sigma-1,2 ligands

Collateral sensitivity was identified by Gottesman and Szakács in the early 2010s. Collateral sensitivity is a “phenomenon in drug-resistant cells whereby the development of resistance in cells to one agent can confer higher sensitivity to an alternate agent than seen in the original (parental) line”. In other words, the resistant cell line is more sensitive to a cytotoxin than the parental line from which it is derived.²⁴ Abate and co-workers reported that the cytotoxicity of sigma-2 ligands²⁵ is increased in multi-drug resistant cells, thus the multiresistant cells are sensitized to the cytotoxic agents. Based on these findings we initiated to test our hit compounds in cancer cells together with their multidrug-resistant counterparts. In order to identify the cytotoxicity difference, two cell lines were selected: Mes-Sa parental and Mes-Sa/Dx5 multidrug-resistant uterine sarcoma cell lines.

Cytotoxicity measurements on MES-SA and MES-SA/Dx5 multiresistant cell lines

In the co-culture system, after trypsinization, suspensions of MES-SA mCherry²⁶ and MES-SA/Dx5 eGFP were tested. Measurements were also performed with MES-SA/Dx5 (*in the Tables 'Dx5'*) cell lines in the presence of the P-gp inhibitor tariquidar (TQ) in order to identify whether the selective toxicity of the compounds (the collateral sensitivity) is linked to the glycoprotein P (P-gp) efflux pump. Our compounds are presumably not interacting with ABCB1, since no significant change in IC₅₀ occurs in the case of tests against cancer cell lines when TQ is present, compared with LCL161, where a significant effect was detected. The two XIAP antagonists – the reference compound LCL161 and particularly our lead compound (CI6-7 SSS) – showed increased sensitivity towards the multidrug-resistant cell line. **Notably, the cytotoxicity of CI6-7 SSS on DX5 cell line was 4.14 μM.**

Table 8. IC₅₀ measurements on MES-SA and MES-SA/Dx5 cell lines (in μM).

	Mes-Sa- mCherry	Mes-Sa- mCherry	Mes-Sa- mCherry	Mes-Sa average IC50	Messa- mCh(TQ)	Dx5- eGFP	Dx5- eGFP	Dx5- eGFP	Dx5 average IC50	Dx5- eGFP(TQ)
Compound	72h	120h	144h	72-120-144h	144h	72h	120h	144h	72-120-144h	144h
CI7-8 2HCl	12.95	13.40	14.34	13.56	12.65	9.04	7.22	10.01	8.75	8.52
LCL161	19.53	21.97	34.61	25.37	16.69	16.95	17.19	17.10	17.08	13.18
CI12-1	1.93	1.98	1.39	1.77	1.96	2.33	2.21	2.11	2.22	1.82
CI7-7 HCl	18.33	19.39	20.78	19.50	18.11	15.54	15.84	17.01	16.13	16.41
CI6-7 SSS	14.96	16.28	13.95	15.06	11.11	3.04	3.25	6.12	4.14	5.65
Values: IC50 in [uM]										
Mes: Human uterus sarcoma drug sensitive cell line										
Dx5: multidrug resistant cell line										
TQ: tariquidar, ABCB1 inhibitor										

Table 9. Collateral sensitivity determination on MES-SA and MES-SA/Dx5 cell lines (IC₅₀ values are shown in μM).

	Mes-Sa average IC50	Dx5 average IC50	Selectivity Ratio (Mes/Dx5)	Dx5-eGFP	Dx5-eGFP(TQ)	Selectivity Ratio (Dx5/Dx5)(TQ)
	72-120-144h	72-120-144h	72-120-144h	144h	144h	144h
CI7-8 2 HCl	13.6	8.75	1.55	10	8.523	1.17
CI7-7 HCl	19.5	16.13	1.21	17.1	13.2	1.29
LCL161	25.4	17.1	1.85	17	16.4	1.04
CI6-7 SSS	15.06	4.136	3.64	6.123	5.649	1.08

Collateral sensitivity measurements revealed that the two sigma 1,2 ligands show moderate collateral sensitivity (Selectivity Ratio - Mes/Dx5 = 1.2-1.5). While the two sigma ligands show moderate collateral sensitivity, our XIAP antagonist lead compound (CI6-7 SSS) indicated increased sensitivity towards the multidrug-resistant cell line (**Selectivity Ratio - Mes/Dx5 = 3.64**).

The presence of the P-gp inhibitor tariquidar (TQ) in a multidrug-resistant cell line (Dx5) does not influence the selective toxicity of the compounds (Selectivity Ratio = $(Dx5/Dx5)(TQ) \sim 1$), which suggests that the observed collateral sensitivity could be linked to other, cell line-specific factor(s).

PAMPA modelling assay

The potential absorption of the compounds was tested by PAMPA test.²⁷

Table 10. Permeability test results of lead compounds

Compound	Calc (nm)	C ₀ (μM)	C _d (μM)	C _a (μM)	C _e (μM)	P _e (cm/s*10 ⁻⁶)	R (%)
Caffeine	270	50	42.4	11.2	29.9	10.4	0.33
LCL161	270	50	40.9	8.6	28	8.2	6.64
CI7-8	310	50	23.7	9.9	18.2	17.5	39.34
CI12-1	295	50	10.4	0.6	6.5	2.3	78.38
CI7-7	310	50	20.5	8.7	15.8	17.8	47.29
CI6-7	275	50	33.1	14.3	25.5	18.2	14.85
Calc: the characteristic absorption peak in UV							
Pe: permeability							
R: recovery							

The results showed that CI7-7, CI7-8 and CI12-1 had high, CI6-7 medium and LCL161 low permeability compared to caffeine as a reference compound. That means that the permeability of our IAP antagonist was increased significantly and the sigma ligand serves as a carrier molecule which might exploit the active transport in the sigma receptor. It is also interesting that LCL161 has lower penetration than our lead CI6-7.

Synthesis of the compounds by applying innovative procedures

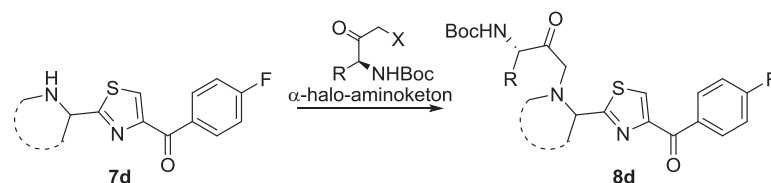
The reactions were carried out according to the best practice of comprehensive organic chemistry in standard chemical laboratory environment following the general rules and conditions of compound preparations.

Analytical methods

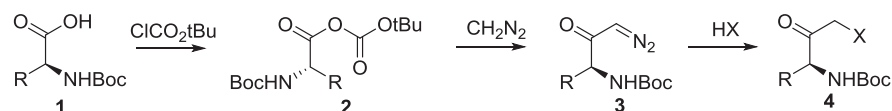
Purity analyses and molecular weight determination of the samples were performed on a Waters 2695 HPLC/ Waters ZQ MS system or on a Waters Acquity H-class/ Qda MS system. The optical purity was determined by chiral HPLC tests applying YMC CELLULOSE-SB 250*4.6, 5 μM column and Heptane-IPA 95-5 eluent at ambient temperature. The structures were confirmed by ¹H, ¹³C and 2D NMR tests at ambient or elevated temperatures.

Preparation α -halo-aminoketones in flow reactor

The first active compound contained $-\text{CH}_2-$ derived structure of amino acids. These types of compounds were prepared by coupling α -halo-aminoketones with the piperidine ring of the intermediate **7d**.



The direct preparation of the reagent from amino acids utilizes *Arndt-Eistert* reaction, which requires dry diazomethane. The safe and effective method worked out by Kappe's group at University Graz²⁸ was adapted in our laboratory for the derivatization of oligopeptides as well.²⁹



The key to the reaction was the special membrane coil Teflon AF-2400, which was permeable for diazomethane and delivered dry diazomethane in the activated amino acid solution. Reaction in continuous conditions gave the required halo-ketone in a safe and simple way, which is illustrated in Figure 27.

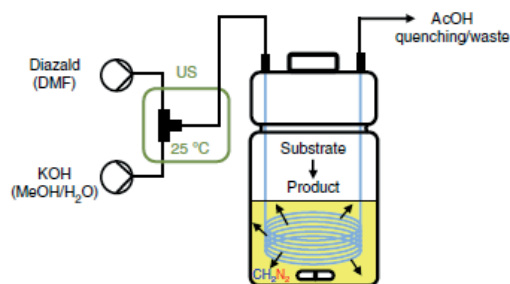


Figure 27. Simplified scheme of the diazomethane flow reactor

Synthesis of XIAP antagonists

Albeit the synthesis of the LCL161 was published by the Novartis researchers,³⁰ several synthetic steps and novel reactions were worked out and used in the preparation of the novel molecules. The synthesis of the compounds is summarized in Figure 28.

The individual routes could be combined since they had several common intermediates. The versatility of the syntheses allowed us to get a high number of different derivatives and complex libraries. The optical isomers of the starting amino acids **1** were

commercially available in pure form and were convenient starting points of the synthesis. The synthetic steps were carried out both with the *S* and the *R* optical isomers in a similar way to get finally the two epimers of the target compounds **10** and **10a**.

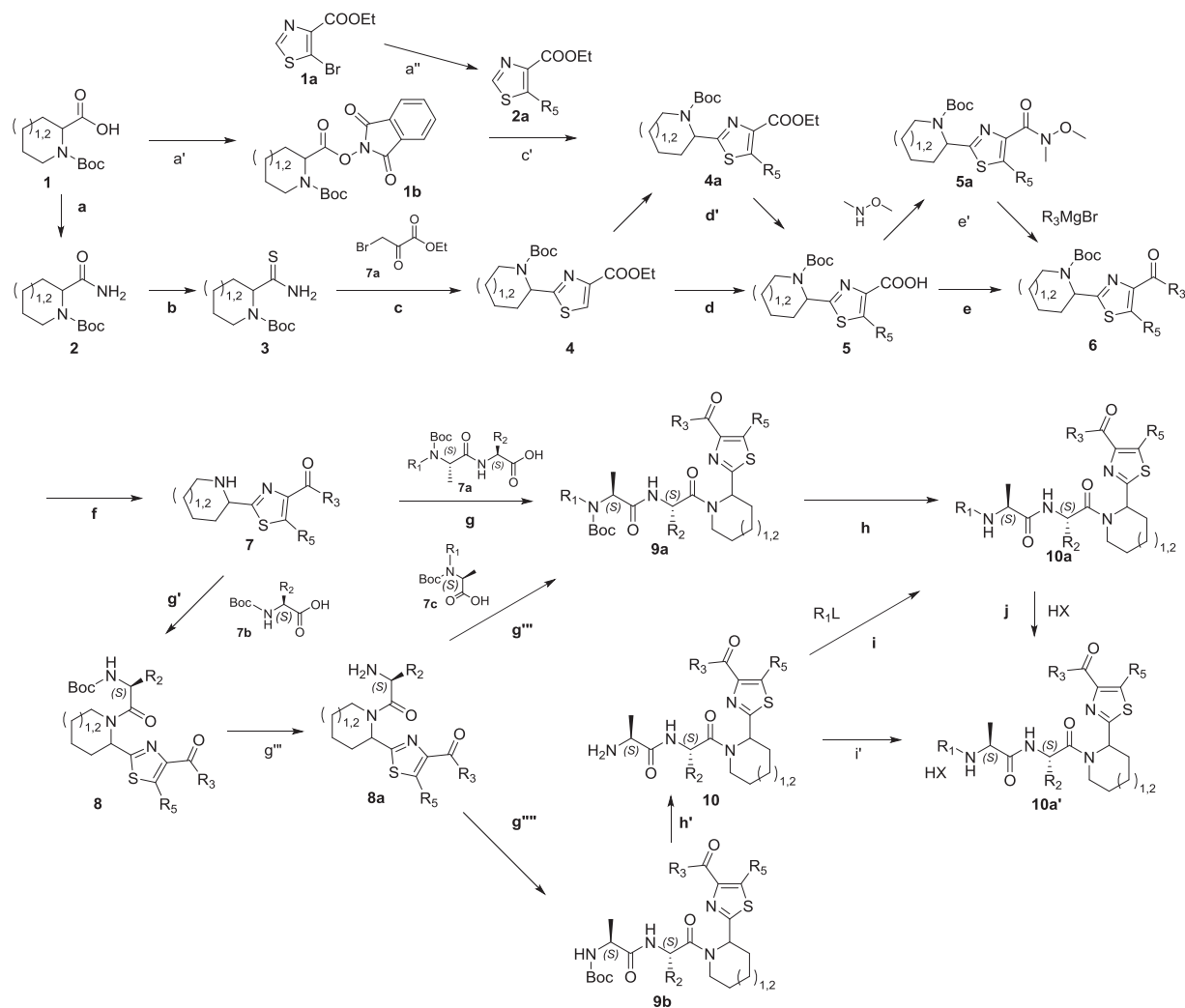


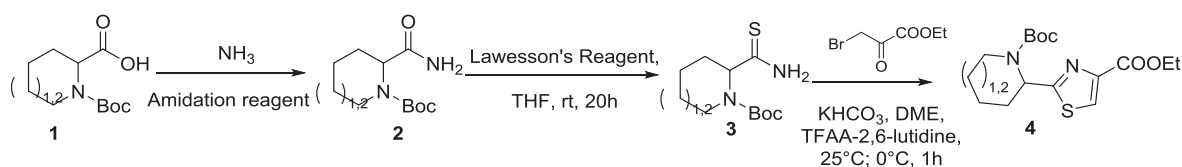
Figure 28. General synthetic scheme of XIAP antagonist library

Stereoselective synthetic methodology was applied to isolate the compounds in optically pure forms, which were confirmed during the synthesis by continuous analytical monitoring and structure confirmations applying chiral HPLC, LCMS and NMR methods. The amide bond was prone to form stable tautomer/rotamer forms, which were elucidated by 2D and heated NMR tests. The syntheses of individual target compounds were basically linear and had around 8 linear steps, thus it had to be started in a relatively big scale to ensure the needed amount of the final compounds. T3P (Propylphosphonic anhydride) as reagent was selected for amidation, which proved to be optimal, since using it in dichloromethane showed high enantio- and diastereo-selectivity and could be washed out from the reaction mixture by

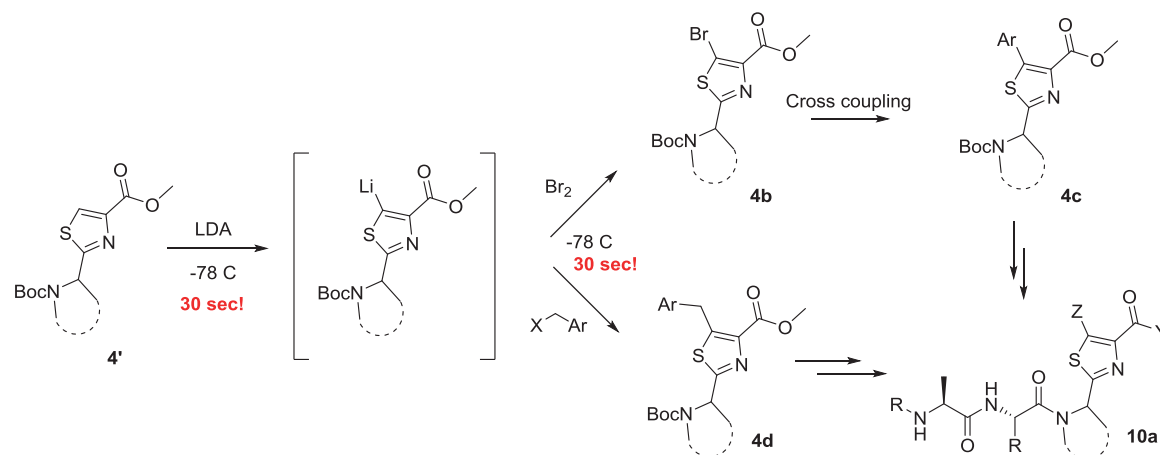
extraction. The products were purified by crystallization or in smaller scale by flash or by preparative HPLC chromatography.

Novel reactions and methodologies for the preparation of substituted thiazoles

Key intermediate thiazole ester **4** was prepared by the reproduction of the LCL161 synthesis; however, the published route was not appropriate for expanding the preparation to get compound libraries, therefore novel reactions were worked out for the substitution of the thiazole-4-carboxylate ring either at *C2* or *C5* position.



In the described approach³¹ compound **1** was transformed to the corresponding amide **2** by applying routine amidation reaction. The formation of thioamide **3** needed careful operation, since over 50°C the molecule racemized. Ring closure of thioamide **3** had to be carried out in neutral conditions otherwise the compound racemized again or lost the Boc protecting group. The classical methodology to transfer intermediate **4** to **4a** thiazole derivatives was based on lithiation reaction.³²



The reaction published in the literature was hardly reproducible since both the lithiation and the transformation of the lithium salt requested quick operations at low temperature, otherwise the sensitive intermediates decomposed. Applying IceCube continuous reactor supplied by ThalesNano, the reaction was carried out stereoselectively in good yields. The reactor set-up is illustrated in Figures 29 and 30.

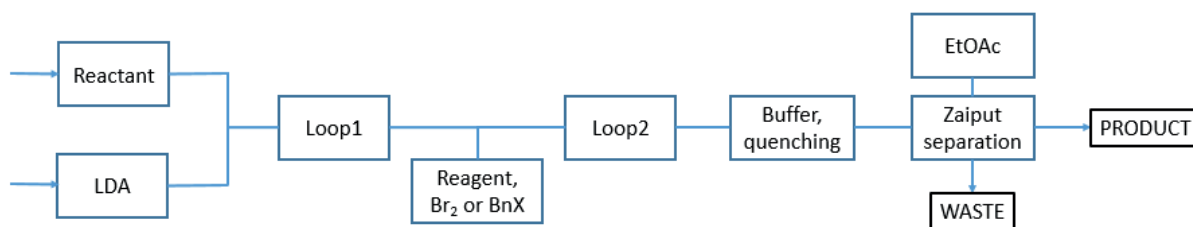
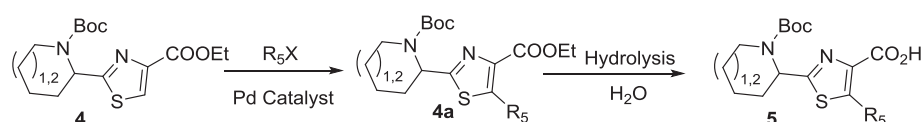


Figure 29. Continuous preparation of 5 substituted thiazole carboxylates using IceCube



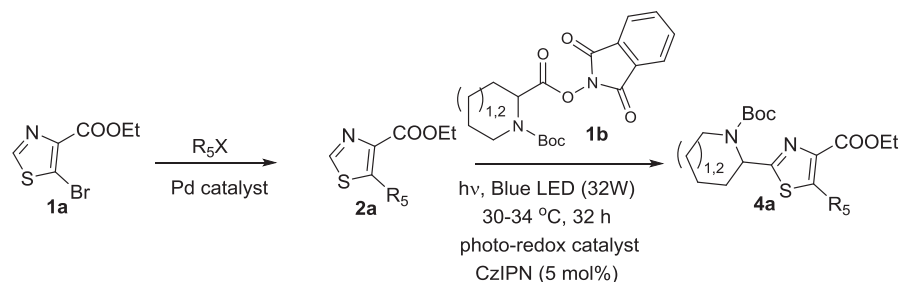
Figure 30. ThalesNano IceCube and PhotoCube reactor

Starting from intermediate **4** a novel reaction was worked out for the direct substitution of the thiazole ring in position *C5* to get the new derivative.³³



Activation of compound **4** using palladium catalyst then substitution with benzyl-halide gave **4a**, which delivered the acid **5** after hydrolysis using mild conditions. The isolated compounds had over 90% ee. Fortunately, after coupling the acid with chiral amino acids the formed diastereomers could be separated and optically pure compounds were isolated.

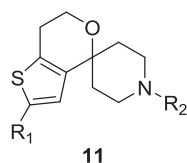
To increase the chemical possibilities a new route was worked out for the substitution of thiazole ring in *C2* position as well. The reaction was developed by applying the *Minisci* photo-redox reaction³⁴ using the photoreactor supplied by ThalesNano Inc (Figure 30). For the successful reaction, 5-bromo-thiazolate **1a** had to be converted to aryl-substituted derivative **2a** applying classical coupling reactions.



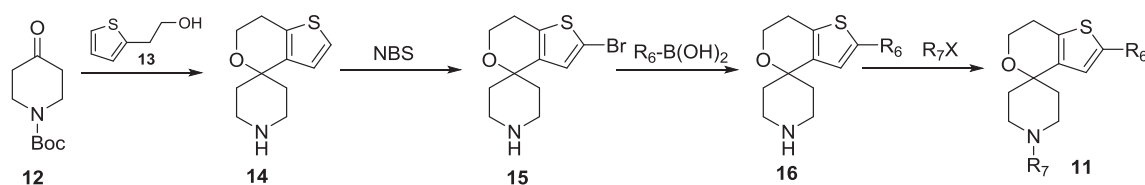
The stereoselective conversion of optically active pipecolinic acid reagent still needs further development.

Synthesis of Sigma receptor ligands

The core structure of the active Sigma Receptor ligand of compound **11** was systematically derivatized and tested to have SAR data and to find correlations to confirm the *in silico* modelling results.



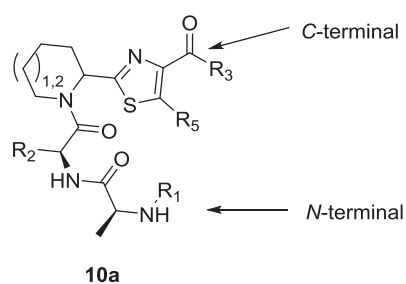
Albeit the general core structure was known and published,³⁵ we worked out a simple method for the synthesis of the skeleton which allowed us to generate a two-dimensional compound library.



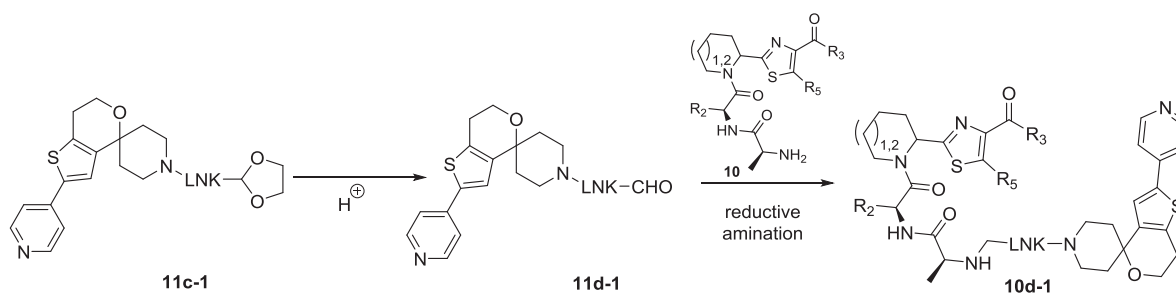
Starting from piperidone **12** the spiro-condensation was carried out in acidic conditions with thiophene-ethanol **13**. The thiophene ring was then selectively brominated with *N*-bromosuccinimide forming the key intermediate **16**, which was transformed in two steps to the **11** derivatives *via* cross-coupling and *N*-substitution reactions. The free amine function of compound **16** allowed us to connect the compound to linkers and IAP binding warheads.

Conjugation of Sigma and IAP binding ligands, preparation the conjugated chimeras

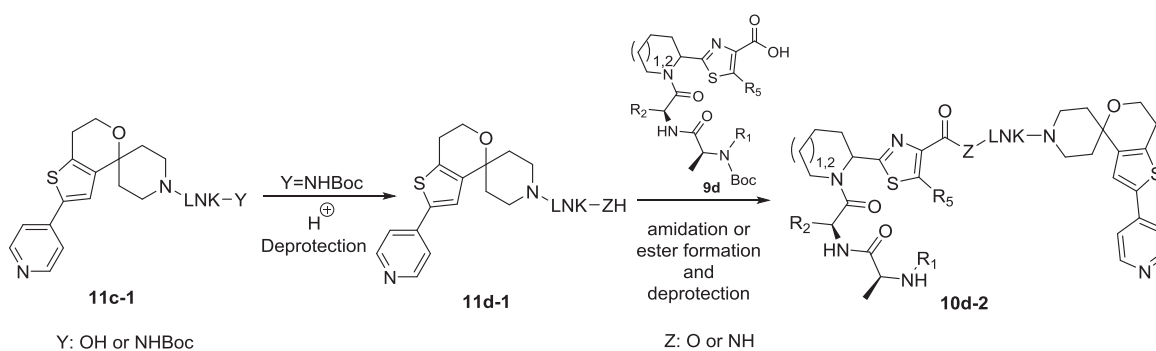
The two types of compounds were connected with linkers to bridge the two active sites resulting in bifunctional chimeras, where the sigma ligand served as a carrier molecule and the IAP antagonist as an apoptosis inducer “warhead”. The IAP molecule had two connection points to conjugate either at the *N*-terminal function in the peptide chain or at the *C*-terminal functional group on the thiazole ring. The compounds were stable both in base or salt forms. The solubility of the compounds was good, however, when it was possible, the salt form was preferred to prepare to increase their physicochemical and ADME properties.



The IAP binding ligands had several asymmetric centers and potential substitution points, thus reductive amination was applied to get the *N*-alkylated chimeras. Reductive amination proved to be the most effective and selective method. The linker-conjugated and protected sigma ligand **11c-1** was then deprotected in acidic conditions and reacted immediately with the IAP ligand in reductive media.



The *C*-terminal compounds, esters or amides, were prepared by coupling the protected thiazol-4-carboxylic acid derivative **9d** with the corresponding amine or alcohol **11c-1**. In the case of the esters mild conditions were applied to avoid the hydrolysis. The prepared conjugates showed high potency and proved the concept that the Lead compound is actively transported and internalized in the cancer cells.



Summary and conclusions

Based on the preliminary cytotoxic screening results and applying our Target Oriented Library platform, we identified novel compounds for two protein targets which play important role in cancer development. The selected targets, the Inhibitor of Apoptosis Protein family

(cIAP1/2 and XIAP) and the Sigma-1,2 receptors are key elements of the apoptosis regulation.

Our best IAP antagonist compound (31 μ M, 72 h, PANC-1) showed two-fold higher cytotoxic activity than the structurally related Novartis compound LCL161, which is under clinical trials. The replacement of the pyrrolidine ring of the reference compound to piperidine (LCL161 \rightarrow CI6_7) resulted in the increase of cytotoxic activity even though previous reports argued against such change.

We also identified and developed novel sigma receptor ligands with promising cytotoxic activities (51 μ M, 72 h, PANC-1). Sigma receptors have also important roles in apoptosis regulation and cell proliferation. They are characteristic motifs on the cancer cell surfaces, thus, are targeted as tumor markers in the cancer diagnosis.

Both structure groups have proper physicochemical properties and were promising starting points for the development of novel anticancer drug leads.

Based on the role played by sigma receptors in the active internalization of the complexed compounds, we connected the apoptosis-inducing XIAP antagonist compound (“warhead”) with sigma-2 ligands serving as a targeting or delivering tool forming a Small Molecule Targeting Drug Conjugate compound. The resulting chimeras, comprising the in-house discovered best IAP antagonist compound and the novel sigma ligand, multiplied the biological activity compared to the parent XIAP antagonist and showed more than one order of magnitude cytotoxicity increase compared to the reference compounds (3.0-6.6 μ M, 72 h, COLO-205, A2058, PANC-1). The results proved our concept that the targeted transport is feasible. The preliminary *in vivo* data showed positive signs of activity, however, further studies are planned to confirm the results. For the novel compound family a patent has already been filed.

During the project execution, we developed a novel 3D docking and virtual screening technology to identify potential IAP antagonists and sigma ligands based on the available protein structures. During the preparation of the novel structures, innovative synthesis technologies were developed and applied, such as novel *CH* activation reaction, flow chemistry, flow photochemistry and membrane technology.

In summary, by the integration of the biology, chemistry and chemoinformatics we set the foundation of a novel integrated drug discovery platform.

Acknowledgement

We thank all past and present members of the ComInnex management and participating teams for their backing and outstanding scientific and experimental contributions that have enabled us to develop the new promising anticancer compounds and innovative chemistry, especially Gellért Sipos and Gergő Ignác for the flow chemistry solutions and for the development of the photoredox reaction, Dániel Kócsi for the development of the new thiazole substitution reaction based on CH activation. We are grateful to NKFIH for the NVKP16-1-2016-0036 grant and the financial support of the project. We thank the ELTE and the Semmelweis teams for their work and the excellent collaboration especially Gábor Mező for his coordination of the grant consortium and for backing the preliminary in vivo tests of our compounds. We appreciate very much and say a big thank you for Schrodinger Co. for letting us to use the state-of-the art modelling and simulation platform free of charge. We thank András Simon for the 2D NMR structure assignment of compounds, Szilárd Tóth and Gergely Szakács for the collateral testing, József Murányi for the PAMPA experiments and József Tóvári for the mouse xenograft tests. We express our thanks for ThalesNano Inc. for supplying us the flow reactors.

References

1. Apoptosis Physiology and Pathology (Eds.: Reed JC, Green DR, Cambridge University Press, 2011)
2. Mohammad RM, Muqbil I, Lowe L, Yedjou C, Hsu H-Y. *Semin Cancer Biol* **35**: 78-103 (2015)
3. Tewari KM, Dhaneshwar SS. *J Cancer Res* **1**: 213 (2012)
4. Lalaoui N, Vaux DL. *F1000Res* **7**: 1889 (2018)
5. Fulda S. *Clin Cancer Res* **21**: 5030-5036 (2015)
6. Cong H, Xu L, Wu Y, Qu Z, Bian T, Zhang W, Zhuang C. *J Med Chem* **62**: 5750-5772 (2019)
7. Tesei A, Cortesi M, Zamagni A, Arienti C, Pignatta S, et al. *Front Pharm* **9**: 711 (2018)
8. Kim FJ, Pasternak GW. *Proc Nat Acad Sci* **114**: 6888-6890 (2017)
9. Riad A, Zeng C, Weng CC, Winters H, Xu K, Makvandi M, Mach RH. *Sci Reports* **8**: 1-12 (2018)
10. Zeng C, Rothfuss JM, Zhang J, Vangveravong S, Chu W, et al. *Anal Biochem* **448**: 68-74 (2014)
11. Longhitano L, Castracani CC, Tibullo D, Avola R, Viola M, et al. *Oncotarget* **8**: 91099 (2017)
12. Hashim YM, Spitzer D, Vangveravong S, Hornick MC, et al. *Mol Oncol* **8**: 956-967 (2014)
13. Zeng C, Weng CC, Schneider ME, Puentes L, Riad A, et al. *Cell Death Discovery* **5**: 1-12 (2019)
14. Naito M, Ohoka N, Shibata N. *Drug Discovery Today: Technologies* **31**: 35-42 (2019)
15. Huang Y-S, Lu H-L, Zhang L-J, Wu Z. *Med Res Rev* **34**: 532-566 (2014)
16. Finlay D, Teriete P, Vamos M, Cosford ND, Vuori K. *F1000Res* **6**: 587 (2017)
17. Kalászi A, Szisz D, Imre G, Polgar T. *J Chem Inf Model* **54**: 1036-1049 (2014)

18. Leeson PD, Young RJ. *Med Chem Lett* **6**: 722-725 (2015)
19. Young RJ, Green DVS, Luscombe CN, Hill AP. *Drug Discovery Today* **16**: 822-830 (2011)
20. Johnson CN, Ahn JS, Buck IM, Chiarparin E, Day JEH, et al. *J Med Chem* **61**: 7314-7329 (2018)
21. Oberdorf C, Schepmann D, Vela JM, Buschmann H, et al. *J Med Chem* **55**: 5350-5360 (2012)
22. Oberdorf C, Schepmann D, Vela JM, Diaz JL, et al. *J Med Chem* **51**: 6531-6537 (2008)
23. Giaever I, Keese CR. *Proc Nat Acad Sci* **81**: 3761-3764 (1984)
24. Szakács G, Hall MD, Gottesman MM, Boumendjel A, et al. *Chem Rev* **114**: 5753-5774 (2014)
25. Niso M, Riganti C, Abate C. *Receptors Clin Invest* **1**: 208-216 (2014)
26. Pape VFS, Tóth S, Füredi A, Szebényi K, Lovrics A, et al. *Eur J Med Chem* **117**: 335-354 (2016)
27. Di L, Kerns EH, Fan K, McConnell OJ, Carter GT. *Eur J Med Chem* **38**: 223-232 (2003)
28. Pinho VD, Gutmann B, Miranda LSM, et al. *J OrgChem* **81**: 5814-5823 (2016)
29. Sipos G, Ignác G, Bertók B (2019). Flow Chemistry Europe Conference, Cambridge (2019)
30. NOVARTIS AG; Thakur J, Yang D, Feng, L. WO2011/18474 (2011)
31. Deng S, Taunton J. *J Am Chem Soc* **124**: 916-917 (2002)
32. Deng S, Taunton J. *Org Lett* **7**: 299-301 (2005)
33. Kócsi D, Bertók B. unpublished results (2019)
34. Varga I, Balogh F, Ignác G, et al. *J Am Chem Soc* **258**: ACS National Meeting, SanDiego (2019)
35. Meyer C, Schepmann D, Yanagisawa S, Yamaguchi J, et al. *J Med Chem* **55**: 8047-8065 (2012)