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Synthesis and biological studies of new piperidino-1,2,3-triazole hybrids with 3-aryl isoxazole side chains.

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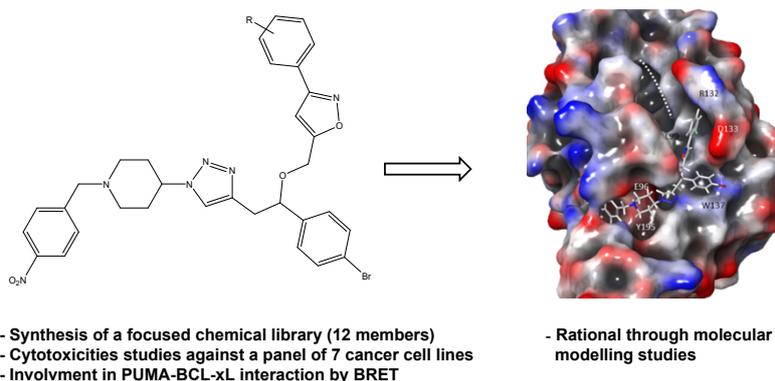
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Graphical Abstract



Highlights: in this paper, we report:

- Design and synthesis of a library of piperidine-triazole hybrids with new aryl-isoxazole side chains,
- Study of their cytotoxicity against seven representative cancer cell lines,
- BRET studies on the best molecule, to demonstrate its implication in PUMA-BCL-xL interaction,
- A rationale to these results through extensive molecular modelling calculations.

Keywords:

- Cancer
- Protein-protein interactions
- PUMA (p53 upregulated modulator of apoptosis)
- Bcl-xL (B-cell lymphoma-extra large)
- BRET (Bioluminescence Resonance Energy Transfer)
- Molecular modelling

Abstract

A small library of new piperidine-triazole hybrids with 3-aryl isoxazole side chains has been designed and synthesized. Their cytotoxicity against a panel of seven cancer cell lines has been established. For the most promising compound, an IC_{50} value of 3.8 μ M on PUMA/Bcl-xL interaction in live cancer cells was established through BRET analysis. A rationale was proposed for these results through complete molecular modelling studies.

The world is regularly faced to new diseases, such as the recent SARS-CoV-2 pandemy. However, at the same time, other diseases continue to develop and cancer in particular remains a leading cause of death worldwide, accounting for nearly 10 million deaths in 2020.¹ One of the hallmarks of cancer is the evasion of apoptosis,² therefore the discovery of molecules able to counteract this effect is a very active area of research.³ Apoptosis is ruled by numerous protein interactions mostly involving members of the BCL-2 family. Of these, the interaction which engage PUMA and BCL-xL has recently been described to be critical in some cancers, wherein BCL-xL is frequently overexpressed and promote resistance to conventional chemotherapy.⁴ Thus, the tight sequestration of PUMA by BCL-xL favors the resistance of cancer cells to many apoptotic triggers.⁵ The molecular interface of this interaction is defined by the BH3 (BCL2-Homology-Domain-3) binding groove, a shallow groove at the surface of BCL-xL, and the BH3 domain of PUMA, a consensus domain shared by BCL2 family members. This domain fits the binding groove with more affinity than other BH3 domains defined within pro-apototic proteins, thereby driving the strong resilience of this interaction.⁵ Inhibitory compounds targeting specifically the binding groove of BCL-xL have been designed, but until recently, the binding of PUMA to BCL-xL was unaffected by these molecules. In our recent publication however, we reported the first molecule **X** that was able to perturb BCL-xL/PUMA interaction. It seems to proceed originally, through an increase of PUMA intrinsic instability, and we have proposed a new mechanism for this action based on extensive molecular docking and molecular dynamic studies.⁶

The proposed binding mode for this triazole-hybrid type molecule⁷, **X** shown in Figure 1, suggests that the inhibitor could be further improved by extending beyond its current site. Actually, the location of the benzylic position suggests the possibility of reaching PUMA's crevice. Toward this goal, we have designed a novel generation of inhibitors. This new chemotype **Y** (Fig. 1C) bears two modifications of the molecule **X**: (i) the incorporation of bulky heteroaromatic groups (phenyl-isoxazoles) in order to fill partially the large PUMA cavity with a balanced hydrophobicity, since the pocket is solvent exposed, (ii) replacement of the methylene linker between the triazole and the phenyl group in **X** by a more flexible ethylene to facilitate a correct orientation of the phenyl-isoxazoles units in **Y**.

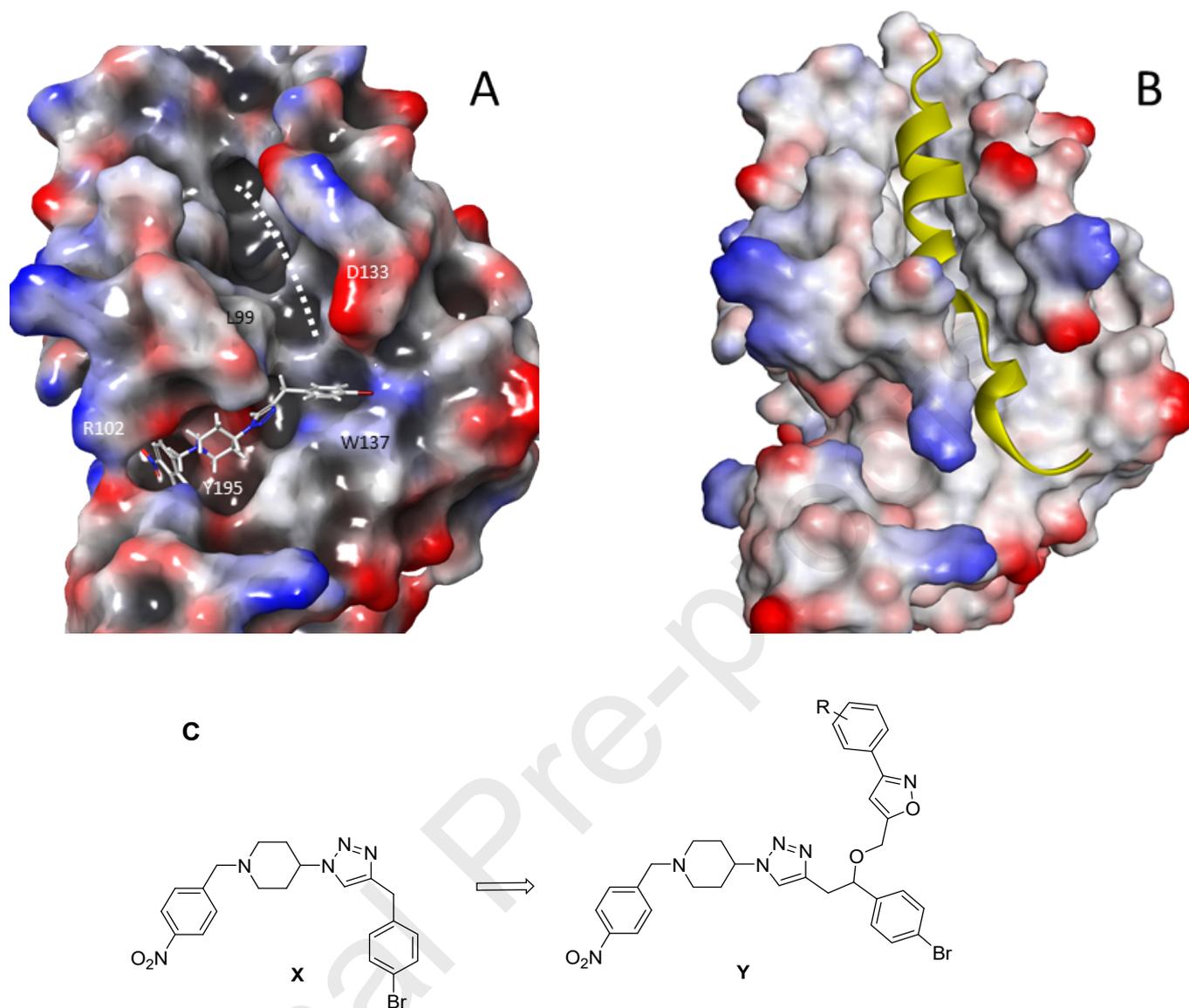
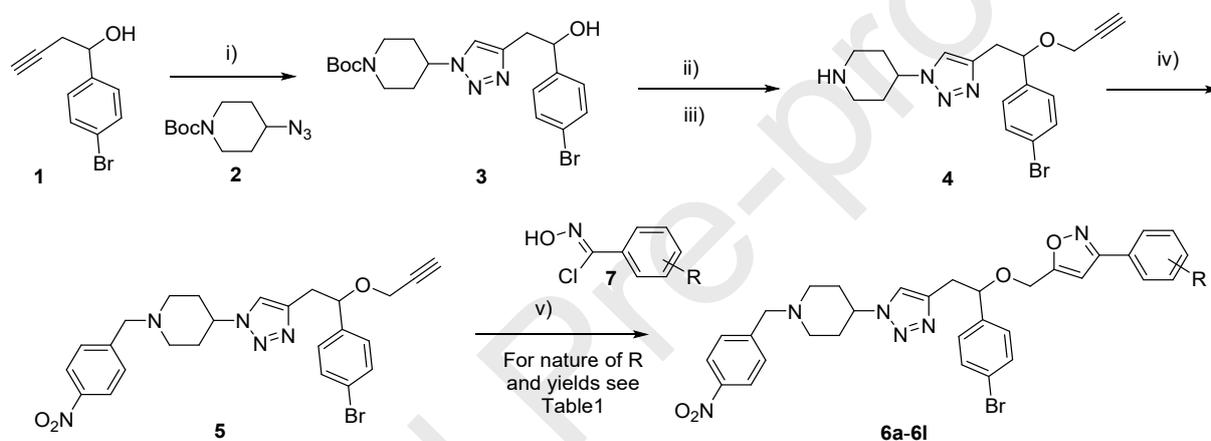


Fig. 1A: Binding mode of the first generation compound **X** in BCL-xL, as suggested by previous molecular simulations.⁶ Several important residues are labelled, and PUMA binding crevice is illustrated as white dotted line. Fig. 1B NMR-based structure of BCL-xL/PUMA complex, in the same orientation B (PDB:2M04).⁸ PUMA is shown as a yellow α -helix. Fig. 1C Proposed transition from the first generation compound **X** to the second series **Y**.

The purpose of this paper is first to report the synthesis of a focused library (with 12 members) of such type-**Y** molecules. Next, these compounds will be evaluated on cytotoxic studies, using a representative panel of seven cancer cell lines. Finally, for the best compound, the precise involvement of BCL-xL/PUMA interaction will be checked by BRET and molecular modelling experiments.

The synthesis (Scheme 1) starts from propargylic alcohol **1**, easily available from *p*-bromo benzaldehyde.⁹ Then, «click reaction»¹⁰ with the, known,¹¹ piperidinoazide **2** gave in good yield the triazole **3**. After propargylation of the alcohol, followed by the deprotection of the *N*-Boc group, the intermediate aminopiperidine **4** was obtained. After reaction with *p*-nitrobenzyl bromide, the key intermediate **5** was obtained in 4 steps and 17% overall yield from **1**. A final classical 1,3 dipolar cycloaddition of alkyne **5** with the nitrile-oxides obtained from chloroximes **7**,^{10d} gave the target molecules **6a-6l** in moderate to good yields (Table 1). All these molecules have spectral and analytical data in agreement with their structures (see experimental section and supplementary information).



Scheme 1. Synthesis of compounds **6a-6l**. Reagents and conditions: (i) **2**, CuSO₄·5H₂O, sodium ascorbate, *t*-BuOH: H₂O, rt, 16h (68%); (ii) Propargyl bromide, NaOH:DCM, TBAI, rt 18h; (iii) TFA, DCM, rt, 18h; (iv) 4-nitrobenzyl bromide, Et₃N, DCM, 40 °C, 16h (40.4%); (v) **7**, Et₃N, THF, reflux (24-65%).

Table-1 Synthesis of the target molecules **6a-6l**.

Entry	Compound	R	% Yield
1	6a	H	65
2	6b	4-Br	63
3	6c	4-F	57
4	6d	4-OMe	51
5	6e	4-CN	25
6	6f	3-Br	55
7	6g	3-F	52
8	6h	3-OMe	24
9	6i	3-CN	30
10	6j	2-Br	62
11	6k	2-F	32
12	6l	2-OMe	26

We performed a cytotoxicity screening of the twelve molecules **6a-6l**, investigating seven representative cancer cell lines. The compounds effect on cell viability was evaluated on hepatocellular carcinoma Huh-7, colorectal adenocarcinoma CaCo2, colorectal carcinoma HCT-116, breast carcinoma MDA-MB 231, breast carcinoma MCF7, prostate carcinoma PC3 and lung carcinoid NCI-H727). Human skin fibroblasts (Fibro) were used as reference for non-tumor cells with Roscovitine and Doxorubicin as positive controls. After 48 h treatment with the new molecules, cells were fixed and the nuclei were stained with Hoechst 33342, and counted using HCS technology (Table 2).

The determination of IC_{50} indicated:

- significant cytotoxicities on three cell lines:
 - o HuH7 for all molecules except **6j**,
 - o CaCO2 for all compounds except **6l**,
 - o MCF7 for all derivatives except **6a**.
- mixed activities on two cell lines MDA-MB468 and PC3,

- a selective cytotoxicity on two cell lines: in the case of MDA-MB-231 and HCT 116, only **6g** and **6k** exhibit potent cytotoxicities.

Alltogether, these results indicate that two most potent derivatives are **6g** and **6k**. However, the first exhibited cytotoxicity for both normal and cancerous cells while the second **6k** exhibited cytotoxicity only for cancer cell lines and not for fibroblasts. Therefore, this last derivative was identified as the most promising molecule and has been considered for further studies.

Table 2. Cytotoxic studies (IC_{50} determination) of the molecules **6**.^[a]

	HuH7	CaCo-2	MDA-MB-231	MDA-MB-468	HCT116	PC3	MCF7	Fibro
	IC_{50} μ M							
DMSO	>25	0.07						
Roscovitine	9	14	17	18	9	11	9	15
Doxorubicine	0.02	0.05	0.04	0.06	0.08	0.08	0.10	0.02
6a	7	7	>25	3	>25	>25	>25	>25
6b	2	3	>25	5	>25	>25	4	>25
6c	2	3	>25	4	>25	>25	7	>25
6d	2	3	>25	9	>25	2	4	>25
6e	5	2	>25	5	>25	3	3	>25
6f	3	5	>25	>25	>25	>25	6	>25
6g	3	5	5	3	6	9	8	10
6h	5	5	>25	>25	>25	>25	7	>25
6i	3	3	>25	>25	>25	>25	2	>25
6j	>25	3	>25	>25	>25	>25	6	>25
6k	2	4	4	4	9	6	8	>25
6l	5	>25	>25	>25	>25	>25	3	>25

^[a] IC_{50} determination of molecules **6** effects on seven representative tumor cell lines and normal Human fibroblast. IC_{50} (μ M) were calculated from dose-response curves after 48 h exposure (mean of triplicates).

To confirm that the mechanism of action of our novel derivative was still that of the parent molecule, i.e. perturbation of BCL-xL / PUMA interaction, we performed BRET experiments with the most active compound **6k**.

Dose responses experiments were performed on MCF7 cell line using A1331852 (Abbvie), a well-known specific antagonist of BCL-xL, as positive control. Due to its poor cytotoxic activity on MCF7 cells, **6a** was used as negative control. Cells were treated overnight with 20 μM of each molecule before being analyzed for their energy transfer ability. Results indicate that **6k** has a reproducible effect on the interaction and diminishes the BRET ratio between PUMA and BCL-xL by almost 10% (Fig. 2a). A dose response curve for **6k** was then established which evaluated the IC_{50} of the molecule at 3.8 μM , close to the IC_{50} found for its cytotoxic effects (Fig. 2b). This value is also very similar to the value obtained for the reference molecule **X** (3 μM).

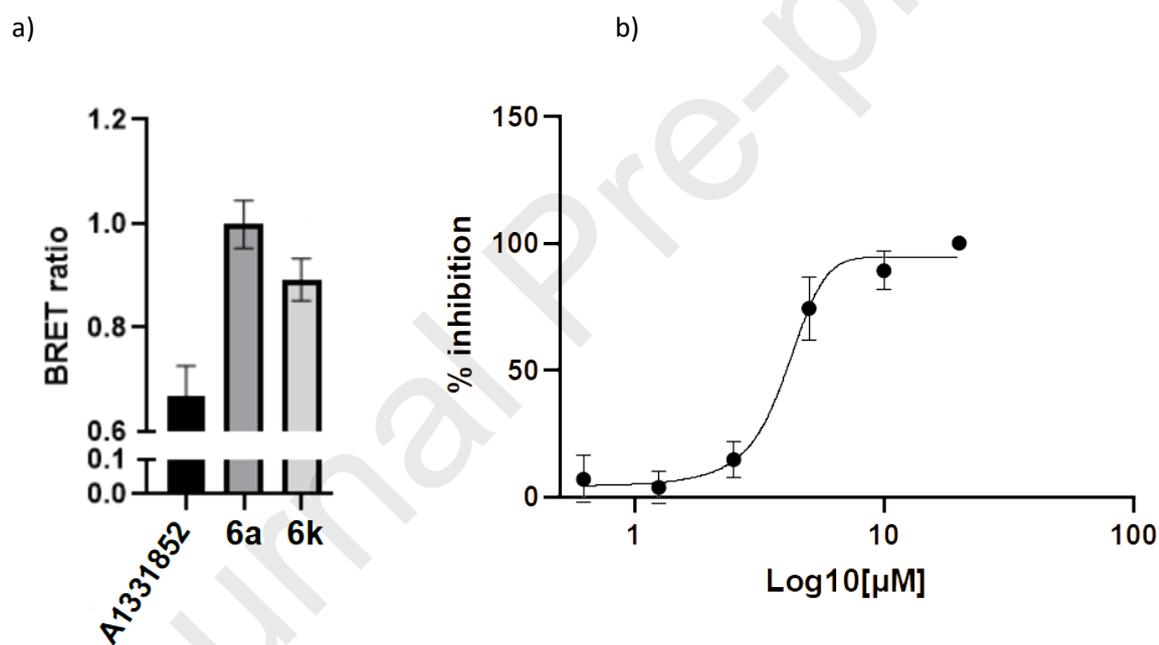


Figure 2 a): BRET studies on molecules **6a** and **6k**: BRET based dose response curve of compound **6k** on PUMA/Bcl-xL interaction in live cancer cells. A1331852 was used as a reference antagonist for Bcl-xL.

Molecular docking of the novel molecules showed that they retain the parent molecule binding mode. Key interactions persist, such as a salt bridge between the piperidine and E96, π -stacking with Y195 and W137 (Fig. 3). As expected, the additional phenyl isoxazole engages into the PUMA's crevice where it forms additional contacts. The isoxazole is sandwiched between sidechain of L99 and backbone of D133. The phenyl is packed with backbone of R132 and L130. Interestingly, its 2-fluoro substituent forms a halogen bond with D133, in agreement with the general higher cytotoxicity of **6k** as compared with the non-halogenated analogue **6a**. Further, molecular dynamic experiment have been performed and they confirmed the relevance of the selected pose (Sup Information; Fig. 1).

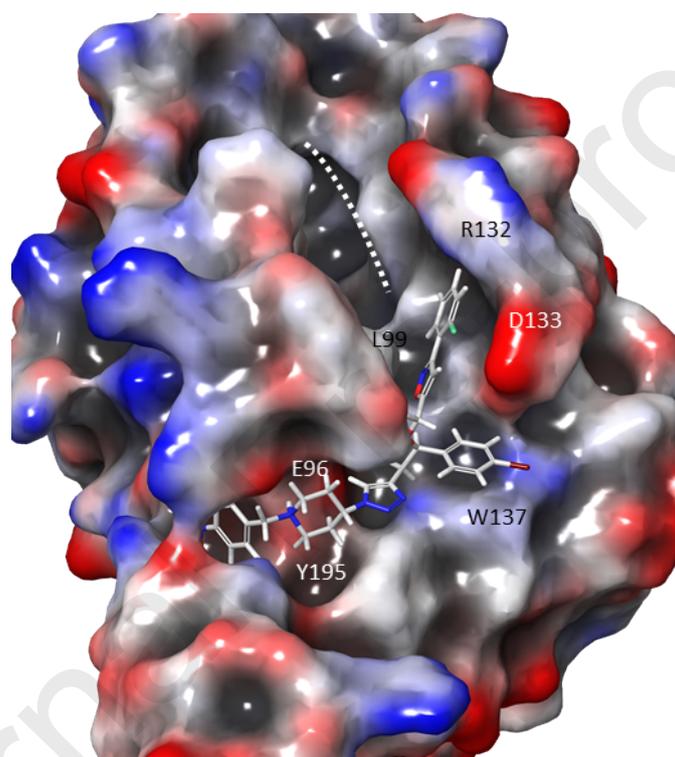


Fig. 3: Proposed binding mode of the new molecule **6k**. Important residues are labelled, and PUMA binding crevice is illustrated as white dotted line.

In summary, based on our recent discovery of the first small organic molecule **X** able to perturb PUMA from BCL-xL, we have designed and synthesized a dozen of new piperidine-triazole hybrids with 3-aryl isoxazole side chains. Cytotoxicity studies identified one of them **6k** as the most attractive derivative with a high activity against a representative panel of seven cancer cell lines. Further, studies by BRET confirmed that this molecule was also active in displacing PUMA from BCL-xL. Extensive molecular modelling studies indicated that the new side chain with the aryl isoxazole moiety should partly fill the BCL-xL groove where PUMA is binding. Thus, corresponding data allowed us to propose a rationale for

the activity of this new molecule and confirm the potentialities of this family of compounds towards perturbation of the strong PUMA-BCL-xL interaction.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Supplementary data

Supplementary data to this article (Complete experimental procedures and analytical data plus copies of the NMR spectra) can be found online at <https://>

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