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In vitro anticancer activity and SAR studies of triazolyl aminoacyl(peptidyl) penicillins†

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Patricia G. Cornier,[a] Carina M. L. Delpiccolo,[a] Florencia C. Mascali,[a] Dora B. Boggián,[a] Ernesto G. Mata,*[a] Mariano G. Cárdenas,[b] Viviana C. Blank[b] and Leonor P. Roguin*[b]

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A library of triazolyl aminoacyl(peptidyl) penicillins was designed, synthesized, and evaluated for their antiproliferative activity against HeLa and B16-F0 cell lines. Structure–activity relationship studies were carried out, and minimal structural requirements were determined. Among the tested compounds, derivatives **7f**, **7p** and **7m** demonstrated the highest anticancer activity and a promising selectivity profile against these two cell lines.

Introduction

The β-lactam ring is the key component of the most widely prescribed antimicrobials such as penicillins, cephalosporins, thienamycins and monobactams.[1] Bacterial resistance to antibiotic drugs has maintained and even increased the interest in the chemistry of β-lactam compounds since continuous effort is required for the development of new drugs with a much broader spectrum of action.[2] Apart from their antibacterial properties, β-lactams also show biological activities[3] that include inhibition of prostate specific antigen,[4] thrombin, human cytomegalovirus protein, human leukocyte elastase, cholesterol absorption[5] and cysteine protease,[6] as well as neuroprotective action.[7] It is also interesting to note that a series of β-lactam derivatives has been recently associated with antiproliferative activity[8] and prodrug for anticancer chemotherapies.[9] Cancer is currently one of the leading causes of death worldwide. Despite great effort to try to control this disease, most of the treatments cannot provide an effective response. Because of the problems encountered in anticancer treatments, such as toxicity and resistance, the development of new and improved chemotherapeutic agents has become a constant challenge for industry and academia.

The discovery and development of novel β-lactams of importance in anticancer research prompted us to develop a library of potentially active hybrids having in their structure a bicyclic β-lactam derivative. Molecular hybridization techniques[10] allow the preparation of new hybrid compounds by conjugation of bioactive molecules and/or pharmacophoric units derived from known bioactive compounds. In this sense, we have linked a penicillin to a peptide *via* a triazole group in order to achieve structures that preserve or even improve the most desirable attributes of the original moieties. The 1,2,3-triazole subunit is found in a large number of biologically active compounds, and also in chemotherapeutic drugs.[11] Although there is not a direct connection between pharmacokinetic properties and cytotoxic effect, the addition of a peptide segment could facilitate transport across the cell membranes and/or achieve effective protein–protein interactions.[12]

In the present study, a library of triazolyl aminoacyl(peptidyl) penicillins was synthesized using an azide–alkyne cycloaddition[13] as the key reaction step. Also, based on the intrinsic properties of the hybrid subunits, we evaluated all compounds as anticancer agents against two cancer cell lines and a preliminary SAR analysis was carried out.[14]

Results and discussion

Chemistry

An efficient and versatile solid-phase methodology was used for the synthesis of the library of triazolyl aminoacyl (peptidyl) penicillins.[15] Target compounds were synthesized in good to high yields for the whole synthetic sequence (Table 1). The key intermediate, immobilized *N*-propiolyl amino acids (peptides) **3**, was synthesized from condensation of different Fmoc protected-amino acids linked to Wang resin **(1)** and propiolic acid in the presence of *N*,*N*-diisopropylcarbodiimide (DIC) as an activating agent (Scheme 1). Then the solid-phase Cu(I)-catalyzed Hüisgen 1,3-dipolar cyclization was carried out using alkynes **3** and penicillin azide **4**,[15] with Cu(I) or $[(CH_3CN)_4Cu]PF_6$ as catalysts, to afford the immobilized conjugates **5**. Final products **7** were obtained treating **5** with TFA (10% in DCM) followed by methylation using diazomethane and purification by column chromatography.

[a]*Instituto de Química Rosario (CONICET – UNR), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, 2000 Rosario, Argentina. E-mail: mata@iquir-conicet.gov.ar*

[b]*Instituto de Química y Fisicoquímica Biológicas (UBA – CONICET), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, C1113AAD, Buenos Aires, Argentina*

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Table 1 Diversity and yields of the synthesized compounds

Entry	Comp.	R^1	R^2	R^3	*n*	AA_1	AA_2	Yield[a]
1	**6b**[b]	H	Br	Br	0	Phe	—	20
2	**6c**[b]	H	Br	Br	0	Val	—	38
3	**7a**	Me	Br	Br	0	Gly	—	44
4	**7b**	Me	Br	Br	0	Phe	—	36
5	**7c**	Me	Br	Br	0	Val	—	50
6	**7d**	Me	Br	Br	0	Gly	Phe	37
7	**7e**	Me	Br	Br	0	Leu	Tyr	30
8	**7f**	Me	Br	Br	0	Leu	Phe	49
9	**7g**	Bn	Br	Br	0	—	—	65
10	**7h**	Me	Br	Br	0	Ile	Phe	40
11	**7i**	Me	Br	Br	0	Val	Phe	38
12	**7j**	Me	Br	Br	0	Pro	Phe	30
13	**7k**	Me	Br	Br	0	Leu	Leu	25
14	**7l**	Me	Br	Br	0	Ile	Tyr	29
15	**7m**	Me	Br	Br	0	Met	Phe	8
16	**7n**	Me	Br	Br	0	Leu	—	51
17	**7o**	Me	Br	Br	0	Tyr	—	20
18	**7p**	Me	H	H	0	Leu	Phe	18
19	**7q**	Me	Br	Br	2	Leu	Phe	34

[a] Overall isolated yield after being released into solution with 10% TFA/DCM followed by esterification with diazomethane, and purification by column chromatography (based on the initial loading level of Wang resin, five to seven reaction steps). Reactions were performed at the 0.1 mmol scale and monitored by the disappearance of the alkyne signal in FT-IR. [b] No esterification was performed in these cases.

In vitro anticancer screening

The antiproliferative activity of all compounds was initially tested *in vitro* at a 20 μM concentration against two tumor cell lines (HeLa: human cervix adenocarcinoma, B16-F0: murine melanoma) (Fig. 1). IC_{50} values, defined as compound concentrations that produce 50% growth inhibition, were obtained from dose–response curves and are summarized in Table 2. This table includes IC_{50} values corresponding to those compounds that at a 20 μM concentration caused a reduction of ≥50% in the growth of one or both tumor cell lines (see Fig. 1). Thus, IC_{50} values corresponding to compounds that inhibited tumor cell proliferation less than 50% at 20 μM in both cell lines were not evaluated. To examine the selectivity against tumor cells, the cytotoxic effect of compounds included in Table 2 was also determined in an epithelial cell line derived from normal mammary gland of mice (NMuMG). In order to make easier the comparison between IC_{50} values obtained in tumor and non-cancer cells, we defined the relative potency (RP) of each compound in a particular tumor cell line as the ratio of the IC_{50} obtained in NMuMG with respect to the IC_{50} value obtained in tumor cells. Thus, the highest this value, the greatest antitumor potency and selectivity is exhibited by a compound. Based on the RP values obtained (Table 2), triazolyl peptidyl penicillins are classified as follows.

*Highly potent and selective (RP ∼ 30): compounds **7f**, **7m** and **7p***: within this group, although the antiproliferative potency (IC_{50} values) of derivative **7f** in both tumor lines was higher than those of derivatives **7m** and **7p**, the lower toxicity of the two latter compounds in NMuMG cells allowed a similar range of RP values to be obtained, suggesting the usefulness of this factor for comparison purposes.

*Moderately active and selective (RP ∼ 10–20): compounds **7b**, **7i** and **7k***: this group of derivatives showed a rather higher efficacy in B16-F0 cells than in HeLa cells.

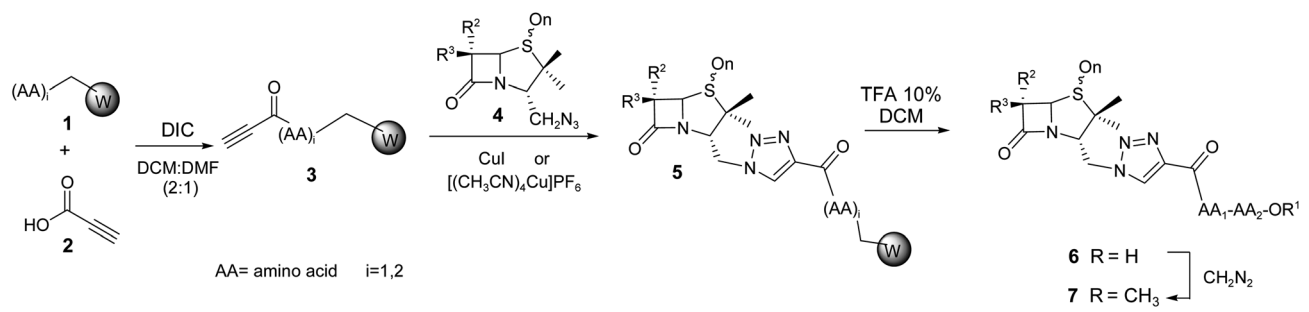
Structure–activity relationship

Based on the information collected in Tables 1 and 2, some structure–activity relationships (SARs) can be established in order to recognize molecular requirements for the antiproliferative activity. Thus, we can deduce that esterification of the carboxylic acid group of the terminal amino acid appears to be important for activity. This is evident for compounds **6b** and **6c** that showed no activity unlike their methyl ester counterpairs **7b** and **7c**.

To determine the importance of the peptidic portion of the molecule, we synthesized and evaluated the "amino acid-free" penicillin derivatives **7g**, **8** and **9** (Fig. 2). These compounds showed very weak or null activity, confirming that the presence of the amino acid moiety plays an important role in maintaining the antitumor activity. In the case of compound **9**, cytotoxicity is high but for both cancer and normal cell lines.

Then, the penicillin segment was evaluated. Compound **10** (Fig. 2) having no penicillin core but having the phenylalanine–leucine dipeptide and the triazole group showed no cytotoxic activity, suggesting that penicillin is also essential for such activity.

Generally speaking, those compounds bearing only one amino acid residue were less active than those having two amino acid residues (compare **7f** with **7b** and **7n**). In addition,



Scheme 1 Solid-phase synthesis of triazolyl aminoacyl (peptidyl) penicillins.

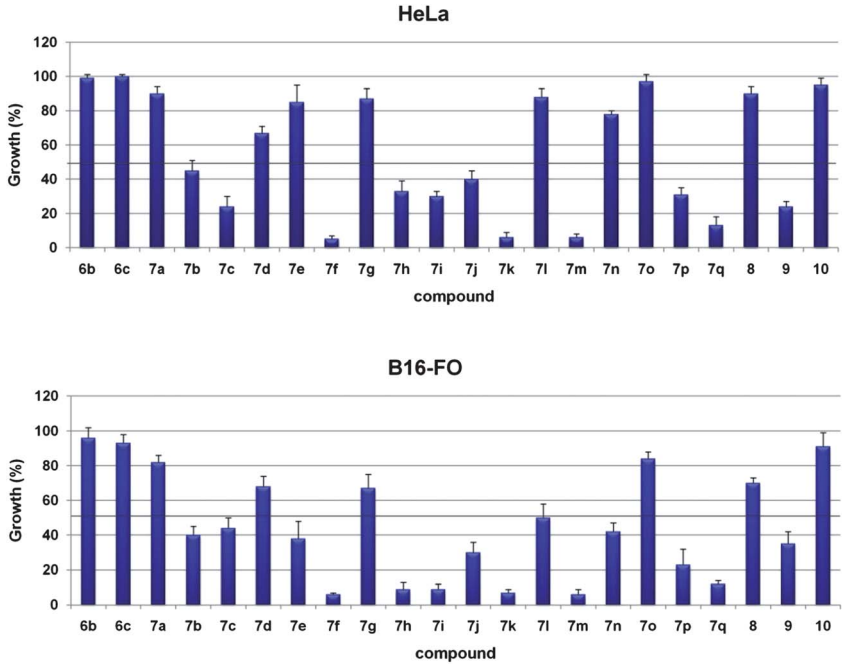


Fig. 1 Effect of different triazolyl aminoacyl (peptidyl) penicillins on the proliferation of HeLa and B16-F0 cell lines. 2×10^4 cells per well (HeLa) or 1×10^4 cells per well (B16-F0) were incubated in the presence or absence of a 20 μM concentration of different compounds for 72 h at 37 °C. Cell proliferation was determined by colorimetric determination of hexosaminidase levels. Results are expressed as the percentage of growth obtained in the absence of compounds (control) and represent means ± SE of three different experiments.

Table 2 Results of the *in vitro* cytotoxic activity of the synthesized compounds

	IC_{50} values[b] (μM)			RP[c]	
Comp.	HeLa[e]	B16–F0[e]	NMuMG	HeLa	B16–F0
7b	14 ± 1	10 ± 1	125 ± 7	9	12.5
7c	12 ± 4	18 ± 4	33 ± 4	3	2
7e	—[a]	22 ± 8	>160	ND	7[d]
7f	3 ± 1	3 ± 1	102 ± 8	34	34
7h	15 ± 2	9 ± 1	83 ± 8	5.5	9
7i	16 ± 3	9 ± 2	170 ± 7	10.6	19
7j	17 ± 2	11 ± 1	55 ± 7	3	5
7k	9 ± 2	4 ± 1	88 ± 5	10	22
7l	—[a]	20 ± 3	95 ± 7	ND	5
7m	10 ± 5	9 ± 2	275 ± 15	27.5	30.5
7n	—[a]	18 ± 2	39 ± 6	ND	2
7p	13 ± 4	10 ± 3	355 ± 7	27	35.5
7q	2 ± 1	2 ± 1	7 ± 4	3.5	3.5
9	12 ± 1	15 ± 3	69 ± 8	6	5

[a] The symbol (—) indicates the compounds that inhibited cell growth less than 50% at a 20 μM concentration. ND not determined. [b] The molar drug concentrations required to cause 50% growth inhibition (IC_{50}) were determined from dose–response curves. Results represent means ± SE of at least three different experiments. [c] The relative potency (RP) of a compound in a tumor cell line was calculated as the ratio of IC_{50} NMuMG/IC_{50} tumor cells. [d] Minimum value. [e] Reference standards: Doxorubicin: 0.68 (HeLa), 8.05(B16-BL6);[17] Cisplatin: 14.31 (HeLa).[18]

within the group containing just one amino acid, the presence of phenylalanine seems to be more effective (compare **7b** with **7a**, **7c**, **7n** and **7o**).

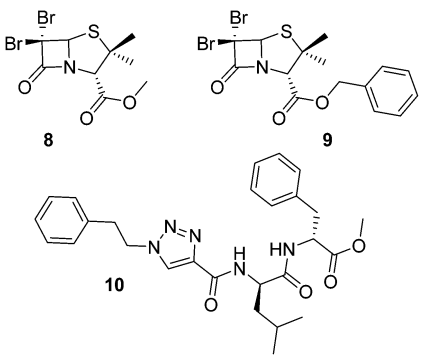


Fig. 2 Synthesized compounds to extend the SAR study.

We then investigated the amino acid moiety adjacent to the triazole group (AA_1); data suggest that residue bulkiness is important for the activity. Glycine, the less bulky amino acid, present in compounds **7a** and **7d**, did not contribute to the antitumor effect. Thus, by increasing the size of the amino acid α-substituent, the activity was improved. Leucine, present in one of the most active compounds (**7f**), seems to be optimal, while its replacement by similar-shaped amino acids like isoleucine (**7h**) or valine (**7i**) reduced the cytotoxic effect in tumor cells. Interestingly, the substitution for methionine (compound **7m**) maintained the antitumor activity comparable to **7h** and **7i**. However, the low toxicity of **7m** in non-tumor cells led to a relative potency similar to **7f**.

Changes in the second amino acid (AA_2) were also significant. Replacement of phenylalanine (**7f**) with tyrosine (**7e**, **7l**)

resulted in partial loss of cytotoxicity. However, when leucine was employed as the second amino acid (**7k**), a slight reduction of biological activity was observed.

To further explore the SAR of this series, modifications of the most active compound **7f** were carried out. Dehalogenated derivative, compound **7p** ($R^2 = R^3 = H$), showed similar relative potency compared to the parent compound **7f**. This interesting finding could be useful for *in vivo* experiments. In addition, the dehalogenated version has a lower molecular weight than its dibromo counterpart (about 500 Da), a feature that correlates better with the requirements for drug oral or epidermal absorption.[16]

Finally, the sulfur oxidation state was evaluated. Interestingly, compound **7q**, which is the sulfone of **7f**, showed good cytotoxic activity against cancer cells but also remarkable inhibitory effects on the growth of normal cells.

Conclusions

A library of triazolyl aminoacyl(peptidyl) penicillins was designed and synthesized. The antiproliferative activity of these compounds was evaluated against HeLa and B16-F0 cell lines, and several of them show selective and high cytotoxic activity. Compounds **7f**, **7p** and **7m** demonstrated the highest anticancer activity and a promising selectivity profile among the tested compounds against these two cell lines with relative potency values around 30.

The SAR study indicates the minimal structural requirements, as the penicillanic core and peptidic portion are crucial for the antitumor activity. Two amino acid residues are better than just a single one, and changes in the shape of amino acid α-substituents could affect activity. Replacement of bromine by hydrogen at position 6 of the penicillanic core maintained the antitumor activity. Results also indicate that sulfone derivatives have low cytotoxic selectivity.

In summary, this study has provided important information about the structure–activity relationships of triazolyl aminoacyl(peptidyl) penicillins as novel antiproliferative structures. We are currently designing, synthesizing and evaluating new derivatives in order to obtain improved analogs and the results will be reported in due course. Moreover, studies on the mechanism of antiproliferative effects, as well as *in vivo* experiments, will be our next objective with the purpose of finding the best candidates for further development of potential chemotherapeutic agents.

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