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A solid- and solution-phase-based library of 2β-methyl substituted penicillin derivatives and their effects on growth inhibition of tumor cell lines†

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We described the design, synthesis and antiproliferative properties of a series of twenty 2β-methyl substituted penicillin derivatives. This analysis includes evaluation against HeLa and MCF-7 human tumor cell lines and LM3 and B16-F0 murine tumor cell lines. The epithelial cell line derived from the normal mammary gland of mice (NMuMG) and the mouse embryo fibroblast cell line (3T3) were used as controls (non-cancer cells).

Introduction

The β-lactam ring is an active agent whose efficiency has been proven in a large number of clinically significant therapeutic areas.[1] Apart from being the key structural element of the most broadly prescribed antibiotics, such as penicillins, cephalosporins, carbapenems and monobactams (Fig. 1),[2] β-lactams have offered a new panorama with the development of some derivatives as potent cholesterol absorption inhibitors, including commercial ezetimibe (Fig. 1).[3] The β-lactam skeleton is also found in prostate specific antigen, thrombin,[4] human cytomegalovirus protein,[5] human leukocyte elastase,[6] cysteine protease,[7] and human fatty acid amide hydrolase[8] inhibitors. This class of compounds has also been considered as peptidomimetic species for mimicking certain properties of proteins.[9] Their non-antibacterial uses and the constant need for new antibiotics to attack the problem of bacterial resistance to traditional drugs have maintained and even increased the interest in the chemistry of β-lactams. Last but not least, β-lactams are key synthons for the preparation of various heterocyclic compounds of biological importance.[10] For example, substituted hydroxy-β-lactams have been used in the semisynthesis of paclitaxel (Taxol) and related compounds from baccatin III.[11]

Interestingly, we and others have recently demonstrated that β-lactam derivatives can also be useful in the field of antiproliferative chemotherapy. In this sense, *N*-thiolated,[12] 1,4-diaryl[13] 1,3,4-triaryl,[14] and polyaromatic[15] 2-azetidinones have been reported as active against cancer cells. Apart from being considered as prodrugs for the ADEPT strategy,[16] cephalosporin derivatives have also shown anticancer activity.[17] Penicillins are a major class of β-lactams and the oldest used as antibacterial agents. We have recently reported antiproliferative studies on a series of triazolyl aminoacyl(peptidyl) penicillins.[18] These hybrid compounds, prepared by azide–alkyne cycloaddition as the key reaction step, were evaluated against cancer cell lines, and several of them show high selectivity and cytotoxic activity.

As part of our efforts to discover new potential anti-cancer drugs, we have synthesized a solid and solution-phase-based

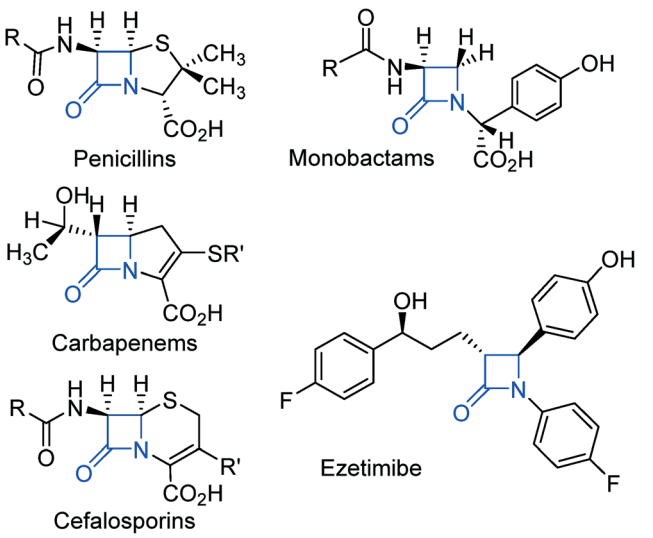

Fig. 1 The β-lactam ring is present in several commercially available drugs.

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† Electronic supplementary information (ESI) available: Experimental procedures, characterisation data, description of cell lines, culture conditions and cell proliferation assay. See DOI: 10.1039/c4md00430b

library of 2β-methyl substituted penicillins and evaluated their *in vitro* antiproliferative activity against four cancer cell lines and two non-cancer cells, and some conclusions on the preliminary structure–activity relationship have been drawn.

Results and discussion

Chemistry

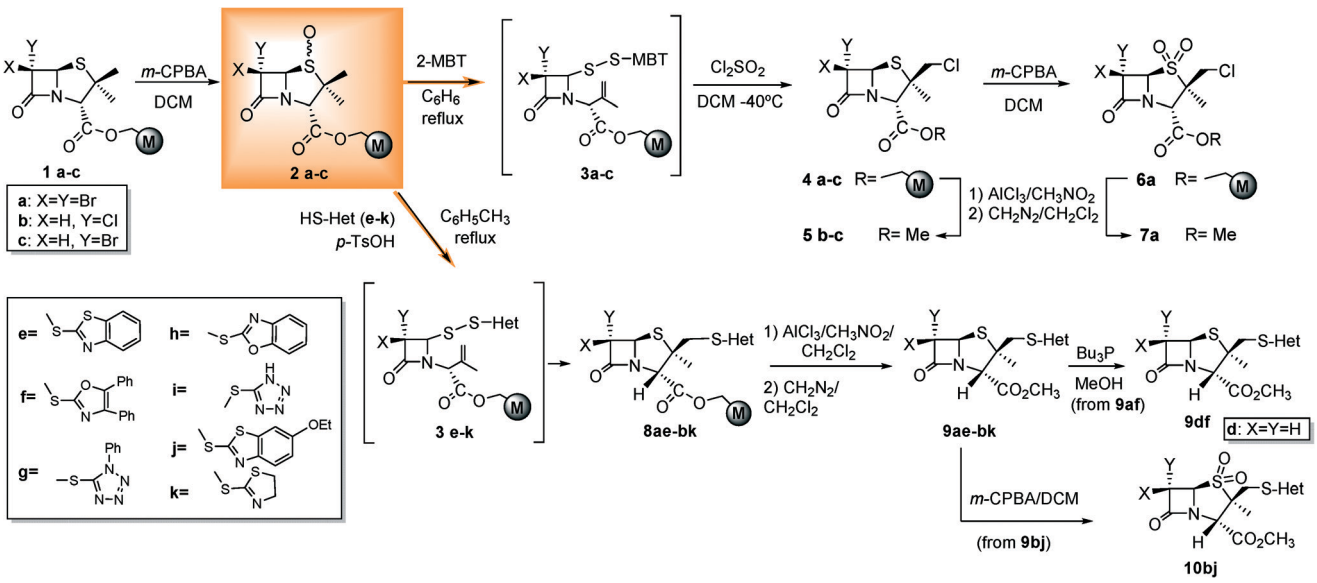
We have developed a convenient solid-phase synthetic sequence for the preparation of 2β-methyl substituted penam derivatives using the commercially available and cost-effective Merrifield resin.[19] The synthesis started with the immobilization of 6,6-dihalo- and 6α-halopenicillanic acids on the resin using standard methodology. Oxidation of the resin-bound penam sulfide (**1**, Scheme 1) under 1.5 equiv. of *m*-chloroperbenzoic acid (*m*-CPBA) leads to the sulfoxides (**2**). The key step for the generation of the 2β-methyl substituted penicillins is the thermal rearrangement of the corresponding sulfoxides.[20] For the generation of the 2β-chloromethyl penam derivatives, 6,6-dihalo- and 6α-halopenicillanate sulfoxides tethered to the Merrifield resin (**2**) were treated with 1.5 equiv. of 2-mercaptobenzothiazole (2-MBT) and heated in refluxing benzene, to give the unsymmetrical monobactam disulfides (**3**). Then, the penam system is re-established by addition of sulfuryl chloride in dichloromethane at −40 °C, affording the immobilized 2β-chloromethyl penicillanates (**4a–c**).[21] Final products were obtained by treatment with aluminum chloride[21] followed by esterification with diazomethane, to give the methyl 6,6-dihalo- and 6α-halo-2β-chloromethyl penicillanates (**5b–c**) in good overall isolated yield (see Table 1). Alternatively, sulfone **7a** was obtained by oxidation of sulfide **4a** with an excess of *m*-CPBA, followed by cleavage and methylation, according to the procedure described above.

In the case of the preparation of 2β-(heterocyclyl)-thiomethyl penam derivatives, immobilized sulfoxides (**2**) were treated with excess of heterocyclic thiols (HS-Het) and catalytic amounts of *p*-toluenesulfonic acid (*p*-TsOH), in refluxing toluene.[22] Under these conditions and in the absence of an external nucleophile, the initially formed disulfide intermediates (**3e–k**) underwent an internal rearrangement, assisted by *p*-TsOH, to yield the 2β-(heterocyclyl)thiomethyl penams (**8ae–bk**). After cleavage, esterification and purification by column chromatography, the 2β-(heterocyclyl)thiomethyl penicillins (**9ae–bk**) were isolated, in good overall yields for the entire synthetic sequence (see Table 1). When **9af** was treated with tributylphosphine (Bu_3P) in methanol,[23] the dehalogenated product **9df** was obtained. On the other hand, sulfide **9bj** was treated with excess of *m*-CPBA to give the corresponding sulfone **10bj** in good yield.

Benzyl ester derivatives were synthesized by a solution-phase strategy (Scheme 2). The corresponding sulfoxide (**11a**) was treated with heterocyclic thiols (HS-Het) and *p*-TsOH, under the conditions described above. The benzyl 6,6-dibromo-2β-(heterocyclyl)thiomethyl penicillanates (**12**) were reduced by Bu_3P to give the dehalogenated derivatives (**13de–dk**). Otherwise, by treating **13de** with excess of *m*-CPBA, sulfone **14de** was obtained. Finally, compound **15a** was synthesized from **11a** according to a literature procedure.[22]

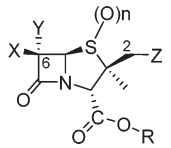
In vitro antiproliferative studies

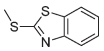
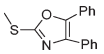
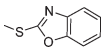
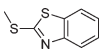
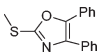
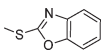
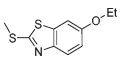
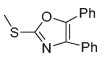
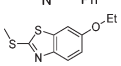
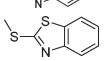
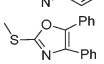
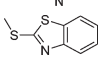
The *in vitro* antiproliferative activity of a library of twenty 2β-methyl substituted penicillin derivatives was initially evaluated at 20 μM concentration against epithelial cells derived from the normal mammary gland of mice (NMuMG) and fibroblast cells from mouse embryo (3T3) (Fig. 2). Based on results obtained for these non-neoplastic cells, only those compounds without toxicity towards non-cancer cells (*i.e.*, those that inhibited less than 50% cell proliferation in either NMuMG cells or 3T3 cells at 20 μM) were selected for



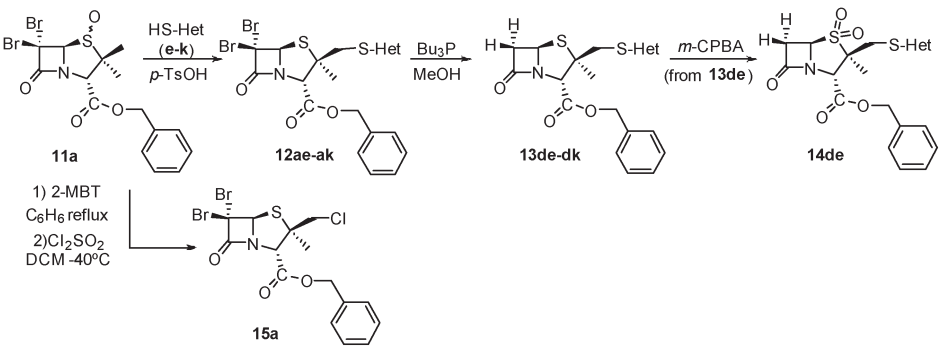
Scheme 1

Table 1 Library of 2β-methyl substituted penicillins



Entry	Compound	Y	X	Z	R	*n*	Yield[a] (%)
1	**5b**	Cl	H	Cl	Me	0	54
2	**5c**	Br	H	Cl	Me	0	48
3	**7a**	Br	Br	Cl	Me	2	37
4	**9ae**	Br	Br		Me	0	52
5	**9af**	Br	Br		Me	0	70
6	**9ag**	Br	Br		Me	0	43
7	**9ah**	Br	Br		Me	0	60
8	**9ai**	Br	Br		Me	0	52
9	**9be**	Cl	H		Me	0	40
10	**9bf**	Cl	H		Me	0	46
11	**9bh**	Cl	H		Me	0	50
12	**9bj**	Cl	H		Me	0	44
13	**9bk**	Cl	H		Me	0	48
14	**9df**	H	H		Me	0	32[b]
15	**10bj**	Cl	H		Me	2	34[c]
16	**13de**	H	H		Bn	0	46[d]
17	**13df**	H	H		Bn	0	17[d]
18	**13dk**	H	H		Bn	0	19[d]
19	**14de**	H	H		Bn	2	11[e]
20	**15a**	Br	Br	Cl	Bn	0	32[d]

[a] Obtained by solid-phase synthesis, unless otherwise stated. Yield after $AlCl_3$ cleavage and esterification with CH_2N_2, and calculated on the basis of the manufacturer's loading of the Merrifield resin. [b] Yield from **9bf** (solution-phase synthesis). [c] Yield from **9bj** (solution-phase synthesis). [d] Yield from compound **11a** (solution-phase synthesis). [e] Yield from **13de** (solution-phase synthesis).



Scheme 2

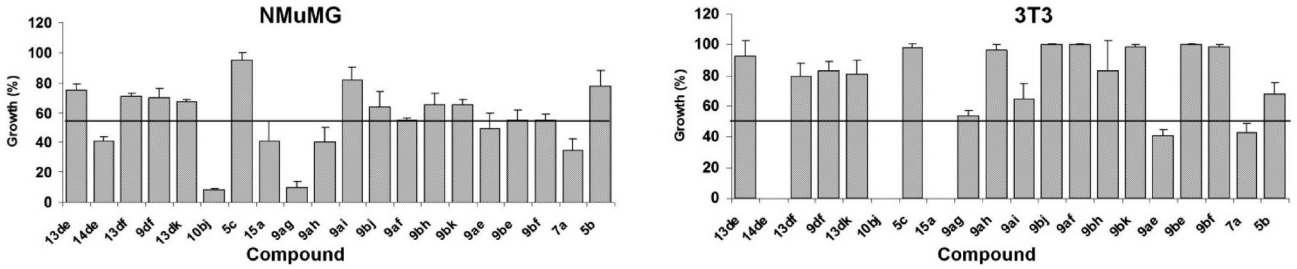


Fig. 2 Effect of synthetic penicillin derivatives on the proliferation of non-neoplastic cell lines. 2×10^4 cells per well (NMuMG and 3T3) were incubated in the presence or absence of 20 μM of different compounds for 72 h at 37 °C. Cell proliferation was determined by colorimetric determination of hexosaminidase levels.[24] Results are expressed as the percentage of growth obtained in the absence of compounds (control) and represented as mean ± S.E.M. of three different experiments. The antiproliferative activity of compounds **14de**, **10bj** and **15a** was not evaluated in 3T3 cells.

further studies. Thus, compounds **14de**, **10bj**, **15a**, **9ag**, **9ah**, **9ae** and **7a** were not tested in tumor cells. The antiproliferative effect of the remaining selected compounds was then determined in two human tumor cell lines (cervix HeLa adenocarcinoma; breast MCF-7 cancer) and two murine cell lines (LM3 mammary adenocarcinoma; B16-F0 melanoma) (Fig. 3). As shown in Fig. 3, the compounds that caused a reduction of ≥50% in the growth of tumor cell lines at 20 μM concentration were: **13de**, **13df**, **9df**, **9bj**, **9af**, **9bk**, **9be** and **9bf** (HeLa cells); **9df** and **9af** (MCF-7 cells); **9bj**, **9af** and **9be** (B16-F0); **9df**, **13dk**, **9bj**, **9af**, **9bk** and **9be** (LM3). IC_{50} values, defined as compound concentrations that produce 50% growth inhibition, were determined from dose–response curves only for these derivatives and are summarized in Table 2. Consequently, IC_{50} values corresponding to compounds that inhibited less than 50% tumor cell proliferation at 20 μM were

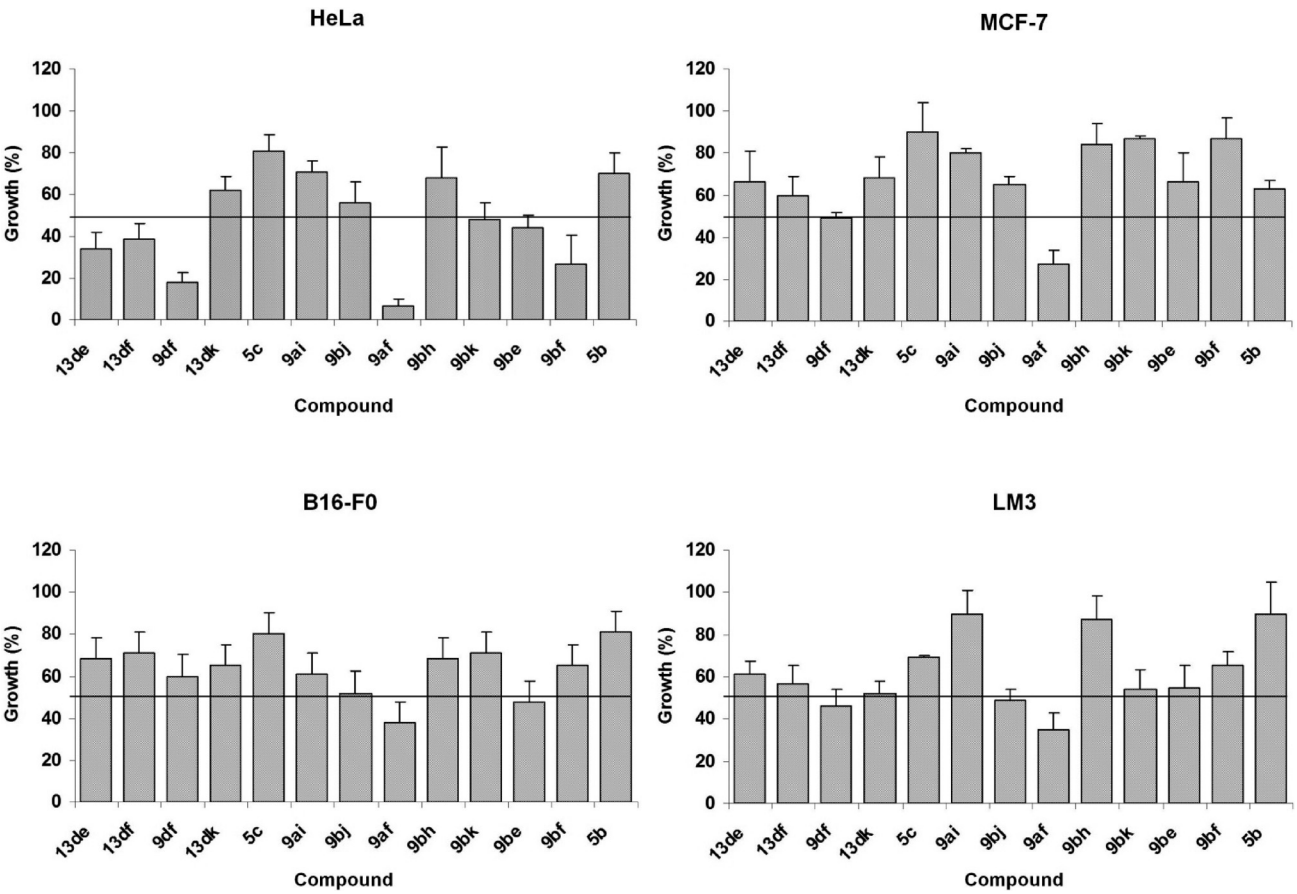
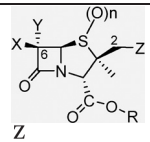
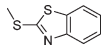
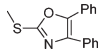
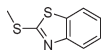
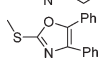
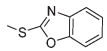
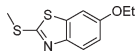
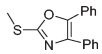
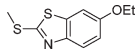
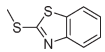
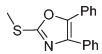
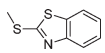


Fig. 3 Effect of some synthetic penicillin derivatives on the proliferation of different human and murine cell lines. 1×10^4 cells per well (B16-F0) or 2×10^4 cells per well (HeLa, MCF-7, LM3) were incubated in the presence or absence of 20 μM of different compounds for 72 h at 37 °C. Cell proliferation was determined by colorimetric determination of hexosaminidase levels.[24] Results are expressed as the percentage of growth obtained in the absence of compounds (control) and represented as mean ± S.E.M. of three different experiments.

Table 2 Molecular structures and antiproliferative effect of the compounds tested



Compound	Y	X	Z	R	*n*	Normal cell lines growth (%)		Tumoral cell lines growth (%)				IC_{50} values[a] (μM)					RP[b]			
						NMuMG	3T3	HeLa	MCF-7	B16-F0	LM3	HeLa[d]	MCF-7	B16-F0[d]	LM3	NMuMG	HeLa	MCF-7	B16-F0	LM3
5b	Cl	H	Cl	Me	0	78 ± 10	68 ± 8	70 ± 10	63 ± 4	81 ± 4	90 ± 15	—	—	—	—	—	ND	ND	ND	ND
5c	Br	H	Cl	Me	0	95 ± 5	98 ± 3	81 ± 8	90 ± 14	80 ± 7	69 ± 1	—	—	—	—	—	ND	ND	ND	ND
7a	Br	Br	Cl	Me	2	35 ± 7	46 ± 6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
9ae	Br	Br		Me	0	49 ± 11	41 ± 4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
9af	Br	Br		Me	0	55 ± 11	100 ± 1	7 ± 3	27 ± 7	38 ± 8	35 ± 8	7 ± 2	18 ± 1	11 ± 3	14 ± 3	31 ± 1	4.4	1.7	2.8	2.2
9ag	Br	Br		Me	0	10 ± 4	54 ± 3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
9ai	Br	Br		Me	0	82 ± 8	65 ± 10	71 ± 5	80±2	61 ± 6	90 ± 11	—	—	—	—	—	ND	ND	ND	ND
9be	Cl	H		Me	0	55 ± 7	100 ± 1	44 ± 6	66 ± 14	48 ± 5	55 ± 10	18 ± 2	—	18 ± 3	20 ± 2	36 ± 6	2	ND	2	1.8
9bf	Cl	H		Me	0	55 ± 4	99 ± 1	27 ± 14	87 ± 10	65 ± 9	65 ± 7	13 ± 4	—	—	—	32 ± 8	2.5	ND	ND	ND
9bh	Cl	H		Me	0	65 ± 8	83 ± 20	68 ± 15	84 ± 10	68 ± 2	87 ± 11	—	—	—	—	—	ND	ND	ND	ND
9bj	Cl	H		Me	0	64 ± 10	100 ± 11	56 ± 10	65 ± 4	52 ± 9	49 ± 5	24 ± 6	—	27 ± 8	24 ± 6	43 ± 4	1.8	ND	1.6	1.8
9bk	Cl	H		Me	0	65 ± 4	99 ± 1	48 ± 8	87 ± 1	71 ± 6	54 ± 9	19 ± 4	—	—	20 ± 1	41 ± 1	2.2	ND	ND	2
9df	H	H		Me	0	70 ± 6	83 ± 6	18 ± 5	49 ± 3	60 ± 5	46 ± 8	8 ± 1	20 ± 1	—	20 ± 1	35 ± 5	4.4	1.8	ND	1.8
10bj	Cl	H		Me	2	8 ± 1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
13de	H	H		Bn	0	75 ± 4	93 ± 10	34 ± 8	66 ± 15	68 ± 2	61 ± 6	11 ± 1	—	—	—	>80	7.3[c]	ND	ND	ND
13df	H	H		Bn	0	71 ± 2	80 ± 8	39 ± 7	60 ± 9	71 ± 5	57 ± 8	20 ± 3	—	—	—	>80	4[c]	ND	ND	ND
13dk	H	H		Bn	0	67 ± 2	81 ± 9	62 ± 7	68 ± 10	65 ± 5	52 ± 6	—	—	—	23 ± 4	>80	ND	ND	ND	3.5[c]
14de	H	H		Bn	2	41 ± 3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
15a	Br	Br	Cl	Bn	0	41 ± 13	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

The symbol (—) indicates the compounds that inhibited less than 50% cell growth at 20 μM concentration. ND: not determined. **9ah** was not tested in tumor cells (% growth, NMuMG: 40 ± 10; 3T3: 97 ± 3). [a] The molar drug concentrations required to cause 50% growth inhibition (IC_{50}) were determined from dose–response curves. Results are represented as means ± SEM of at least three different experiments. [b] The relative potency (RP) of a compound in a tumor cell line was calculated as the ratio of IC_{50} NMuMG to IC_{50} tumor cells. [c] Minimum value. [d] Reference standards: doxorubicin: 0.68 (HeLa), 8.05 (B16–BL6); cisplatin: 14.31 (HeLa).[25]

not evaluated. In order to establish the selectivity of the tested compounds, we decided to compare the IC_{50} values obtained in tumor cells with those obtained in NMuMG cells, the non-cancer cell line more susceptible to the cytotoxic effect of penicillin derivatives. Therefore, 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 to the IC_{50} value obtained in tumor cells. The higher this value, the greater the antitumor potency and selectivity is exhibited by a compound (Table 2). Based on RP values obtained, the most potent and selective penicillin derivatives (RP ~4–7) were compound **13de** followed by compounds **13df**, **9df** and **9af**. Within this group, compounds **13de** and **13df** are selective for HeLa cells, although **13de** showed a higher potency than **13df**. Furthermore, compounds **9df** and **9af**, showing a similar RP to compound **13df**, also exerted a slight antiproliferative effect in other tumor cell lines. In addition, compound **13dk** was moderately effective in LM3 cells. It should be considered that since compounds **13de**, **13df** and **13dk** could not be tested at concentrations higher than 80 μM due to their lower solubility in the culture medium, the RP values reported correspond to the minimal difference between the efficacy in NMuMG and tumor cells.

Compounds **9bj**, **9bk**, **9be** and **9bf** showed a slight and similar antitumor potency (RP ~2). However, although **9bj** and **9be** were effective in HeLa, B16-F0 and LM3 cells, **9bk** was selective for HeLa and LM3 cells, and **9bf** only for HeLa.

Compounds **5c**, **9ai**, **9bh** and **5b**, which were tested for being not cytotoxic in non-cancer cells (Fig. 2), showed no antiproliferative effect in the tumor cell lines herein studied.

Structure–activity relationships

Analysis of the data obtained in this study allowed us to draw some significant conclusions about the structure–activity relationships of this type of molecule, regarding their antiproliferative activity.

As shown in Table 2, derivatives having 2-mercaptobenzothiazole (**9be**, **9bj** and **13de**), 2-mercapto-4,5-dihydrothiazole (**9bk** and **13dk**) and 2-(methylthio)-4,5-diphenyloxazole (**9af**, **9bf**, **9df**, **13df**) at the 2β-methyl position of the penam nucleus showed the highest antiproliferative activity against cancer cells. Oxidation of the thiazolidine sulfur atom is directly related to cytotoxicity in normal cells (NMuMG). Thus sulfone derivatives **14de** and **10bj** affect the growth of normal cells NMuMG (inhibit approximately 60% and 90% growth rate, respectively) to a greater extent than sulfide derivatives **13de** and **9bj** (25% and 35%, respectively).

We have also noted that the introduction of a chlorine atom at the 2β-methyl position of the penam nucleus is detrimental to antitumor activity since those derivatives were either cytotoxic against normal cells or innocuous to both normal and tumor cells (**5c**, **15a**, **7a** and **5b**). The presence of the tetrazole heterocycle at the 2β-methyl position (**9ag** and **9ai**) is remarkably unfavorable for the antitumor activity of these compounds. Furthermore, the incorporation of a phenyl group at position 3 of the tetrazole ring showed an increase in cytotoxicity against normal cells; **9ag** inhibits 90% of cell growth in NMuMG and about 50% in 3T3 cell lines, while **9ai** inhibits those cell lines by approximately 20% and 35%, respectively.

Regarding position 6 of a penam nucleus, the presence of the halides appears to have no influence on the cytotoxic activity. Thus, dibromo derivative **9af** did not show any significant difference in activity compared to its dehalogenated analogue **9df**. No significant differences were observed in cytotoxicity between the two carboxylic acid protective groups of 2β-methyl penicillins, benzyl and methyl esters. This is exemplified by similar activity values obtained for compound **13dc** and its analogue **9dc**.

Conclusions

In conclusion, a series of 2β-methyl substituted penicillin derivatives were synthesized and the *in vitro* cytotoxic activities against four human tumor cell lines and two normal cells were evaluated. Some of the tested compounds showed satisfactory antitumor activity, especially compounds **13de**, **13df**, **9df** and **9af** which displayed higher values and good relative potencies. We have explored the structure–activity relationships and some general conclusions have been drawn. 2β-[(Benzothiazol-2-yl)thio]methyl and 2β-[(4,5-diphenyl-oxazol-2-yl)thio]methyl have shown to be the most promising penam derivatives of this series.

Our work demonstrates the possibility of obtaining new antitumor leaders by simple chemical transformations from a given molecular framework. The use of 2β-methyl substituted penicillin derivatives may help to discover new related compounds with potent and selective cell growth inhibitory activity which could be further tested as potential therapeutic agents for cancer treatment.

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