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Doxorubicin prodrug on the basis of *tert*-butyl cephalosporanate sulfones

Grigory Veinberg,* Irina Shestakova, Maxim Vorona, Iveta Kanepe, Ilona Domrachova and Edmunds Lukevics

Latvian Institute of Organic Synthesis, 21 Aizkraukles Street, Riga, LV 1006, Latvia

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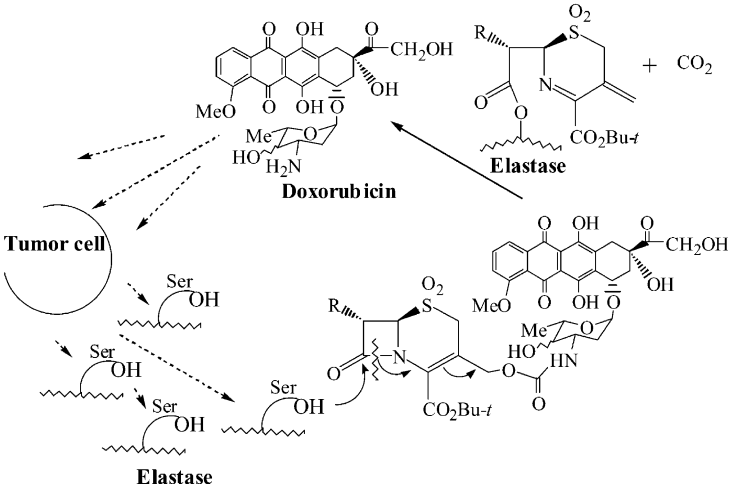
Abstract—Doxorubicin–cephalosporin prodrug adapted to the development of elastases for the liberation of parent drug was synthesized on the basis of cephalosporanate sulfone esters.

The design of doxorubicin containing prodrugs in general is focused on the creation of specific carriers selectively liberating cytotoxic agent in organism sites affected by cancer and preventing healthy ones from unnecessary toxic exposure. There are various approaches to solve this problem one of which presumes the covalent attachment of doxorubicin amino group to 3-carbonyloxymethyl moiety of cephalosporanic acids. The employment of monoclonal antibody (MoAb)-β-lactamase conjugate system to split cephalosporin molecule according to a specific mechanism confines the spread of expelling drug by the region chosen for antitumor treatment.[1–3]

Presented investigation was targeted at the exploration of the alternative type of doxorubicin–cephalosporin prodrug, which structural design was adapted to the involvement of elastases usually generated during metastasizing process (Scheme 1).[4]

Such a concept predetermined the selection of cephalosporanate sulfone esters acting as irreversible or



Scheme 1. The concept of elastase mediated splitting of doxorubicin-cephalosporin prodrug.

* Corresponding author. Tel.: +371-755-5946; fax: +371-755-0338; e-mail: veinberg@osi.lv

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reversible elastase inhibitors for the role of the drug carrier.[5] Target prodrugs **6a**,**b** containing an urethane bridge between doxorubicin and cephalosporin parts were obtained by three steps conversion of *tert*-butyl deacetoxycephalosporanate sulfones (**2a**,**b**) into their 3-(4-nitrophenoxy)carbonyloxymethyl derivatives **5a**,**b** capable to condense with **1**, employing already developed synthetic pathways (Scheme 2).[1,6,7]

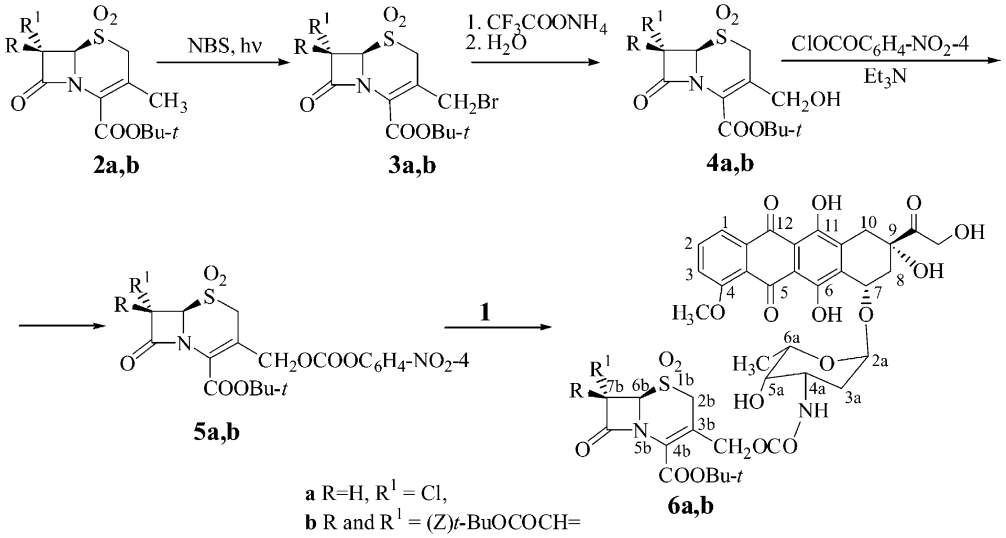
According to the HPLC data **6a** and **6b** did not contain even the traces of the parent drug. They were also stable at pH 7.5 in phosphate buffer at least for 24 h. Their treatment with slightly basic water solutions provided hydrolytic splitting of β-lactam ring and liberation of doxorubicin proved by TLC and HPLC.

Cytotoxic activity of doxorubicin, starting deacetoxycephalosporanate sulfones **2a**,**b** and prodrugs **6a**,**b** in vitro was tested on standard monolayer tumor cell lines: MG-22A (mouse hepatoma), HT-1080 (human fibrosarcoma), B16 (mouse melanoma) and Neuro 2A (mouse neuroblastoma) (Table 1).[8] Obtained IC_{50} data evidenced that prodrugs **6a**,**b** being less cytotoxic than doxorubicin (**1**) were much more potent in this respect than starting cephalosporanates **2a**,**b**.

The in vivo treatment of rapidly growing Sarcoma S-180 tumor transplanted in male JRC mice with prodrugs at small 1.5 mg/kg/day dosages failed.[8] The increase in a dosage up to 5 mg/kg/day positively changed the effectiveness of the treatment, which resulted in 30% and 22% tumor growth inhibition by prodrugs **6a** and **6b** reaching 80% and 60% of doxorubicin activity respectively (Table 2).

In the case of a more slowly growing melanoma B16 transplanted in male C57BL/6 mice the inhibition of tumor growth was achieved even at small dosage of prodrug **6a** (Table 2, Nos 1–3). Its enlargement up to 2 and 5 mg/kg/day increased the effectiveness of both prodrugs **6a** and **6b**, especially on the eleventh and fourteenth day (Table 2, Nos 4–6). However their final tumor inhibiting effect was 2 and 4 times weaker than in the case of doxorubicin. Specially carried out experiments showed that tumor growth in a control group treated with physiologic solution (PS) containing 2% DMSO was comparatively 10–15% faster than in the control group treated only with PS. The agarose additions to both solutions did not influence this process. It enabled to presume and then to prove experimentally (Table 2 Nos 7, 8) that the curing properties of **6a** could be more impressive if its effectiveness was estimated against the control group treated only with PS without DMSO additive.

A significant difference in the activity demonstrated by **6a** and **6b** during their in vivo testing against melanoma



Scheme 2. The synthesis of doxorubicin–cephalosporin prodrugs.

Table 1. Cytotoxic properties of doxorubicin, prodrugs and starting deacetoxycephalo-sporanate sulfones in vitro

Compd	Concentrations providing 50% cell killing effect IC_{50} (μg/mL)							
	HT-1080		MG-22A		B16		Neuro 2A	
	CV[a]	MTT[b]	CV	MTT	CV	MTT	CV	MTT
1	0.17	0.31	<0.001	0.73	0.001	<0.001	0.004	<0.001
2a	6	6	6	2	3	2	2	2
2b	8	7	3.4	3.0	>100	>100	>100	>100
6a	0.02	0.08	0.03	0.04	0.03	0.04	0.001	0.008
6b	0.4	0.5	0.07	0.07	0.09	0.5	0.04	0.04

[a] CV, crystal violet coloration.
[b] MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide coloration.

Table 2. Antitumor effect of doxorubicin and prodrugs in vivo

Nos	Compd[a]	Dosage[b] (mg/kg/day)	Tumor growth inhibition GI on fixed date (%)			
			S-180		B 16	
			7	9	11	16
1	**1**	1.5	7	28	70	60
2	**6a**	1.5	No effect		75	26
3	**6b**	1.5	No effect		13	−49[c]
4	**1**	5.0	35	37	65	75
5	**6a**	5.0	72	30	86	39
6	**6b**	5.0	−10[c]	22	11	16
7	**1**	2.0			25	70
8	**6a**	2.0[d]			86	56

[a] Doxorubicin was administered in physiological solution (PS, 0.9%NaCl, pH 6.2); prodrugs **6a**,**b** in PS containing 2% DMSO and 0.3% agarose additive.
[b] Administration schedule: 1, 2, 3, 4, 7 days.
[c] Negative data indicate the activation of tumor growth.
[d] The treatment of control group with physiological solution.

B16 evidences about the more pronounced involvement of doxorubicin in the curing process in the case of **6a**. That is why at the present stage of comparative biological studies we assume that compound **6a** to more extent than **6b** meets the requirements of the prodrug according to the mechanism proposed.

It is well known that one of the most serious toxic consequences of doxorubicin treatment is connected with its irreversible cardiotoxic action. That is why there were carried out the comparative studies of physiologic condition of cardiomyocytes after injections of doxorubicin and prodrugs in healthy mice and in mice with transplanted tumor. In both series of experiments cardiomyocytes were isolated from the treated animals on the 9th day,[10] and changes in the amount of their intracellular peptides and mitochondrial redox activity against control cells taken from the untreated animals were analysed using Coomassie Brilliant Blue (CB) and MTT coloration, respectively.

According to the CB test there was not observed a substantial difference in the amount of peptides in cardiomyocytes after the treatment of healthy mice with doxorubicin (**1**) and prodrugs **6a**,**b** at the same dosages. Contrary to it the MTT test revealed 60% decrease in mitochondrial redox activity after the long term exposure of cardiomyocytes to **1** contrasting with its 15% and 46% reduction in the case of **6a** and **6b** (Table 3). Such a specificity of the MTT test correlates with the known ability of doxorubicin to inhibit the oxidation of mitochondrial palmitate serving as a major source of energy in cardiomyocytes .[12] At higher dosages a similar decrease and even stimulation of redox activity in cardiomyocytes was observed after doxorubicin and **6b** administration.

Similar trends were detected in cardiomyocytes isolated from mice with transplanted Sarcoma S-180 and treated with doxorubicin and prodrugs **6a**,**b**. According to the MTT test they were characterized with abnormally high level of redox activity exceeding 2–3 times the respective one observed in healthy mice, however, preserving the same tendencies in the changes of its intensity (Table 3). Such an increase in redox activity regardless to the chemical nature of administered compounds could be explained as a specific response of cardiomyocytes to pathologic alterations caused by tumor transplantation.

Table 3. Physiological changes in cardiomyocytes after the treatment of male JRC mice with doxorubicin and prodrugs

Compd	Dosage[a] (mg/kg/day)	Physiologic changes in cardiomyocytes[a]			
		Healthy mice[b]		Mice with transplanted Sarcoma S-180[b]	
		CB	MTT	CB	MTT
1	1.5	83±6	40±1	90±6	114±10
6a	1.5	92±4	85±6	106±16	225±12
6b	1.5	87±2	54±6	92±4	270±12
1	5.0	60±6	39±4	81±6	130±10
6a	5.0	90±6	87±4	70±5	230±21
6b	5.0	75±4	102±7	90±4	360±12

[a] Administration schedule: 1, 2, 3, 4, 7 days.
[b] Cells isolated on the 9th day of experiment.

Obtained data evidence that the application of *tert*-butyl 7α-chlorocephalosporanate sulfone as a prodrug carrier provided **6a** with antitumor efficacy comparable with the parent drug both in vitro and in vivo. Its considerable lower cardiotoxicity correlates with the anticipated difference in the concentrations of prodrug splitting elastases in metastasizing and healthy organs. As a result their relatively smaller amount in animals heart prevented cardiomyocytes from unnecessary exposure to toxic doxorubicin.

References and notes

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7. Doxorubicin hydrochloride (69.5 mg, 0.12 mM) water solution (10 mL) was neutralized with 5% Na_2CO_3 and antibiotic was extracted with dichloromethane (50 mL). Organic phase was evaporated and the residue was redissolved in THF (4 mL). Obtained solution was added to

7α-chloro-3-(4-nitrophenyloxycarbonyloxymethyl)cephalosporanate 1,1-dioxide **5a** (63 mg, 0.12 mM) in THF (4 mL). After stirring for 24 h at room temperature target 7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-hydroxyacetyl-1-methoxy-10-[2,3,6-trideoxy-3-[[4-*tert*-butoxycarbonyl-7α-chloro-1,1-dioxo-ceph-3-em-3-yl]methoxycarbonylamino]-α-L-*lyxo*-hexopyranosyl]oxy]-5,12-naphtacenedione (**6a**) was isolated using column chromatography on Silicagel Merck (70–230 mesh) and dichloromethane–methanol (60:1) as eluent from fractions characterized with R_f 0.51 giving 74 mg (68%) of red crystalline powder. IR spectra: cm^{-1}: 3500, 3400, 1810, 1720, 1620, 1580 cm^{-1}. HPLC analysis: Symmetry C18 (3.9×150 mm), $v = 1$ mL/min. Mobil phase: acetonitrile (0.1 M)–phosphate buffer (pH 2.5) (70:30). Detector: UV 254 nm. Purity 97%. Retention time: 6.55 min.

^{1}H NMR ($CDCl_3$), δ: 2.05–2.18 (2H, m, 8-H_2); 3.60–3.95 (4H, m, 10-H_2, 9-$COCH_2O$); 4.05 (3H, s, 4-OCH_3); 4.45 (1H, br.s $CH_2O\underline{H}$); 5.20–5.28 (1H, m, 7-H); 5.50 (1H, m, 9-H); 7.35 (1H, d, 3H); 7.63 (1H, t, 2H); 8.12 (1H, br.s, 1H); 13.20 (1H, br.s, 11-OH); 13.96 (br.s, 6-OH) naphthacene fragment. 1.24 (3H, d, 6a-CH_3); 1.75–1.90 (2H, m, 3a-H_2); 3.62 (1H, br.s, 5a-H); 3.78 (1H, br.s, 4a-H); 4.09 (1H, s, 5a-OH); 4.21 (2H, d, 6a-H); 5.20–5.28 (1H, m, 2a-H) pyranosyl fragment. 1.50 (9H, s, t-C_4H_9); 2.40 (3H, s, $OCOCH_3$); 2.86, 3.18 (2H, AB-q, $J = 19$, 2b-H_2); 4.62, 5.02 (2H, AB-q, $J = 14$, 3b-CH_2OCO); 4.78 (1H, br.s, 6b-H); 5.24 (1H, br.s, 7b-H) cephem fragment.

7,8,9,10-Tetrahydro-6,8,11-trihydroxy-8-hydroxyacetyl-1-methoxy-10-[2,3,6-trideoxy-3-[[4-*tert*-butoxycarbonyl-7(Z)-*tert*-butoxycarbonylmethylene-1,1-dioxo-ceph-3-em-3-yl]methoxycarbonylamino]-α-L-*lyxo*-hexopyranosyl]oxy]-5,12-naphtacenedione (**6b**) was prepared from doxorubicin base and *tert*-butyl 3-(4-nitrophenyloxycarbonyloxy)methyl-7(Z)-*tert*-butoxycarbonylmethylenecephalosporanate 1,1-dioxide (**5b**) in the same manner as **6a**. Yield 84%. R_f 0.68 dichloromethane-methanol (60:1). IR spectra: cm^{-1}: 3400, 1790, 1720 cm^{-1}. HPLC analysis: Symmetry C18 (3.9×150 mm), $v = 1$ mL/min. Mobil phase: acetonitrile (0.1 M): phosphate buffer (pH 2.5) (70:30). Detector: UV 254 nm. Purity 95%. Retention time: 3.82 min.

^{1}H NMR ($CDCl_3$), δ: 2.00–2.18 (2H, m, 8-H_2); 3.74–4.10 (4H, m, 10-H_2, 9-$COCH_2O$); 4.03 (3H, s, 4-OCH_3); 4.44 (1H, br.s $CH_2O\underline{H}$); 5.24 (1H, m, 7-H); 5.52 (1H, br.s, 9-H); 7.18 (1H, d, 3H); 7.78 (1H, t, 2H); 8.03 (1H, br.s, 1H); 13.26 (1H, br.s, 11-OH); 14.00 (br.s, 6-OH) naphthacene fragment. 1.24 (3H, d, 6a-CH_3); 2.00–2.18 (2H, m, 3a-H_2); 3.63 (1H, br.s, 5a-H); 3.74 (1H, br.s, 4a-H); 4.06 (1H, s, 5a-OH); 4.20 (2H, d, 6a-H); 5.22 (1H, m, 2a-H) pyranosyl fragment. 1.55 (9H, s, *t*-C_4H_9); 2.38 (3H, s, $OCOCH_3$); 2.96, 3.11 (2H, AB-q, $J = 19$, 2b-H_2); 4.72, 5.07 (2H, AB-q, $J = 14$, 3b-CH_2OCO); 5.52 (1H, br.s, 6b-H); 6.54 (1H, br.s, =CH–) cephem fragment.

8. Methodology of in vitro and in vivo antitumor assays is presented in ref 9.
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