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Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 16 (2006) 3727–3730

Novel class of cyclophosphamide prodrug: Cyclophosphamide spiropiperaziniums (CPSP)

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Received 4 January 2006; revised 17 April 2006; accepted 20 April 2006
Available online 6 May 2006

Abstract—A novel class of cyclophosphamide spiropiperaziniums was synthesized and evaluated for their in vivo anti-cancer activities against S_{180} and H_{22}. Most of them exhibited definite activities. Especially, compounds **8b** and **8k** showed good anti-cancer activities, meanwhile, **8k** also showed much lower toxicity than CP. Several interesting structure–activity relationships were revealed.

Cyclophosphamide (CP, **1**) is a widely used anti-cancer agent that is dependent on cytochrome P_{450} metabolism for its therapeutic effectiveness. The pharmacology and chemistry of CP have been extensively studied and reviewed.[1–3] During the process of metabolism, CP liberates two important metabolites, phosphoramide mustard (PM, **2**) and acrolein. And then, PM is transported into cell to interfere with DNA that shows the anti-tumor activities. On the contrary, acrolein is responsible for hemorrhagic cystitis, a side effect observed during CP therapy. To reduce the side effect and find more potent anti-cancer drug, numerous modifications for CP have been performed over 50 years.[4–19] However, these extensive structure–activity relationship studies failed to produce better drugs than CP. Analysis of the known works revealed that nearly all the modifications were focused on the left part of CP, just as cyclic and acyclic phosphoramidate alkylating agents (**3** and **4**), and the mustard group still remained perfectly, because it was considered as a very key pharmacophore.

Nevertheless, spirobromine (**5**) with dispirotripiperazinium structure is also an alkylating agent that exhibited not only excellent anti-cancer activities and broad spectra, but also quite low toxicity (LD_{50} = 1980 mg/kg).[20] Furthermore, some recent works have been reported that the drug molecules incorporating quaternary ammonium moiety exhibited high in vivo target-function for cartilage, for example compounds **6** and **7**.[21]

Our group has been interested in the biological activities and synthesis of piperazine quaternary ammonium salts all along.[22,23] Inspired by above results, we envisioned that changing the mustard group of CP into spiropiperazinium structure might be an effective way for the modification of CP. Herein, we disclose the first report of novel class of cyclophosphamide spiropiperaziniums (CPSP, **8**), and some of them showed significant in vivo anti-tumor activities and low toxicity.[24]

According to our previously described procedure,[25] the compounds **8a**–**l** were prepared in good yields by the reaction of CP with the corresponding cyclic secondary amines or acyclic secondary amines in the presence of $NaHCO_3$ refluxing for 5–10 h (Scheme 1). The compound **8k** was treated with potassium bromide and potassium iodide, respectively, to give the corresponding compounds **8m** and **8n** (Scheme 2). All the compounds were purified by recrystallization and identified by NMR and elemental analysis.

Preliminary anti-tumor activities of all the newly synthesized compounds **8a**–**n** were assessed in vivo against Sarcoma 180 (S_{180}) and hepatocyte sarcoma 22 (H_{22})

Keywords: Prodrug; Cyclophosphamide; Cyclophosphamide spiropiperaziniums; Anti-cancer; Synthesis.
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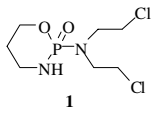
doi:10.1016/j.bmcl.2006.04.049



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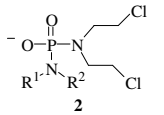

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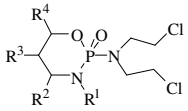

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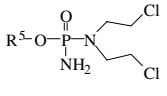

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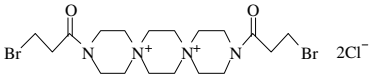
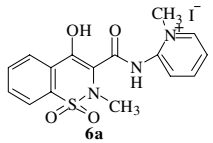
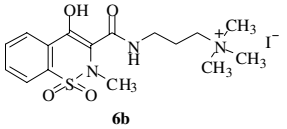


Spirobromine 5



6a



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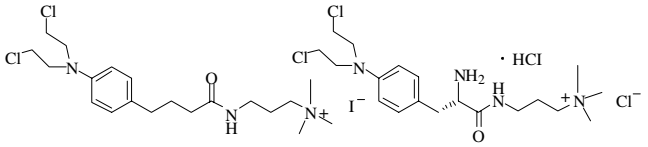
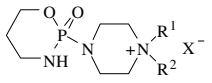


7a

7b



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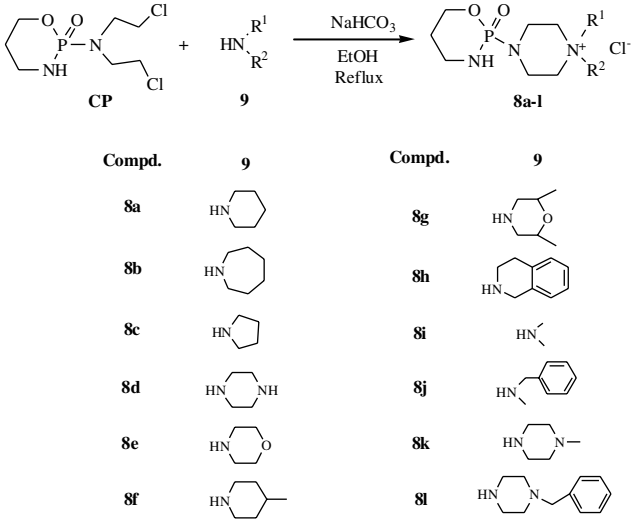


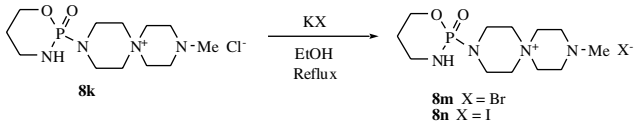
CP 9 8a-l

Compd.	9	Compd.	9
8a	HN	8g	HN O
8b	HN	8h	HN
8c	HN	8i	HN
8d	HN NH	8j	HN
8e	HN O	8k	HN N–
8f	HN	8l	HN N

Scheme 1. Synthesis of compounds **8a**–**l**.

in mice at the dose of 30 mg/kg, and the CP was used as control. The results are summarized in Table 1.

Because this kind of compound was designed as prodrug principle, all of them showed very poor in vitro



8k 8m X = Br 8n X = I

Scheme 2. Synthesis of compounds **8m** and **8n**.

Table 1. Preliminary anti-cancer activities of **8** against S_{180} and H_{22} (ip)

Compound	Dose (mg/kg)	Inhibition rate against S_{180} (%)	Inhibition rate against H_{22} (%)
CP	30	72.94***	72.02***
8a	30	39.86*	12.79
8b	30	35.98**	48.43**
8c	30	26.66	17.26
8d	30	10.70	8.64
8e	30	−27.61	32.49*
8f	30	−13.30	32.25*
8g	30	46.63**	20.60
8h	30	18.87	—
8i	30	33.47**	28.79*
8j	30	−3.28	−6.63
8k	30	42.31**	41.66***
8l	30	33.70**	—
8m	30	18.72	11.60
8n	30	29.41*	16.19

Mice (10 mice in group) were implanted subcutaneously (sc) with tumor cells, and the drugs were dosed (mg/kg) intraperitoneally (ip) one time daily for successive 10 days, the dissected sarcoma weights (g) were obtained precisely, and inhibition rates and *P* value were calculated using normal saline as control. Inhibition rate (%) = the average weight of control group (g)—the average weight of experiment group (g)/the average weight of control group (g) × 100%.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

anti-tumor activities. However, as shown in Table 1, most of them displayed definite in vivo activities against S_{180} and/or H_{22}. Especially, the compounds **8b** and **8k** showed the inhibition rates of 35.98% and 42.31% for S_{180}, and 48.43% and 41.66% for H_{22}, respectively.

From the biological results of compounds **8a**–**c**, it is found that size of terminal spirocycle significantly influences the activity against H_{22}. Such as, compound **8b** (seven-member ring) shows highest anti-cancer activity (48.43%); compounds **8a** (six-member ring) and **8c** (five-member ring) only show weak activities. However, its effect on the activity against S_{180} is slight.

In an attempt to explore the influence of incorporating heteroatom in the moiety of terminal spirocycle on the activity, the 9-CH_2 in **8a** was, respectively, replaced with –NH, O, –NMe, and –$NCH_2C_6H_5$ to afford the corresponding compounds **8d**, **8e**, **8k**, and **8l**. Biological results indicated that introduction of 9-NH (**8d**, 10.70% against S_{180} and 8.64% against H_{22}) led to a dramatic decrease of activity; however, 9-N-substituted analogues **8k** (9-NCH_3, 42.31% against S_{180} and 41.66% against H_{22}) and **8l** (9-$NCH_2C_6H_5$, 33.70% against S_{180}) showed similar to or better activities than

8a. It is suggested that substitution of hydrogen at 9-NH in compound **8d** with suitable group would improve the activity. Comparing with **8a**, after replacing 9-CH_2 with 9-O, compound **8e** exhibited better activity against H_{22} than **8a** and complete loss of activity against S_{180}.

Compounds **8f** and **8g**, methyl-substituted derivative of **8a** and dimethyl-substituted derivative of **8e** at carbon atoms of terminal ring, respectively, show interesting biological results. Comparing the **8f** with **8a**, introduction of methyl group led to the loss of activity against S_{180} (**8a**, 39.86%; **8f**, −13.30%) and increase of activity against H_{22} (**8a**, 12.79%; **8f**, 32.25%); on the contrary, for compound **8g**, after introduction of two methyl groups, the activity against S_{180} (**8e**, −27.61%; **8g**, 46.63%) was enhanced significantly and the activity against H_{22} (**8e**, 32.49%; **8g**, 20.60%) was slightly decreased. These results should be contributed from the change of conformation caused by introduction of substitute. Compound **8h** with benzospirocycle showed far lower biological activity (18.87% for S_{180}) than **8a** (**8a**, 39.86%), also demonstrating that the suitable conformation is very critical for the activity. Additionally, it is worth to note that compound **8g** showed the highest activity against S_{180} among the tested compounds. Because compound **8g** has two kinds of stereo-structures, *trans*-**8g** and *cis*-**8g**, one of them may be having more potent activity.

Though only the moiety of anion is different among compounds **8k**, **8m** and **8n**, they showed different activities against both S_{180} and H_{22}, just as, for S_{180}: **8k** (Cl^-, 42.31%) > **8n** (I^-, 29.41%) > **8m** (Br^-, 18.72%); for H_{22}: **8k** (Cl^-, 41.66%) > **8n** (I^-, 16.19%) > **8m** (Br^-, 11.60%). These results reveal that the anion of quaternary ammonium also influences the anti-tumor activity markedly.

As compared with spiropiperazinium derivatives of CP, we also synthesized two unspiropiperazinium derivatives of CP, **8i** ($R^1 = R^2 = Me$) and **8j** ($R^1 = Me$, $R^2 = C_6H_5CH_2$). Compound **8i** exhibited significant activity against both S_{180} (33.47%) and H_{22} (28.79%). However, compound **8j** did not show any activity. This result demonstrates that unspiropiperazinium derivatives of CP still have activity as long as selecting suitable substitutes connected with quaternary nitrogen atom.

Among the tested compounds, the most potent compounds are **8b** and **8k**. Therefore, we selected them to further study for their in vivo activities against S_{180} and H_{22} at three doses of 15, 30, and 60 mg/kg, and the results are summarized in Table 2. Both compounds **8b** and **8k** showed excellent dose–activity relationships. Though their activities are slightly weaker than that of CP, **8b** and **8k** did not significantly cause the body weight loss comparing with the NS group even at the dose of 60 mg/kg. However, the CP group's mice have lost the weight greatly than NS group.

In order to further examine their toxicity, the LD_{50} of compounds **8b** and **8k** were also tested and the CP as control. The result from Table 3 shows that the LD_{50} of **8k** (1202 mg/kg, ip) is more than three times higher and the LD_{50} of **8b** (239 mg/kg) is slightly lower comparing with that of CP (387 mg/kg).

In summary, we have designed and synthesized a novel class of cyclophosphamide spiropiperaziniums and evaluated for their in vivo anti-tumor activities against S_{180} and H_{22}. Most of compounds showed definite anticancer activity. Among them, compounds **8b** and **8k** are most potent which retained the similar anti-tumor activity as CP, meanwhile, the latter **8k** also showed much lower toxicity than CP. These results demonstrate that changing mustard group of CP into suitable spiropiperazinium structure is an effective way for the modification of CP. Several structure–activity relationships have been revealed that would be valuable for us to develop more potent anti-cancer agents.

Table 3. LD_{50} of CP, **8b**, and **8k** (ip)

	CP (mg/kg)	**8b** (mg/kg)	**8k** (mg/kg)
LD_{50}[a]	387.4 ± 33.8	239.4 ± 13.9	1202.0 ± 27.0

[a] Drugs were dosed (mg/kg) intraperitoneally (ip) to mice (five groups, 10 mice each) at different concentrations for successive 7–10 days. Death was recorded to calculate the LD_{50} value.

Table 2. Anti-cancer activities of **8b** and **8k** against S_{180} and H_{22} (ip)

Compound	Dose (mg/kg)	Inhibition rate against S_{180} (%)	Inhibition rate against H_{22} (%)	Weight (g) (X ± SD)
NS	—	—	—	12.09 ± 3.10
CP	30	78.85***	68.87***	8.58 ± 1.78
8b	15	38.16*	17.19	13.86 ± 3.03
	30	35.98**	48.43**	12.22 ± 1.69
	60	53.36**	51.3**	10.68 ± 2.00
8k	15	37.50*	24.43	10.88 ± 2.13
	30	42.31**	41.66**	12.02 ± 3.00
	60	55.22***	38.04*	13.10 ± 2.61

Mice (10 mice in group) were implanted subcutaneously (sc) with tumor cells, and the drugs were dosed (mg/kg) intraperitoneally (ip) one time daily for successive 10 days, the dissected sarcoma weights (g) were got precisely, and inhibition rates and *P* value were calculated using normal saline as control. Inhibition rate (%) = the average weight of control group (g)—the average weight of experiment group (g)/the average weight of control group (g) × 100%.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

Acknowledgment

This work was supported by the funds of National Natural Science Foundation of China (NSFC 20472008).

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