Study on Anti-Proliferative Activity in Cancer Cells and Preliminary Structure–Activity Relationship of Pseudo-Peptide Chiral Thioureas

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In our previous studies, we have shown that thiourea compounds containing phosphate esters have potent antitumor activity and can be used as a novel strategy for the development of antitumor agents. Herein, a series of novel phosphonate thioureas **5–38** have been synthesized, which were fully characterized by ^{1}H NMR, ^{13}C NMR spectrum, elemental analysis. Three human cancer cell lines (Bcap-37, BGC-823, and PC-3) have been used to investigate these compounds' antitumor activities. After the summarization of the structure–activity relationships, we found that the variation of R, R_1, and R_2 in these novel phosphonate thioureas contribute to the antitumor activities. All these SAR-guided efforts may lead to novel antitumor drugs in the market in the near future.

Keywords: Thiourea, Phosphonate, Pseudo-peptide, Antitumor agents, Structure–function relationship, Human cancer cell lines

Introduction

The thioureas belong to the urea derivatives, which demonstrate a wide range of bioactivities including antimicrobial,[1,2] antiviral,[3,4] and antitumor activities.[5–8] Among the urea derivatives, the thiourea derivatives are most if not all attractive for the scientist according to their diverse anticancer activity against various leukemias and solid tumors.[9,10] Besides, the increasing evidence show that the thioureas derivates are potent anticancer agents because of their potent inhibition against DNA topoisomerase,[11–13] protein tyrosine kinase (PTKs),[14–18] receptor tyrosine kinases (RTKs),[19] carbonic anhydrase[20] and sirtuins,[21,22] etc. These important findings will continuously encourage people to pursue the novel design of thiourea compounds as anticancer drug candidates. Previously, people show that the thioureas conjugated with amino acids can generate series of newly bioactive molecules.[10] By using the combination principles of medicinal chemistry, we also developed pseudo-peptide thiourea derivatives containing α-aminophosphonate moiety as potential anticancer agents,[23] which generally consist of three parts, like α-aminophosphonate, thiourea, and amino acid. Based on our previous results, we are sure that thiourea and α-aminophosphonate are very important pharmacophores in these derivatives. Additionally, fluorinated derivatives of *D*-IIe and *L*-IIk show the comparable cytotoxic potency with Adriamycin (ADM) in PC-3 cells and 6,7-dimethoxy-*N*-(3-bromophenyl)-4-aminoquiazoline (PD153035) in BGC823 cells, respectively.[23] It is most likely to discover more potent anticancer agents by further varying the part of amino acid and keeping the parts of α-aminophosphonate and thiourea unchanged. Thus, We would like to update a series of newly synthesized phosphonate thioureas (Figure 1), their anticancer activities and structure–activity relationships.

Herein, a series of novel phosphonate thioureas **5–38** have been synthesized, which were fully characterized by ^{1}H NMR, ^{13}C NMR spectrum, elemental analysis. Three human cancer cell lines (Bcap-37, BGC-823, and PC-3) have been used to investigate these compounds' antitumor activities. All these novel phosphonate thioureas have demonstrated comparable antitumor activities compared to a commercial anticancer drug, Adriamycin (ADM). After the summarization of the structure–activity relationships, we found that the variation of R, R_1, and R_2 in these novel phosphonate thioureas contribute to the antitumor activities.

Experimental

Materials. The reagents used were analytically pure, from Aldrich or Acros (Waltham, MA, USA), and were treated in a conventional manner prior to use. The melting points were determined by the X-4 type microcalorimetric analyzer (the thermometer is not corrected). IR data were measured by a Shimadzu IR Prestige-21 type Fourier transform infrared spectrometer (manufactured by Shimadzu Corporation,

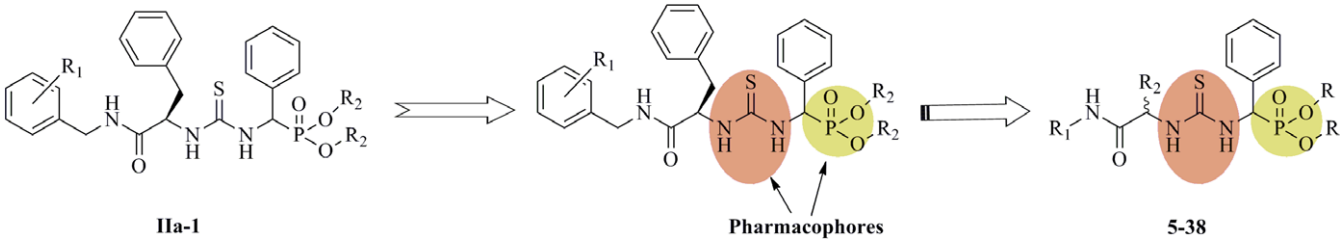

Figure 1. The design of novel phosphonate thioureas.

Kyoto, Japan) (KBr tablet); ^{1}H, ^{13}C, ^{19}F, ^{31}P NMR data were measured by JEOL-ECX400 type 400 (101, 162, 376) MHz NMR (Chiyoda, Japan) (TMS as internal standard, $CDCl_3$ or DMSO-d6 as solvent); elemental analysis data were determined by Elementar Vario type element analyzer; optical rotation was determined by WZZ-2A automatic polarimeter (Shanghai, China). TLC analysis was performed using silica gel GF254.

PC-3, BGC-823, and Bcap-37 cells were adherent cells and cultured in RPMI 1640 containing 10% (V/V) fetal bovine serum (FBS) and high glucose DMEM medium at 37°C, 5% CO_2 in a saturated humidity incubator, 2 days for a culture medium, 4–6 days pass a generation, take the logarithmic growth phase cells as the experimental object.

Synthesis. The Boc-protected amino acid **2** (5 mmol, 1.0 equiv) and the condensing agent benzotriazole-*N, N, N′, N′*-tetramethyluronium hexafluorophosphate (HBTU, 5 mmol, 1.0 equiv) were added to a 100 mL dry three-necked flask, then injection 30 mL freshly distilled dry CH_2Cl_2. After stirring at room temperature for 5 min, *N, N*-diisopropylethylamine (DIPEA, 10 mmol, 2.0 equiv) and amine **1** (5.5 mmol, 1.1 equiv) were added thereto. The reaction mixture was stirred at room temperature, after 0.5 h, the reaction system gradually became clear from the turbidity and the TLC was followed by the disappearance of the amine. When the reaction was complete, the reaction solution was dissolved in 40 mL of CH_2Cl_2 then transferred to a 150 mL separatory funnel and washed with 1 N HCl (3 × 20 mL), and the organic phase dried over anhydrous sodium sulfate and concentrated to give a pale yellow oil. The yellowish oil was dissolved with 30 mL of CH_2Cl_2 and transferred to a 100 mL three-necked flask, injected 75 mmol of trifluoroacetic acid (TFA) under ice bath and stirred for 1–2 h at room temperature. The reaction solution was diluted with 30 mL of methylene chloride and then washed with a saturated aqueous solution of sodium carbonate (3 × 50 mL), the aqueous layer was extracted with dichloromethane (3 × 50 mL) and the combined organic phases were dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure. The crude product was purified by flash column chromatography on silica gel (elution solvent: 4% MeOH/CH_2Cl_2, V/V) to afford intermediate **3**.

Dissolved intermediate **3** (1 mmol, 1.0 equiv) into THF (10 mL) and anhydrous DIPEA was added to the solution. After stirring at room temperature for 5 min, *O, O*-dialkyl isothiocyanate (phenyl) methyl phosphonate **4** (1 mmol, 1.0 equiv) was added slowly using a constant pressure dropping funnel. After completion of the dropwise addition, the reaction was stirred at room temperature for 1–2 h. The crude product was purified by column chromatography (developing solvent, dichloromethane/methanol) to give the target compound as a yield of 82–98%. All the target compounds were confirmed by IR, ^{1}H NMR, ^{13}C NMR and elemental analysis.

Anti-proliferative activity in cancer cells. The inhibitory rate of the compounds on cancer cells PC-3, BGC-823, and Bcap-37 was determined by MTT colorimetric method with DMSO as the reference. The logarithmic growth phase cells were inoculated into 96-well culture plates at 2×10^4 cells/mL per well in 100 μL of RPMI 1640 or DMEM medium containing 10% FBS, the rightmost column as a blank control group, pulsed serum RPMI 1640 medium without cells. The cells were incubated for 24 h in a saturated humidity incubator at 37°C and 5% CO_2. Removing the medium, added 200 μL complete medium with different concentrations of the compounds per well, blank control group per hole plus 200 μL complete medium, noted that the final concentration of DMSO in the medium cannot exceed 0.1%. According to the exposure time of the experimental requirements, removed the supernatant, then added 100 μL/well of 0.5 mg/mL MTT. After incubation for 4 h, added 10 μL/well of 10% SDS. After 10 h at 37°C, the crystals were fully dissolved and removed for micro oscillation 5 min, placed at room temperature for 30 min, then measured the OD values at the wavelength of A595. The results were analyzed by SPSS software and the cell viability and inhibition rate were calculated.

Results and Discussion

According to our previous report,[23] the synthetic route of novel phosphonate thioureas **5–38** was shown in Figure 2. We started from different substituted benzylamine analogues **1** and t-butyloxy carbonyl (Boc)

protected α-amino acid **2** to synthesize the compound **3** under the conditions of adding the coupling reagent *O*-benzotriazol-1-yl-*N, N, N′,N′*-tetramethyluronium hexafluorophosphate (HBTU) and the racemization inhibitor of 1-hydroxybenzotriazole (HOBt), followed by deprotection of trifluoroacetic acid (TFA). The desired phosphonate thioureas **5–38** were achieved by the addition of the isothiocyanate **4**[23] to the intermediate **3** in tetrahydrofuran (THF) at room temperature.

By this general protocol,[23] we have introduced different amino acids with *L*- or *D*- configuration into the thiourea pseudo-peptides, such as phenylalanine (R_2 = benzyl, Bn), leucine (R_2 = *iso*-butyl, *i*Bu), *tert*-leucine (R_2 = *tert*-butyl, *tert*-Bu). On the other hand, we conjugated benzyl amine (-R_1 = benzyl, Bn) or fluorinated benzyl amine (R_1 = *ortho*-fluorinated benzyl, *o*-FBn, or *para*-fluorinated benzyl, *p*-FBn) to C-terminal of these amino acids. Besides, we have also investigated the influence of different α-aminophosphonates, like ethyl (R = Et), *n*-propyl (R = *n*-Pr), *iso*-propyl (R = *i*-Pr), and *n*-butyl (R = *n*-Bu) α-aminophosphonates on antitumor activities. Eventually, we obtained 34 novel thiourea pseudo-peptides in total with excellent yields. The structure, yield and melting point of all the synthesized compounds are given in Table 1. All the synthesized compounds were tested by ^{1}H NMR, ^{13}C NMR, IR, and elemental analysis (see Appendix S1, Supporting information).

Three human cancer cell lines of PC-3, BGC-823, and Bcap-37, which are prostate cancer, stomach cancer, and breast cancer cells, respectively, were used to test the antitumor activity of phosphonate thiourea **5–38** with the final concentration of 10 μM by comparing with the listed anticancer drugs, adriamycin (ADM). As shown in Table 2, was measured by the MTT method, phosphonates thiourea **5–38** showed good anti-proliferative activities for PC-3, BGC-823, and Bcap-3 with a range of 12.6–73.4%, 11.1–89.1%, and 13.6–77.2%, respectively. Compounds **6**, **14**, and **25** demonstrate moderate to potent inhibitory activities (from 51.2 to 89.1%) among tested three human cancer cell lines. Compounds **6**, **14**, **23**, **25**, and **34** showed more than 50% inhibition against PC-3 cells, while compounds **6**, **14**, and **25** showed more than 50% inhibition against both of BGC-823 and Bcap-37 cells. The rest compounds only showed lower inhibition against these human cancer cell lines. Notably, compound **25** exhibited the comparable inhibitory potency to that of ADM against stomach cancer cell line, BGC-823 (89.1 vs. 93.8% at 10 μM).

Table 1. Structure, yield and melting point of novel thiourea pseudo-peptides.

Compd.	R_1	R_2	R	Yield (%)	Mp (°C)
5	Bn	*L*-Bn	Et	95	57–59
6	Bn	*D*-Bn	Et	94	56–58
7	Bn	*L*-Bn	*n*-Pr	93	42–44
8	Bn	*D*-Bn	*n*-Pr	92	43–44
9	Bn	*L*-Bn	*i*-Pr	90	50–52
10	Bn	*D*-Bn	*i*-Pr	93	49–51
11	Bn	*L*-Bn	*n*-Bu	86	—
12	Bn	*D*-Bn	*n*-Bu	84	—
13	*o*-FBn	*L*-Bn	Et	96	42–43
14	*o*-FBn	*D*-Bn	Et	94	41–42
15	*o*-FBn	*L*-Bn	*n*-Pr	90	—
16	*o*-FBn	*D*-Bn	*n*-Pr	92	—
17	*o*-FBn	*L*-Bn	*i*-Pr	93	39–40
18	*o*-FBn	*D*-Bn	*i*-Pr	87	38–40
19	*o*-FBn	*L*-Bn	*n*-Bu	87	—
20	*o*-FBn	*D*-Bn	*n*-Bu	86	—
21	*p*-FBn	*L*-Bn	Et	96	55–57
22	*p*-FBn	*D*-Bn	Et	95	53–55
23	*p*-FBn	*L*-Bn	*n*-Pr	93	44–45
24	*p*-FBn	*D*-Bn	*n*-Pr	87	43–45
25	*p*-FBn	*L*-Bn	*i*-Pr	96	47–48
26	*p*-FBn	*D*-Bn	*i*-Pr	90	46–49
27	*p*-FBn	*L*-Bn	*n*-Bu	89	—
28	*p*-FBn	*D*-Bn	*n*-Bu	82	—
29	Bn	*L*-*i*Bu	Et	94	43–45
30	Bn	*L*-*i*Bu	*n*-Pr	89	—
31	Bn	*L*-*i*Bu	*i*-Pr	95	51–53
32	Bn	*L*-*i*Bu	*n*-Bu	86	—
33	*p*-FBn	*L*-*i*Bu	Et	94	31–32
34	*p*-FBn	*L*-*i*Bu	*i*-Pr	90	43–44
35	Bn	L-*tert*Bu	Et	98	157–159
36	Bn	L-*tert*Bu	*n*-Pr	93	171–172
37	Bn	L-*tert*Bu	*i*-Pr	96	176–177
38	Bn	L-*tert*Bu	*n*-Bu	98	122–124

Encouraged by this, we picked up compounds **6**, **14**, **23**, and **25** further testing its effectiveness at various concentrations. As shown in Table 3, the inhibition of these four compounds against PC-3 cells was very potent. The IC_{50} value of compounds **6**, **14**, **23**, and **25** were 11.3, 6.1, 15.6,

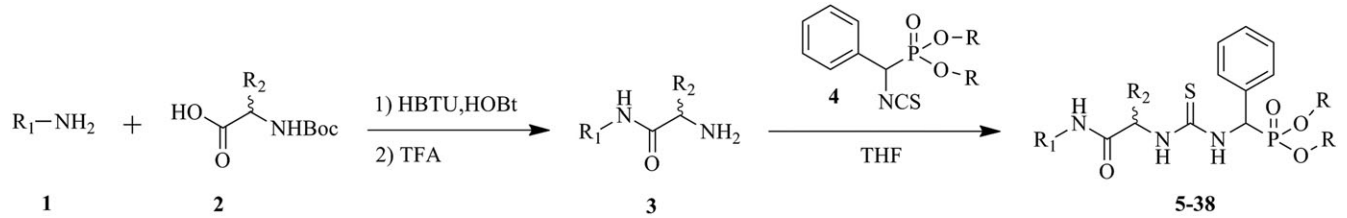

Figure 2. The synthetic route of novel phosphonate thioureas.

Table 2. Anti-proliferation activities of phosphonate thioureas **5–38** against three human cancer cell lines.[a]

Compd.[b]	Inhibition ratio (%)		
	PC-3[c]	BGC-823[d]	Bcap-37[e]
5	20.1 ± 5.1	18.3 ± 8.1	16.9 ± 10.9
6	55.9 ± 7.3	67.8 ± 10.0	52.5 ± 4.3
7	33.5 ± 3.3	35.7 ± 4.5	35.4 ± 4.7
8	30.8 ± 5.2	24.4 ± 11.5	49.1 ± 7.2
9	50.1 ± 8.3	27.8 ± 4.0	34.5 ± 6.5
10	30.5 ± 4.9	29.0 ± 10.9	46.6 ± 8.9
11	22.8 ± 2.4	21.1 ± 11.6	24.4 ± 4.1
12	21.7 ± 2.3	22.7 ± 12.9	22.9 ± 8.4
13	19.3 ± 3.4	30.4 ± 3.8	25.9 ± 4.7
14	73.4 ± 1.7	54.6 ± 3.7	77.2 ± 3.9
15	18.1 ± 5.1	11.1 ± 26.3	44.7 ± 9.2
16	30.6 ± 2.3	11.8 ± 24.3	17.2 ± 4.8
17	26.8 ± 2.5	47.9 ± 5.0	50.4 ± 18.1
18	14.3 ± 1.1	51.6 ± 5.0	27.8 ± 8.1
19	22.5 ± 2.3	31.2 ± 21.7	32.1 ± 7.8
20	18.0 ± 4.4	30.1 ± 21.7	34.0 ± 7.4
21	27.9 ± 2.9	22.9 ± 7.4	26.1 ± 11.7
22	32.4 ± 5.3	44.5 ± 12.6	35.0 ± 8.6
23	56.7 ± 6.7	21.0 ± 6.3	24.0 ± 16.4
24	30.6 ± 7.3	53.9 ± 0.1	13.6 ± 8.4
25	58.6 ± 8.1	89.1 ± 1.2	53.1 ± 9.2
26	34.8 ± 2.3	20.8 ± 14.7	27.0 ± 5.8
27	19.7 ± 3.7	26.1 ± 9.5	48.4 ± 6.7
28	33.6 ± 0.3	33.2 ± 4.2	35.8 ± 7.3
29	31.8 ± 2.2	—	—
30	16.2 ± 0.6	—	—
31	34.4 ± 1.3	—	—
32	18.3 ± 0.7	—	—
33	49.4 ± 4.2		
34	51.2 ± 3.1	61.5 ± 0.6	47.9 ± 4.1
35	26.9 ± 1.3	—	—
36	12.6 ± 3.4	—	—
37	29.1 ± 2.0	—	—
38	15.7 ± 1.4	—	—
ADM	90.5 ± 10.2	93.8 ± 2.1	92.7 ± 4.3

[a] MTT assay.
[b] 10 μM for all tested compounds.
[c] Prostate cancer cells.
[d] Stomach cancer cells.
[e] Breast cancer cells.

13.0 μM, respectively. They all presented the approximate inhibition with that of PD153035 against PC-3 cells (IC_{50} = 13.7 μM). The best one was compound **14**, which showed the highest inhibitory activity (IC_{50} = 6.1 μM) close to that of ADM against PC-3 cells (IC_{50} = 3.2 μM). Additionally, the inhibition of compound **25** was a slightly better compared to that of commercial anticancer drug, PD153035 against both PC-3 and BGC-823 cells. The IC_{50} value of compound 25 against PC-3 cells was 13.0 μM compared to the 13.7 μM of PD153035, while the IC_{50} value of compound 25 against BGC-823 cells was 4.8 μM compared to the 6.9 μM of PD153035.

Table 3. IC_{50} values of selected compounds in PC-3 and BGC-823.[a]

Compound	IC_{50} (μM)[b]
6	11.3 ± 0.9[c]
14	6.1 ± 0.1[c]
23	15.6 ± 1.9[c]
25	13.0 ± 2.1[c]
ADM(adriamycin)[d]	3.2 ± 0.8[c]
PD153035[d]	13.7 ± 1.4[c]
25	4.8 ± 0.0.4[e]
PD153035[d]	6.9 ± 1.1[e]

[a] The data represented the mean of three experiments in triplicate and were expressed as means ±SD.
[b] The IC_{50} value was defined as the concentration at which 50% survival of cells was observed.
[c] For PC-3 cells.
[d] Control drug.
[e] For BGC-823 cells.

Conclusion

According to the data from Table 2, we summarized the structure–activity relationship (SAR) of this series of novel thiourea pseudo-peptides shown in Figure 3. In general, the configuration of amino acid in the desired compounds was not obviously effective on the antitumor activity. However, *D*-configurations in certain cases show better inhibition (more than three folds) than that of L-configurations in these three cancer cell lines (**6** vs. **5** or **14** vs. **23** in Table 2). The desired compounds when *D*- or *L*- phenylalanine conjugated demonstrated better cellular inhibitions compared with those conjugated with *L*-leucine or 4-methyl *L*-leucine. In general, in the *D*-configuration, when R_1 = Bn or *o*-FBn, the compounds containing ethyl group of phosphate ester presented better anti-proliferation activities in tested cell lines than those containing bulky group like propyl, *iso*-propyl or butyl (**6** vs. **8**, **10,** and **12** or **14** vs. **16**, **18,** and **20** in Table 2). However, in the *L*-configuration, when R_1 = *o*-FBn or *p*-FBn, the compounds containing *iso*-propyl group showed better anti-proliferation

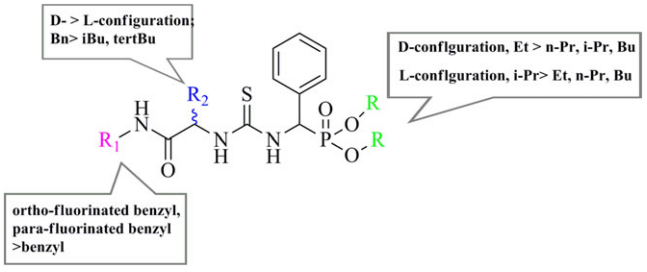

Figure 3. The structure–activity relationship (SAR) of these phosphonate novel thioureas.

activities in tested cell lines than other compounds containing ethyl, propyl, and butyl (**17** vs. **13**, **15,** and **19** or **25** vs. **21**, **23,** and **27** in Table 2); When R_1 = Bn, the tendency is observed only in the case of PC-3 cells (**9** vs. **5**, **7,** and **11** in Table 2). In most cases, the compounds conjugated with fluorinated benzyl amine (R_1 = *o*-FBn and *p*-FBn) demonstrated the comparable anti-proliferation activities to that of those compounds with non-fluorinated benzyl (R_1 = Bn). But compound **14** (R_1 = *o*-FBn) has the best inhibitory activity on PC-3 (73.4% inhibition at 10 μM, Table 2) and Bcap-37 cells (77.2% inhibition at 10 μM, Table 2). Compound **25** (R_1 = *p*-FBn) show the best anti-BGC-823 cells activity (89.1% inhibition at 10 μM, Table 2).

In conclusion, the configuration of α-amino acids, the types of amino acids and the alkyl structures of phosphonates in these compounds all have an influence on the anti-proliferative activity in cancer cells. The presence of fluorine-containing groups in the amino acid amide structure may enhance the anti-cancer activity of these compounds.

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Conflict of Interest. The authors declare no conflict of interest.

Supporting Information. Additional supporting information is available in the online version of this article.

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