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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Antitumor activities of some new 1,3,2-oxaza- and 1,3,2-diazaphosphorinanes against K562, MDA-MB-231, and HepG2 cells

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Abstract New X-substituted 1,3,2-oxazaphosphorinanes, where X = NHC_6H_5 (**1**), $NHC_6H_4S(O)_2NH_2$-4 (**2**), $NHC_6H_4OCH_3$-4 (**3**), $NHC_6H_4NO_2$-4 (**4**), $OC_6H_4CH_3$-4 (**5**), $NHC(O)C_6H_4NO_2$-4 (**6**), plus one X-substituted 1,3,2-diazaphosphorinane, where X = $NHC_6H_4S(O)_2NH_2$-4 (**7**), were synthesized and characterized by NMR, IR spectroscopy, and elemental analysis. The antitumor activities of these compounds, cyclophosphamide (CP), sulfanilamide (SA), and two X-substituted 5,5-dimethyl-1,3,2-diazaphosphorinanes, where X = NHC_6H_5 (**8**) $OC_6H_4CH_3$-4 (**9**), were evaluated by cell culture on K562, MDA-MB-231, and HepG2 cell lines using MTT cell proliferation assay. The IC_{50} values for CP and compounds **1**–**9** were in the range of 0.06 μM (for inhibition of HepG2 cells by compound **3**) to 3.17 μM (for inhibition of HepG2 cells by compound **8**). It was found that compounds **2** and **7** containing sulfonamide substituent and also SA itself are the best candidates for antitumor activity very close to CP.

Keywords 1,3,2-Oxazaphosphorinane · Antitumor activity · MTT assay · K562 · MDA-MB-231 · HepG2

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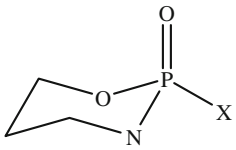
Introduction

Oxazaphosphorinanes are widely used as antineoplastic and immunosuppressive agents that are characterized by a relatively high oncologic specificity (Chen and Waxman, 1995). Cyclophosphamide (CP) prodrug is an oxazaphosphorinane that is activated by liver cytochrome P450 enzymes by a metabolic pathway that ultimately yields the alkylating agent phosphoramide mustard (Schwartz and Waxman, 2001). The parent compound is inactive in vitro and in vivo and exerts its biologic activity through metabolites, mainly phosphoramide mustard, by hepatic microsomal enzymes (Huang *et al.*, 2000). The alkylating metabolite(s) can bind to a variety of molecules including amino acids, proteins, and peptides, but the most important binding site is DNA where cross-linking occurs (Murata *et al.*, 2004). CP is effective against a wide spectrum of malignancies such as, leukemia, lymphoma, breast, lung, prostate, and ovarian cancers (Moore 1991). In fact, CP is one of the most extensively used chemotherapeutic agents in the treatment of many types of cancer that has been the focus of research efforts to understand its mode of action and to develop analogs with improved function (Camerman *et al.*, 1983). The phosphorus-containing heterocycles such as 1,3,2-oxazaphosphorinanes are also an essential part of phosphorus chemistry indicating unique conformational behavior (Bentrude *et al.*, 1986; Setzer *et al.*, 1989; Bentrude *et al.*, 1988). A great number of studies have been carried out on various monocyclic and fused bicyclic and tricyclic 1,3,2-oxazaphosphorinane derivatives about their synthesis, bioactivity, and conformational analyses (Viljanen *et al.*, 1998; Martinek *et al.*, 2000; Bentrude *et al.*, 1984; Ludeman *et al.*, 1986). Modifications of CP have led to the design and synthesis of many cyclic analogs (Li *et al.*, 2003; Gholivand *et al.*, 2005a, b; Ludeman *et al.*, 1979) to

Fig. 1 The structure of cyclophosphamide (CP)



X = $N(CH_2CH_2Cl)_2$

obtain relationships between structure and antitumor activities. It has been shown that the steric and electronic properties of the X substituent (Fig. 1) exert strong effects on the conformational equilibrium of oxazaphosphorinane ring (Martinek *et al.*, 2000).

To obtain compounds with comparative antitumor activities against K562, MDA-MB-231, and HepG2 cell lines to those of CP and to explore whether there are correlations between the steric and electronic properties of the substituents and inhibitory effect, a new series of 1,3,2-oxazaphosphorinanes and 1,3,2-diazaphosphorinanes were designed, synthesized, and characterized. The antitumor activities of these compounds, CP and sulfanilamide (SA) to inhibit the K562, MDA-MB-231, and HepG2 cells growth have been evaluated in vitro using MTT cell proliferation assay. Using spectroscopic data, IC_{50} and log *P* values, the antitumor activities were compared and described.

Results and discussion

Spectroscopic study

The structures of 1,3,2-oxazaphosphorinanes and 1,3,2-diazaphosphorinanes studied in this work are presented in Fig. 2. A summary of the spectroscopic data of these compounds is listed in Table 1. The phosphorus chemical shift in CP is at the most downfield region (11.53 ppm) compared to those of compounds **1**–**9**. The phosphorus atom in **4** (containing *para*-nitrophenyl moiety) is more deshielded than the P atom in **6** (containing nitrobenzamide moiety). In fact, among compounds **1**–**9**, the $\delta(^{31}P)$ of **6** reveals the most upfield shift (−2.16 ppm). The ^1H NMR spectra show different chemical shifts for the H_{axial} and $H_{equatorial}$ atoms of CH_2 groups (Table 2). These diastereotopic protons suggest a single chair conformation for six-membered ring at room temperature and exclude flexible boat forms in which pseudorotation would tend to average the chemical shifts and coupling constants (Eliel and Hutchins, 1969). This was confirmed by the X-ray crystal structures of their analogs (Gholivand *et al.*, 2005a, b). The axial hydrogen atoms are at downfield relative to their equatorial counterparts (Jackman and Sternhell, 1969). Furthermore, the axial H resonances are shifted downfield for **1**–**6** as compared to those of **7**–**9** (Table 2). $^3J_{(P,C)aliphatic}$ coupling constants in these molecules present greater values than $^2J_{(P,C)aliphatic}$ coupling constants. Compound **5** revealed the coupling of phosphorus atoms with carbonyl group ($^2J_{(P,C=O)} = 6.8$ Hz). IR spectra indicate that ν(P=O) varies from 1232 cm^{-1} in **3** to 1171 cm^{-1} in **8**.

The orientation of P=O double bond in axial or equatorial position depends on the changes in ^{31}P chemical shifts, proton chemical shift due to 1,3 diaxial interactions, and ^{13}C chemical shift arising from the shielding effect of oxygen (Bentrude *et al.*, 1986). Thus, it is suggested that the P=O bond is in an axial position in compounds **1**–**6**

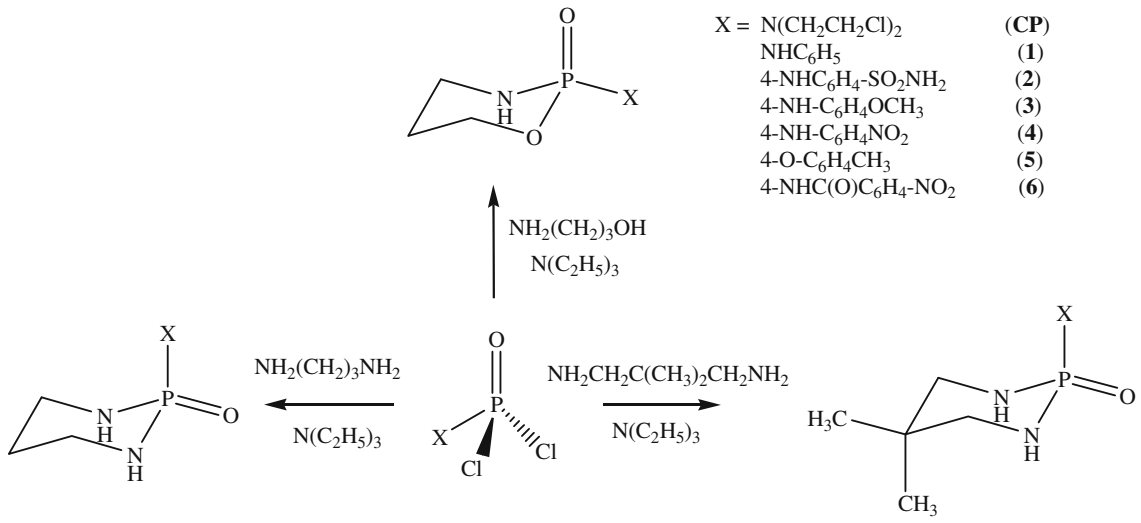


Fig. 2 The preparation pathways for analogs **1**–**9**

Table 1 Some spectroscopic data of cyclophosphamide (CP) and compounds **1–9**

Compound	$\delta(^{31}P)$/ppm	$^{2}J_{(P,Caliphatic\text{-}O)}$/Hz	$^{2}J_{(P,Cal\text{-}N)}$/Hz	$^{3}J_{(P,Cal)}$/Hz	$^{3}J_{(P,Car)}$/Hz	$\nu_{(P=O)}$/cm^{-1}	Ref.
CP	11.53	6.9	2.8	6.3, 3.8	–	1223	–
1	2.41	7.04	3.1	7.5	7.3	1231	[a]
2	1.51	7.2	2.5	7.5	7.2	1222	[a]
3	3.06	7.0	–	7.4	6.7	1232	[a]
4	0.68	7.3	2.7	7.6	7.5	1223	[a]
5	−0.87	7.7	3.5	7.0	4.7	1218	[a]
6	−2.16	7.7	–	7.4	9.2	1229	[a]
7	5.13	–	–	7.6	6.7	1178	[a]
8	5.60	–	3.3	7.7	6.7	1171	Gholivand *et al.* (2007b)
9	8.69	–	4.0	7.8	4.5	1198	Gholivand *et al.* (2007b)

al aliphatic, *ar* aromatic

[a] This work

while it places in an equatorial (or pseudo-equatorial) position in **7–9**. Therefore, the antibonding P–N orbital, when the P=O bond is axial, is suitably aligned for stabilizing overlap with a neighboring electron single pair on N(1) or O(3), an interaction which is not available when the P=O bond is equatorial. The interaction between the P–N antibonding orbital and the lone pair of the oxygen has been reported (Bentrude *et al.*, 1991) to play a substantial role in stabilization of chair conformation. It was shown that the P=O bond in CP places in a pseudo-axial position (Camerman and Camerman, 1973). Also, the crystal structure of a 1,3,2-benzoxazaphosphorinane in which the P=O bond demonstrates a pseudo-axial orientation has been reported (Gholivand *et al.*, 2007a).

Structure–activity relationships

As indicated in Fig. 2, the rationale design in this study is the replacement of $X = N(CH_2CH_2Cl)_2$ group in CP by *para*-substituted aniline, benzoyl, or phenol moieties in compounds **1–9**. Also, one of the endocyclic NH groups was replaced with its *para*-element oxygen atom in **7–9**. The effects of these structural changes on the conformational and biological properties are of our interest.

The antiproliferative activities of CP analogs **1–9** were compared in three types of human cancer cell lines by means of a colorimetric microculture assay technique (MTT assay), and the corresponding mean IC_{50} values are reported in Table 3. All the derivatives showed potent antitumor activities against three cell lines in micromolar concentrations. The cells were exposed to various concentrations of the compounds for 24, 48, and 72 h, and the inhibition percentages of cell growth (*I*) were measured for various times after exposure. The IC_{50} values (μM) after 48 h of incubation for three cell lines and also the log *P* values (which is a measure of molecular lipophilicity) as well as log *D* (computed at pH 7.4 by CSlog D software) for CP, SA (sulfanilamide), and compounds **1–9** are provided in Table 3. The plots of IC_{50} (μM) versus compounds obtained after exposure for 24, 48, and 72 h are shown in Figs. 3, 4, and 5 exhibiting the increase of IC_{50} from 24 to 48 and 72 h in most of the cases. This means that the antitumor activities are reduced due to further exposure to the cancer cell lines. Thus, the main inhibition occurs during 24 h. Also, the plots of mean IC_{50} (μM) against concentration (mM) after 48 h exposure to K562 cancer cells were presented in Figs. 6, 7, and 8 indicating the enhancement of IC_{50} with increasing of the antitumor concentration. This behavior was also observed for MDA-MB-231 and HepG2 cell lines.

The structure–activity relationships (SAR) is base on this definition that the physico-chemical properties that affect the biological activities of a molecule are of three major types: electronic, steric, and hydrophobic and other factors, such as hydrogen bonding, polarizability, dipole moment, topology, and steric effects of substitutions play less important roles (Hansch and Leo, 1995). The SAR studies were performed on phosphoramidate compounds and in their investigations, they have reported linear relationships between $\log(1/IC_{50})$ and log *P*, $\delta(^{31}P)$ and *para*-substituent constants (Ghadimi *et al.*, 2008, 2009). In the following sections, the effects of several parameters on the SAR are discussed.

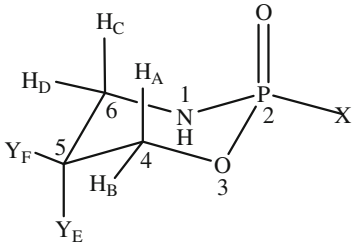
Effect of sulfonamide substitution

In evaluation of the activities of the heterocyclic ring compounds, we found that sulfanilamide is also an anticancer agent with the average IC_{50} value of ~0.12 μM in three cell lines. It should be mentioned that the inclusion of this amide on a phosphorus atom as in compounds **2** and **7** can increase the cytotoxicity against cancer cells.

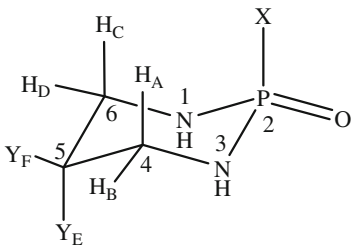
Table 2 The C, H, and Y chemical shifts of cyclophosphamide (CP) and compounds **1–9**

δ(CH), $^{2}J_{(P,C)al}$	δ(CH), $^{2}J_{(P,C)al}$	δ(CH), $^{3}J_{(P,C)al}$	δY_F (J_{PF})	δY_X (J_{PE})	δNH_Y (J_{PNH})	δH_C (J_{PD})	δH_D (J_{PC})	δH_A (J_{PB})	δH_B (J_{PA})	Compound
40.55 (2.8)	67.25 (6.8)	25.50 (6.3)	1.69–1.72 (m)		4.65 (3.6)	3.14 (m)	3.06 (m)	4.17 (m)	4.14 (m)	CP
40.67 (3.1)	67.95 (7.0)	26.30 (7.5)	1.80 (m)	1.60 (m)	4.98 (5.5)	3.11 (m)		4.11 (2.5)	4.20 (m)	**1**
40.57 (2.5)	68.25 (7.2)	26.10 (7.5)	1.79 (s)	1.57 (13.6)	5.19 (–)	3.11 (m)		4.10 (m)	4.21 (m)	**2**
40.66 (s)	67.76 (7.0)	26.30 (7.4)	1.74 (m)	1.55 (14.0)	4.88 (4.2)	3.1 (m)		4.10 (m)	4.18 (m)	**3**
40.47 (2.7)	68.49 (7.3)	25.95 (7.6)	1.81 (m)	1.62 (s)	5.37 (m)	3.13 (m)		4.13 (m)	4.27 (m)	**4**
40.67 (3.5)	69.70 (7.7)	25.80 (7.0)	1.82 (m)	1.61 (m)	5.48 (m)	3.12(m)		4.31 (m)		**5**
40.48 (s)	69.30 (7.7)	25.59 (7.4)	1.86 (m)	1.67 (13.9)	5.36 (s)	3.23	3.11 (22.0)	4.33 (m)		**6**
41.71 (s)	–	26.75 (7.6)	1.41–1.61 (m)		4.46 (s)	2.99–3.06 (m)				**7**
41.86 (3.3)	–	27.03 (7.7)	1.50 (m)	1.40 (m)	4.26 (m)	3.02 (m)				**8**
42.04 (4.0)	–	26.25 (6.8)	1.52 (m)	1.46 (m)	4.78 (m)	3.03 (m)				**9**

al aliphatic



CP, **1-6**



7-9

Diazaphosphorinanes **8** and **9** are much less toxic among these compounds. Hence, it could be concluded that 1,3,2-diazaphosphorinanes are often less toxic than 1,3,2-oxazaphosphorinanes as this is seen from the IC_{50} values in Table 3. The reason may be the easier opening of the aliphatic oxazaphosphorinane ring and P–O bond cleavage than the diazaphosphorinane ring and P–N bond breaking. A comparison of the IC_{50} values discloses that molecules **2** and **7** have much efficient activities than other diazaphosphorinanes. These higher activities are in consistent with their log *P* values which are greater for these compounds. It is very interesting that the IC_{50} values of molecules **2** and **7** are nearly the same that is most probably due to the presence of sulfonamide (SA) moiety with its close anticancer activity to CP in these derivatives.

Electronic effects of substituents

The chemical reactivity is dependent on one or both of two factors: steric and electronic effects (Hansch *et al.*, 1963). A comparison of analogs **1**, **3**, and **4** indicates that due to the electronic effects, the phosphorus atom in **3** is the most positive one. The substituents at *para* position of the phenyl ring in compounds **1**, **3**, and **4** are H, OCH_3, and NO_2

Table 3 The in vitro cytotoxic activity (IC_{50}, μM) of the compounds **1–9** toward human tumor cell lines after 48 h and their log *P*, log *D* values

Compound	Mean IC_{50} K562	Mean IC_{50} MDA-MB-231	Mean IC_{50} HepG2	log *D*	log *P*
CP	0.15	0.09	0.24	0.05	1.12
SA	0.20	0.09	0.08	−0.38	1.47
1	0.55	0.83	0.60	0.16	0.29
2	0.15	0.17	0.14	−0.70	1.88
3	0.20	0.23	0.06	0.46	−0.71
4	0.89	0.09	0.15	0.50	−0.53
5	0.71	0.87	0.59	0.28	0.76
6	0.67	0.27	0.57	0.52	−0.45
7	0.19	0.11	0.13	−1.00	1.56
8	3.11	2.04	3.17	−0.31	−0.03
9	1.44	0.23	2.20	0.22	0.12

CP cyclophosphamide, *SA* sulfanilamide

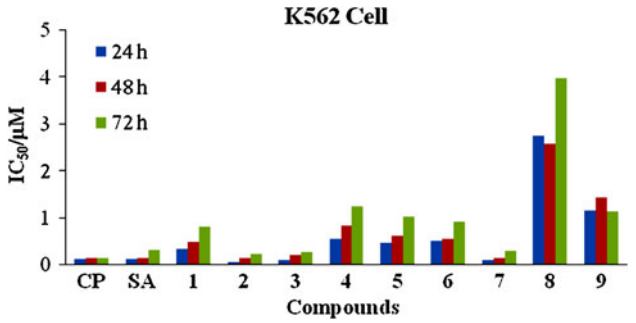


Fig. 3 The plot of IC_{50} (μM) against compounds to inhibit K562 cells

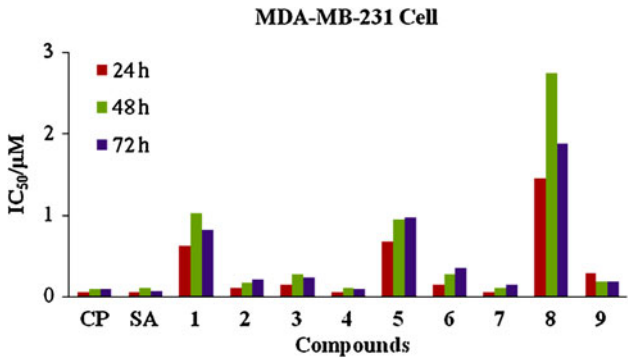


Fig. 4 The plot of IC_{50} (μM) against compounds to inhibit MDA-MB-231 cells

groups that are neutral, electron-donating, and electron-withdrawing groups, respectively. Results indicate that the $\delta(^{31}P)$ values appear at more down field from **4** to **1** and **3** that means a more electron donating group gives a more positive phosphorus atom. The IC_{50} values of these

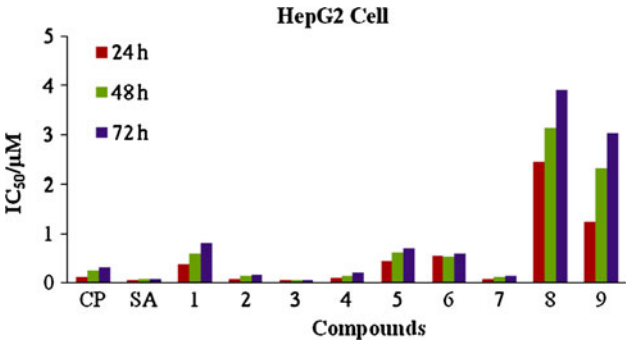


Fig. 5 The plot of IC_{50} (μM) against compounds to inhibit HepG2 cells

compounds to inhibit K562 cells exhibit the same trend, i.e., reduction from **4** to **1** and **3** (Table 3). Similarly, $\delta(^{31}P)$ is more positive in compound **7** (containing electron-withdrawing *p*-NH_2SO_2 moiety) than in compound **8** (containing neutral H atom). The IC_{50} values of **8** are greater than those of **7** against all three K562, MDA-MB-231, and HepG2 cell lines. Figure 9 represents the plot of log ($1/IC_{50}$) against $\delta(^{31}P)$ for compounds CP and **1**, **4–7** exhibiting a nearly linear relationship. This linear correlation diagram is not obtained for all compounds **1–9** that shows the inhibition mechanism is dependent to several parameters and one of them is $\delta(^{31}P)$.

Lipophilicity effect

As known, the lipophilicity of a compound plays a pivotal role during its penetration into the cells and enhances the activity. The log *P* values of the compounds **1–9** were calculated using Hyperchem 7.0 software (Table 3). Typically, partition coefficients in the order of 100–1000 (log $P = 2$–3) are required for efficient passive diffusion process (Taylor, 1996). The results demonstrate that compounds **2** and **7** both of them containing sulfonamide moiety as well as SA itself have the maximum lipophilicity values (∼1.5–2.0). The log *D* values of SA and compounds **2**, **7** are the most negative ones. Thus, the log *D* values cannot be measure of toxicity, and the log *P* values are more informative and applicable. Considering this matter plus the IC_{50} values, it may be suggested that compounds **2** and **7** as well as SA are the best candidates for anticancer activity very close to CP. It is noteworthy that the high log *P* value of 1.47 for sulfonamide (SA) confirms that presence of this substituent in compounds **2** and **7** cause their cytotoxic effects. In fact, it has been indicated that sulfonamide derivatives possess many biological activities such as anticancer, antibacterial, hypoglycemic, diuretic, anti-carbonic anhydrase, and anti-thyroid (Ghorab *et al.*, 2009; Abbate, *et al.*, 2004). Figure 10 presents the plot of log ($1/IC_{50}$) against log *P* for

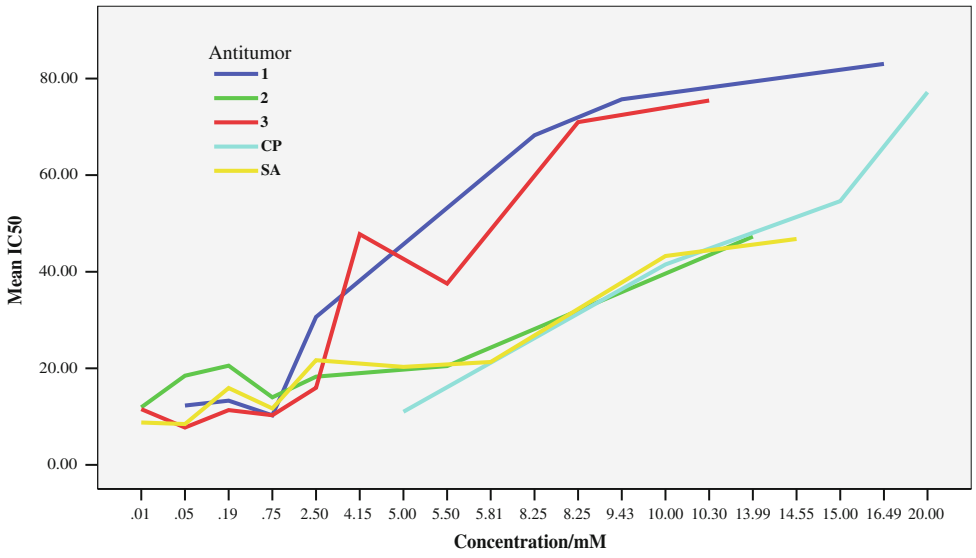


Fig. 6 The plot of IC_{50} (μM) against concentration (μM) to inhibit K562 cells for CP, SA, and compounds **1**–**3**

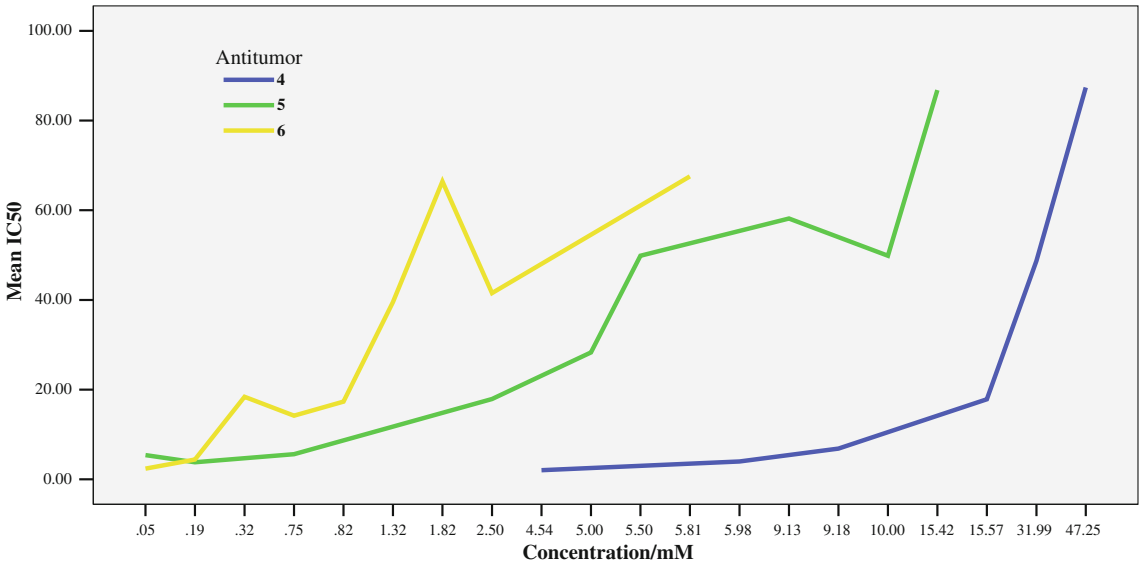


Fig. 7 The plot of IC_{50} (μM) against concentration (μM) to inhibit K562 cells for compounds **4**–**6**

compounds CP, SA and **1**, **2**, **4**, **6**, **7** which is a nearly linear diagram. Similar to the electronic effect, the linear behavior between lipophilicity and inhibition potency is not obtained for all compounds **1**–**9**. This means that the inhibition mechanism does not just depend on log *P*, but several parameters can affect it.

In summary, results of bioassay demonstrate that attachment of sulfonamide to the phosphorus atom in 1,3,2-oxaza-, 1,3,2-diazaphosphorinanes **1**–**9** does lead to potent and selective antitumor activities against the studied K562, MDA-MB-231, and HepG2 cell lines in compounds **2** and **7**. Moreover, the electronic features of the substituents, lipophilicity as well as conformational properties can affect the inhibition potencies of CP and its analogs.

Material and methods

All of the chemicals and solvents for syntheses were prepared from Fluka and Merck companies. The K562, MDA-MB-231, and HepG2 cell lines were purchased from Pasteur Institute of Iran (Tehran, Iran). Melting points were determined on an electrothermal apparatus. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on a Bruker (Avance DRS) 500 spectrometer. ^{1}H, ^{13}C, and ^{31}P chemical shifts were determined relative to TMS and 85% H_3PO_4, respectively, as external standards. IR spectra (KBr pellets) were obtained with a Shimadzu, IR-60 model spectrometer. Elemental analysis was performed using a Heraeus CHN-O-RAPID instrument.

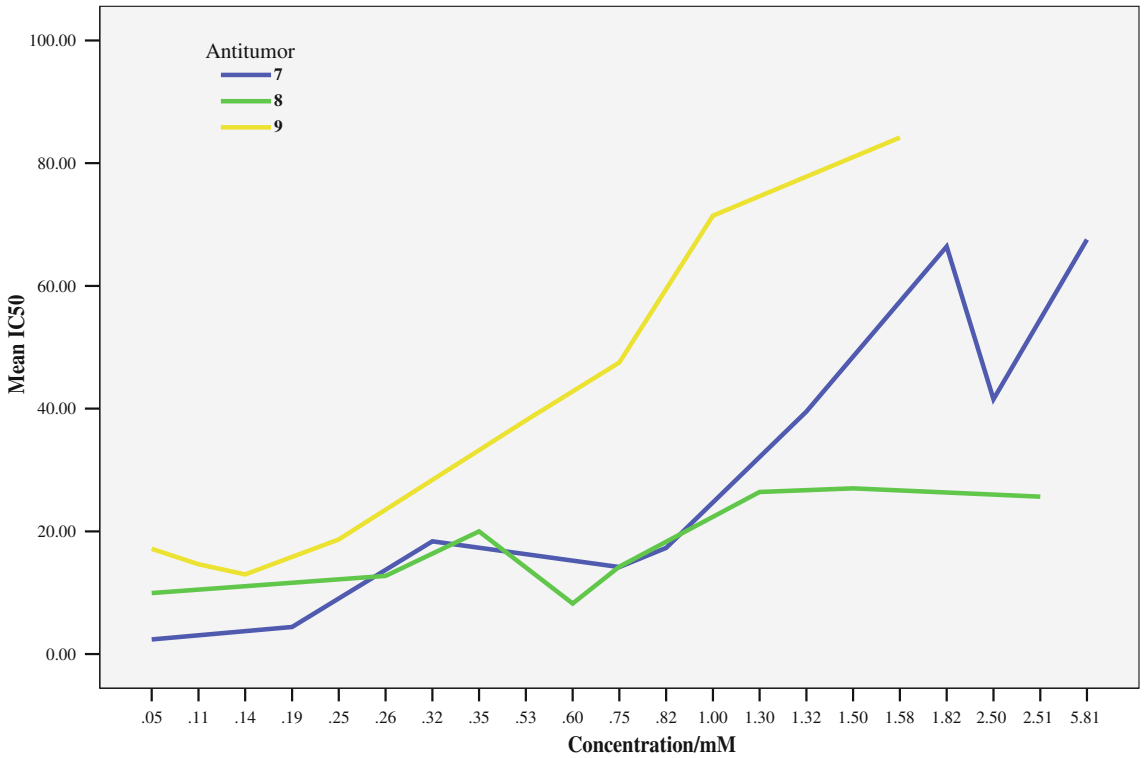


Fig. 8 The plot of IC_{50} (μM) against concentration (μM) to inhibit K562 cells for compounds **7–9**

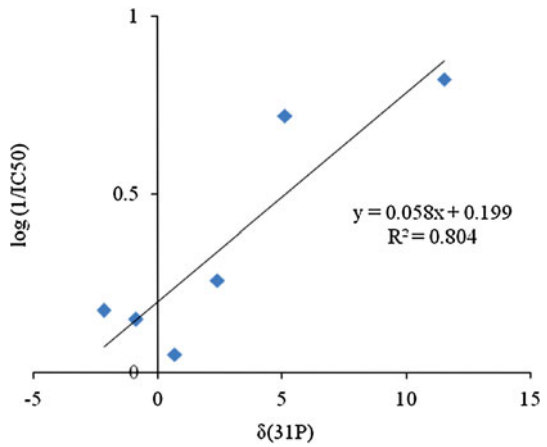


Fig. 9 The plot of log ($1/IC_{50}$) against $\delta(^{31}P)$ for compounds CP, **1**, and **4–7**

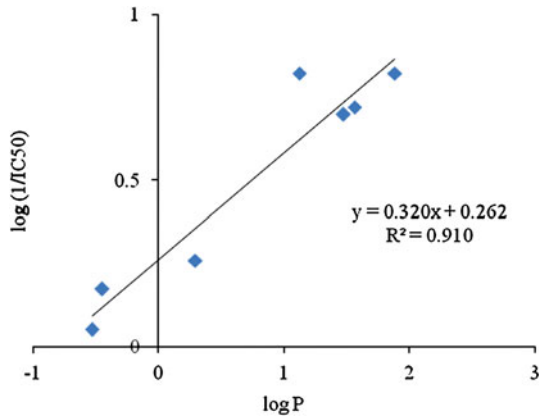


Fig. 10 The plot of log ($1/IC_{50}$) against log *P* for compounds CP, SA, and **1**, **2**, **4**, **6**, **7**

Synthesis (general procedure)

To a solution of 10 mmol of corresponding phosphoramidic dichlorides (*N*-4-phenylsulfonylphosphoramidic dichloride, *N*-4-nitrophenylphosphoramidic dichloride, *N*-nitrobenzoylphosphoramidic dichloride (Amirkhanov *et al.*, 1997), *N*-phenylphoramidic dichloride, 4-methoxyphenyl phosphoramidic dichloride (Cates and Lemke, 1974), and 4-tolyl-dichlorophosphate (Tolkimth, 1959) in dry CCl_4, a mixture of 10 mmol of related diamine (propane-1,3-diamine, 2,2-dimethylpropylene diamine and 3-aminopropane-1-ol) plus 10 mmol triethylamine was added dropwise at about 0°C and the reaction mixture stirred for 10 h. Then the precipitate was filtered and washed with distilled water and dried.

2-(*N*-phenyl)-1,3,2-oxazaphosphorinane-2-oxide

$C_6H_5NHP(O)[NHCH_2CH_2CH_2O]$ (**1**)

Yield: 89%, m.p. 185°C. Anal. Calc. for $C_9H_{13}N_2O_2P$: C, 50.95%; H, 6.18%; N, 13.20%. Found: C, 50.94%; H, 6.19%; N, 13.25%. ^{1}H NMR (d_6-DMSO): δ 1.54–1.58 (m, CH_2), 1.77 (m, 1H, CH_2), 3.21 (m, 1H, CH_2), 4.11 (m, 1H, CH_2),), 4.19 (m, 1H, CH_2), 4.98 (m, 1H, NH_{cyclic}), 6.79 (t, $^3J_{(H,H)} = 7.3$ Hz, 1H), 7.04 (d, $^3J_{(H,H)} = 7.8$ Hz, 2H, CH), 7.15 (m, 2H), 7.51 (d, $^2J_{(P,NH)} = 10.65$ Hz 1H, NH_{sulf}). ^{13}C NMR (d_6-DMSO): δ 26.30 (d, $^3J_{(P,C)} = 7.53$ Hz, 1C, CH_2), 40.68 (d, $^2J_{(P,C)} = 3.08$ Hz, 1C, CH_2–N), 67.98 (d, $^2J_{(P,C)} = 7.0$ Hz, 1C, CH_2–O), 117.23 (d, $^3J_{(P,C)} = 7.3$ Hz, CH–Ar), 119.80 (s), 128.64 (s), 145.07 (s). ^{31}P NMR (d_6-DMSO): δ 2.41 (s). IR (KBr), υ (cm^{-1}): 3235 (NH), 3090, 1601, 1497, 1429, 1371, 1338, 1284, 1231 (P=O), 1202, 1125, 1034, 998, 933 (P–N), 863(P–N), 747, 691, 617, 497.

2-(*N*-4-phenylsulfonyl)-1,3,2-oxazaphosphorinane-2-oxidez

4-$NH_2SO_2C_6H_4NHP(O)[NHCH_2CH_2CH_2O]$ (**2**)

Yield: 49%. Anal. Calc. for $C_9H_{14}N_3O_4PS$: C, 37.11%; H, 4.84%; N, 14.43%. Found: C, 37.21%; H, 4.89%; N, 14.35%. ^{1}H NMR (d_6-DMSO): δ 1.60 (d, $^3J_{(H,H)} = 13.7$ Hz, 1H, CH_2), 1.78(b, 1H, CH_2), 3.095 (b, 2H, CH_2), 4.12 (t, $^3J_{(H,H)} = 10.9$ Hz, 1H, CH_2), 4.23(d, $^3J_{(H,H)} = 10.4$ Hz, 1H, CH_2), 5.19 (d, $^2J_{(P,NH)} = 4.8$ Hz, 1H, NH_{amine}), 7.07 (s, 2H, NH_{sulf}), 7.14 (d, $^3J_{(H,H)} = 8.0$ Hz, 2H, CH), 7.63 (d, $^3J_{(H,H)} = 8.0$ Hz, 2H), 8.05 (d, $^2J_{(P,NH)} = 10.5$ Hz 1H, NH_{amine}). ^{13}C NMR (d_6-DMSO): δ 26.15 (d, $^3J_{(P,C)} = 7.47$ Hz, 1C, CH_2), 40.58 (d, $^2J_{(P,C)} = 2.54$ Hz, 1C, CH_2), 68.29 (d, $^2J_{(P,C)} = 7.2$ Hz, 1C, CH_2), 116.50 (d, $^3J_{(P,C)} = 7.2$ Hz, 2C, CH), 126.83 (s), 135.09 (s), 145.59 (s). ^{31}P{^{1}H} NMR (d_6-DMSO): δ 1.51 (s). IR (KBr), υ (cm^{-1}): 3340 (NH), 3210 (NH), 2940, 1597, 1500, 1467, 1319(s), 1222 (P=O), 1202, 1156 (SO_2), 1094, 1044, 982, 932 (P–N), 830 (P–N), 751, 590, 540.

2-(*N*-4-methoxy-phenyl)-1,3,2-oxazaphosphorinane-2-oxide

4-$CH_3OC_6H_4NHP(O)[NHCH_2CH_2CH_2O]$ (**3**)

Yield: 70%, m.p. 185–186°C. Anal. Calc. for $C_{10}H_{15}N_2O_3P$: C, 49.59%; H, 6.24%; N, 11.57%. Found: C, 49.59%; H, 6.27%; N, 11.55%. ^{1}H NMR (d_6-DMSO): δ 1.55 (d, $^2J_{(H,H)} = 13.5$ Hz, 1H, CH), 1.74 (m, 1H, CH_2), 3.03 (m, 2H, CH_2), 3.65(s, OCH_3), 4.14 (m, 2H, CH_2–O), 4.88 (d, $^2J_{(P,NH)} = 4.2$ Hz, 1H, NH), 6.75 (d, $^3J_{(H,H)} = 8.2$ Hz, 2H, Ar–H), 6.97 (d, $^3J_{(H,H)} = 8.2$ Hz, 2H, Ar–H), 7.25 (d, $^2J_{(PNH)} = 10.0$ Hz, 1H, $NH_{exocyclic}$). ^{13}C NMR (d_6-DMSO): δ 26.32 (d, $^3J_{(P,C)} = 7.35$ Hz, 1C, CH_2), 40.66 (s), 55.15(s), 67.8 (d, $^2J_{(P,C)} = 6.98$ Hz, 1C, CH_2), 114.08(s), 118.67(d, $^3J_{(P,C)} = 6.7$ Hz, 2C, CH), 135.07 (s), 153.28 (s). ^{31}P{^{1}H} NMR (d_6-DMSO): δ 3.06 (s). IR (KBr), υ (cm^{-1}): 3275 (NH), 1510, 1457, 1278, 1232 (P=O), 1209, 1178, 1127, 1030, 977, 947 (P–N), 816 (P–N), 760, 587, 492.

2-(*N*-4-Nitrophenyl)-1,3,2-oxazaphosphorinane-2-oxide

4-$NO_2C_6H_4NHP(O)[NHCH_2CH_2CH_2O]$ (**4**)

Yield: 67%. Anal. Calc. for $C_{10}H_{16}N_3O_4P$: C,43.96%; H,5.90%; N,15.38%;. Found: C, 43.99%; H,5.95%; N, 15.39%. ^{1}H NMR (d_6-DMSO): $\delta = 1.62$ (d, $^3J_{(P,H)} = 12.6$ Hz, 1H, CH_2), 1.80 (m, 1H, CH_2), 3.12 (m, 2H, N–CH_2), 4.12 (m, 1H, O–CH_2), 4.26 (m, 1H, O–CH_2), 5.38 (m, 1H, NH_{cyclic}), 7.18 (d, $^3J_{(H,H)} = 9.0$ Hz, 2H, Ar–H), 8.10 (d, $^3J_{(H,H)} = 9.0$ Hz, 2H, Ar–H), 8.50 (s, 1H, NH). ^{13}C NMR (d_6-DMSO): $\delta = 25.95$(d, $^3J_{(P,C)} = 7.6$ Hz, 1C, CH_2), 40.47 (d, $^2J_{(P,C)} = 2.7$ Hz, 1C, N–CH_2), 68.49(d, $^2J_{(P,C)} = 7.3$ Hz, 1C, O–CH2), 116.57 (d, 2C, $^3J_{(P,C)} = 7.47$ Hz, CH), 125.2 (s), 139.81 (s), 149.48 (s). ^{31}P{^{1}H} NMR (d_6-DMSO): $\delta = 0.68$ (s). IR (KBr), υ (cm^{-1}): 3335, 3170, 2870, 1598, 1517, 1477, 1340, 1254, 1223 (P=O), 1100, 1039, 985, 928 (P–N), 843, 782, 745, 541, 479.

2-(4-Methyl-phenoy)-1,3,2-oxazaphosphorinane-2-oxide

4-$CH_3C_6H_4OP(O)[NHCH_2CH_2CH_2O]$ (**5**)

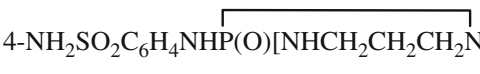
Yield: 47%, m.p. 84–85°C. Anal. Calc. for $C_{10}H_{14}NO_3P$: C,54.32%; H,7.46%; N,5.76%;. Found: C, 54.31%; H, 7.43%; N, 5.73%. ^{1}H NMR (d_6-DMSO): $\delta = 1.61$ (m, 1H, CH_2), 1.83 (m, 1H, CH_2), 2.26 (s, 3H, CH_3), 3.11 (m, 2H, N–CH_2), 4.31 (m, 2H, O–CH_2), 5.29 (m, 1H, NH), 7.09 (d, $^3J_{(H,H)} = 8.3$ Hz, 2H, Ar–H), 7.15 (d, $^3J_{(H,H)} = 8.3$ Hz, 2H). ^{13}C NMR (d_6-DMSO): $\delta = 20.21$ (s, 1C, CH_3), 25.85 (d, $^3J_{(P,C)} = 7.04$ Hz 1C, CH_2), 40.68 (d, $^2J_{(P,C)} = 3.5$ Hz, 1C, CH_2), 69.73 (d, $^2J_{(P,C)} = 7.66$ Hz, 2C, CH_2), 119.75 (d, $^3J_{P,C} = 4.67$ Hz, 2C, CH), 129.92 (s, 2C, CH), 133.26 (s,1C, C), 148.70(d, $^2J_{(P,C)} = 6.78$ Hz, 1C, C). ^{31}P{^{1}H} NMR (d_6-DMSO): $\delta = -0.87$ (s). IR (KBr), υ (cm^{-1}): 3230, 2925, 1737, 1437, 1257, 1218 (P=O), 1166, 990, 932, 910 (P–N), 869 (P–N), 818, 763, 682, 634, 479.

2-(*N*-4-Nitrobenzueil)-1,3,2-oxazaphosphorinane-2-oxide

(4-NO_2)$C_6H_4CONHP(O)[NHCH_2CH_2CH_2O]$ (**6**)

Yield: 75%, m.p. 219°C. Anal. Calc. for $C_{10}H_{12}N_3O_5P$: C,42.11%; H,4.24%; N,14.73%;. Found: C, 42.07%; H,4.33%; N,14.68%. ^{1}H NMR (d_6-DMSO): δ 1.67 (d, $^2J_{(H,H)} = 13.9$ Hz, 1H), 1.86 (m, 1H), 3.11 (dm, $^3J_{(P,H)} = 22.0$ Hz, 1H), 3.23 (t, 1H), 4.33 (m, 2 H), 5.36 (s, 1 H, NH_{amine}), 8.15 (d, $^3J_{(H,H)} = 8.2$ Hz, 2 H), 8.30 (d, $^3J_{(H,H)} = 8.2$ Hz, 2 H), 9.85 (s, 1 H, NH_{amide}). ^{13}C NMR (d_6-DMSO): δ 25.59 (d, $^3J_{(P,C)} = 7.4$ Hz), 40.48 (s), 69.30 (d, $^2J_{(P,C)} = 7.7$ Hz), 123.38 (s), 129.54 (s), 139.00 (d, $^3J_{(P,C)} = 9.2$ Hz), 149.47 (s), 167.13 (s). ^{31}P{^{1}H} NMR (d_6-DMSO): $\delta = -2.16$ (s). IR (KBr, cm^{-1}): 3230 (NH), 3105 (NH), 2870, 1686 (C=O), 1515, 1465, 1343, 1260, 1229 (P=O), 1202, 1100, 988, 880 (P–N), 852 (P–N), 715, 498.

2-(*N*-4-phenylsulfonyl)-1,3,2-diazaphosphorinane-2-oxide



4-$NH_2SO_2C_6H_4NHP(O)[NHCH_2CH_2CH_2NH]$ (**7**)

Yield: 69%, m.p. 193.5°C. Anal. Calc. for $C_9H_{15}N_4O_3PS$: C, 37.24%; H, 5.21%; N, 19.30%. Found: C, 37.21%; H, 5.18%; N, 19.25%. ^{1}H NMR (d_6-DMSO): δ 1.54 (s, 2 H, CH_2), 3.04 (d, $^3J_{(P,H)} = 24.9$ Hz, 2H, CH), 3.15 (s, 2H, CH), 4.46 (s, 2 H, NH_{amine}), 6.80 (b, 2H, NH_{sulf}), 7.19 (d, $^3J_{(H,H)} = 8.4$ Hz, 2H), 7.58 (d, $^3J_{(H,H)} = 8.4$ Hz, 2H), 7.66 (d, $^2J_{(P,NH)} = 9.8$ Hz 1H, NH_{amide}). ^{13}C NMR (d_6-DMSO): δ 26.78 (d, $^3J_{(P,C)} = 7.6$ Hz, 1C, CH_2), 41.71 (s), 116.45 (d, $^3J_{(P,C)} = 6.7$ Hz, 2C, CH), 126.49 (s), 134.21 (s), 146.63 (s). ^{31}P{^{1}H} NMR (d_6-DMSO): δ 5.13 (s). IR (KBr), υ (cm^{-1}): 3320 (N–H), 3195(N–H), 2930, 1588, 1490, 1468, 1332, 1305, 1178 (P=O), 1143 (SO_2), 1086, 918 (P–N), 825 (P–N), 536.

Cell culture and growth inhibition assay

In order to study the degree of selectivity of the cytotoxic activity of the compounds under investigation, assays using healthy cells (Human Peripheral Blood Mononuclear Cells) were carried out on some compounds. The compounds selected were those that showed activity in tumor cells.

Isolation and culture of human peripheral blood mononuclear cells lymphocytes

Separation of lymphocyte was done according to the following procedure (Noble *et al.*, 1968): defibrinated or anticoagulant-treated blood is diluted with an equal volume of phosphate buffered saline (PBS) and layered carefully over Ficoll-Paque PLUS (without intermixing) in a centrifuge tube. After a short centrifugation at room temperature (typically at 400 rpm for 30–40 min), lymphocytes together with monocytes and platelets are harvested from the interface between the Ficoll-Paque PLUS and sample layers. This material is then centrifuged twice in balanced salt solution to wash the lymphocytes and to remove the platelets.

In vitro antitumor assays

For evaluation of antiproliferative activity of CP and compounds **1–9**, three types of cells, i.e. K562 cells (5×10^3 cells/well), MDA-MB-231 cells (2.5×10^3 cells/well), and HepG2 cells (3×10^3 cells/well), were plated in 96-multiwell microtiter plate for 24 h before treatment with the test compounds to allow attachment of cell to the wall of the plate. Cells were maintained in RPMI medium (Gibco BRL) supplemented with 10% fetal bovine serum, 2% L-glutamine (Gibco BRL), penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in an atmosphere humidified with 5% CO_2 and 95% air. Cells were seeded in 96-well cell culture plates and treated on the second day with the candidate drugs.

Test compounds stock solutions were prepared by dissolving of each compound in DMSO and then aliquots of stock solutions further diluted with sterile RPMI to the required concentration, such that the total DMSO concentration did not exceed 2%. At this concentration, DMSO was found to be nontoxic to the cells tested. As a control, a solution containing 2% DMSO in RPMI was used. Various concentrations of compounds **1–9** were added to each well in triplicate at 37°C with 5% CO_2 in the incubator for 24, 48, and 72 h. After incubation for the indicated times, the cell number was estimated by the MTT assay as described in the literature (Mosmann, 1983). After treatment, 20 µl of aqueous MTT solution (5 mg/ml) was added and incubated for 3–4 h at 37°C in an atmosphere humidified with 5% CO_2 and 95% air. After incubation, the medium/MTT mixtures were removed, and formazan crystals were dissolved in 200 µl DMSO/well. Optical densities at 540 nm were measured with an ELIZA plate reader (Labsystems Multiscan, MS). The IC_{50} values of compounds **1–9**, CP, and sulfonamide (SA) for 50% cell death were measured after 48 h of incubation. Statistical analyses were performed using GraphPad prism software, version 5 (GraphPad software) so that using nonlinear regression modeling, one IC_{50} value was calculated from each set of triplicate wells.

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