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Original article

Reactions of purines-containing butenolides with L-cysteine or *N*-acetyl-L-cysteine as model biological nucleophiles: a potent mechanism-based inhibitor of ribonucleotide reductase caused apoptosis in breast carcinoma MCF7 cells

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Abstract

Thiols are the most reactive nucleophilic reagents among the biological models investigated. The reactivity of butenolides **1a**–**c**, **2**–**4**, and **6**–**8** toward L-cysteine, a model biological nucleophile, was studied spectrophotometrically. The rates of the reactions were measured and correlated with antitumour activity of these molecules. *N*-Acetylcysteine addition product **5**, resulting from the treatment of butenolide **4** with glutathione precursor, *N*-acetyl-L-cysteine, was isolated. Unlike purine-containing γ-(*Z*)-ethylidene-2,3-dimethoxybutenolides **1a**–**c**, **4**, **6**, and **7**, adduct **5** and butenolides **10**–**12** did not exhibit inhibitory activity against murine leukemias (L1210 and P388), breast carcinoma (MCF7), and human T-lymphoblasts (Molt4/C8 and CEM/0) cell lines. As such, the biological activity of purine-containing butenolides can be attributed to their adenine moiety as a recognition site as well as their reactivity towards the cysteine residues of functional proteins forming covalent bond via reverse Michael type addition. Adenine-containing phosphonothioanhydride derivative **8** was also synthesised. Its reaction with *N*-acetyl-L-cysteine produced *N*,*S*-diacetylcysteine and thiophosphonate **9**. Compound **9** did not exhibit anticancer activity; yet its precursor **8** displayed the most pronounced inhibition on all the examined malignant tumour cell lines. In the presence of L-cysteine, cytotoxicity of **4** and **8** was decreased, whereas glutathione addition more influenced on the cytotoxicity of **8**. It was found that adenine-containing phosphonothioanhydride **8** functions as a significant irreversible inactivator of the *Escherichia coli* ribonucleoside diphosphate reductase. After treatment of MCF7 cells with compound **8**, fluorescence microscopy demonstrated the presence of nucleus shrinkage or segmentation. This apoptotic morphology, however, was not pronounced in the presence of glutathione or dithiotheritol.

Keywords: Butenolides; Adenine; L-cysteine; Biological nucleophiles; Antitumoral activity; Ribonucleotide reductase; Apoptosis

1. Introduction

The biological importance of unsaturated lactones is well known [1–7]. In particular, the γ-alkylidenebutenolide skeleton [8,9] is a useful entity that is present in natural products such as fibrolides [10], dihydroxerulin [11], and protoanemonin [12,13]. A number of sesquiterpene unsaturated lactones exhibited growth inhibitory activity in vivo against animal tumour systems and in vitro against cells derived from human carcinoma of the nasopharynx (KB) [14]. Their reactivity toward thiols and amines suggested that the antiinflammatory and antineoplastic activity of these compounds may result from alkylation of nucleophilic centres (i.e. transcription factor NF-kB subunits, p50

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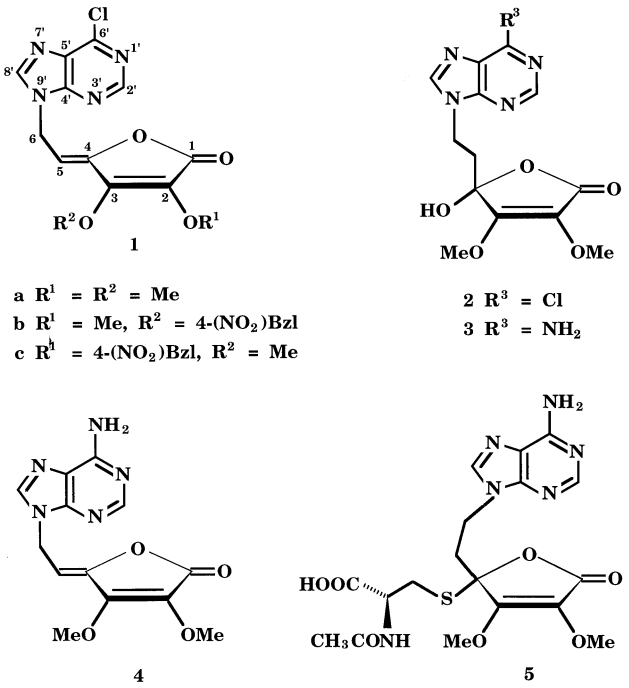

Fig. 1. Purine-containing butenolides **1**–**4** and *N*-acetyl-L-cysteine adduct **5**.

and p65, which contain cysteine residues in their DNA binding domains [15] or L-γ-glutamyl-L-cysteinyl-glycine = glutathione = GSH [16]) in a biological system [14,17].

On the other hand, inhibition of enzymes (i.e. ribonucleotide diphosphate reductase (RDPR) [18–21] and *S*-adenosyl-L-homocysteine hydrolase (AdoHcy hydrolase) [22]) that are crucial to metabolic pathways involved in cell division is an attractive concept for the design of rational chemotherapeutic agents [23]. RDPR in mammalian systems provide the only de novo pathway to the 2′-deoxynucleotide components of DNA [24]. RDPR is composed of two homodimeric subunits R1 and R2. R1 contains the active site for the reduction of both purine and pyrimidine substrates and possess five cysteines which are required for catalysis [25]. Reaction of a substrate with cysteine residues of this protein may obstruct the route to replication of the genetic material for cancer cell division.

We have recently reported the synthesis and biological activities of purine-containing butenolides **1**–**4** (Fig. 1) and **6** (Fig. 2) [26]. Among these bioactive compounds, only adenine-containing γ-(*Z*)-ethylidene-2,3-dimethoxybutenolide **4** was found to be a notable time-dependent 'enzyme–substrate intermediate' inacti-

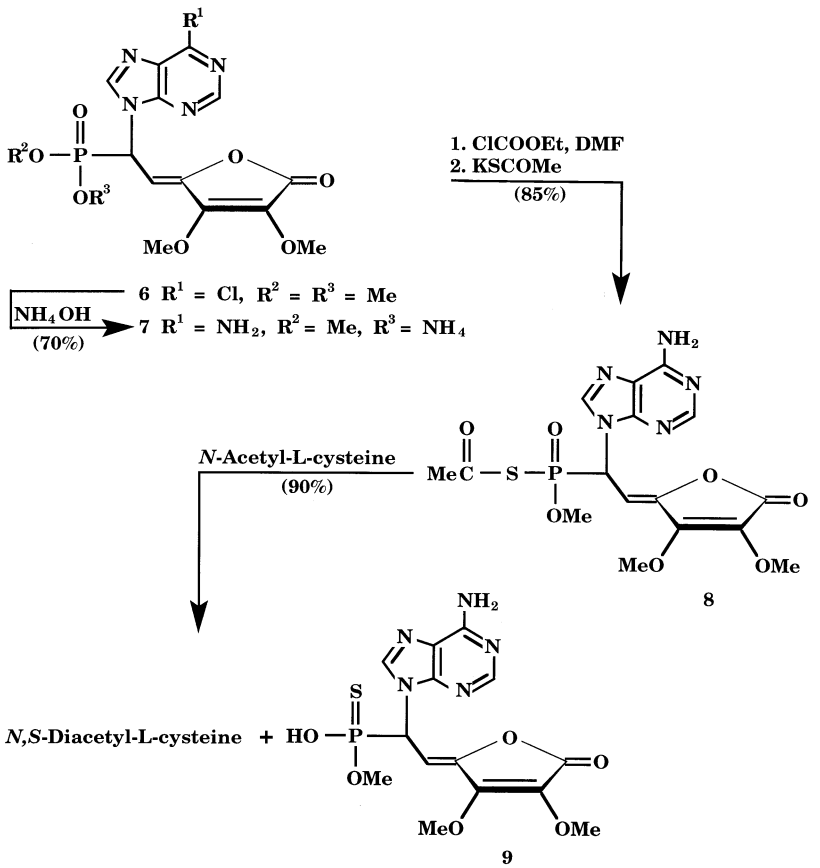

Fig. 2. Synthesis of adenine derivatives of phosphonobutenolide **7**, butenolide-containing phosphonothioanhydride **8**, and thiophosphonobutenolide **9**.

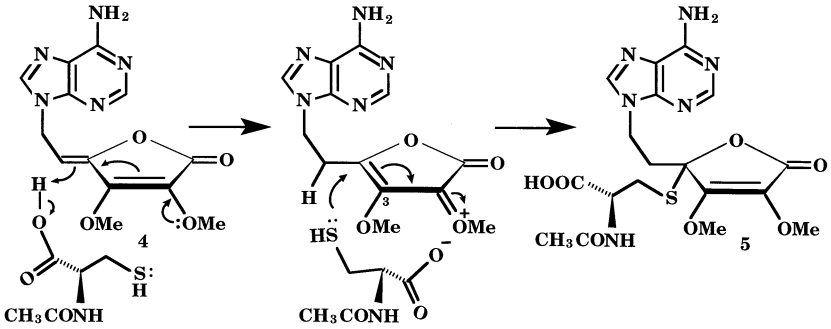

Fig. 3. Reverse Michael-type reaction via protonation and oxonium ion formation followed by *N*-acetyl-L-cysteine addition.

vator (inactivation rate constant ($k_2 = 0.052$ min^{-1}), dissociation constant ($K_i = 5.68$ μM), and derived second order rate constant ($k_2/K_i = 9.15 \times 10^3$ M^{-1} min^{-1}) of AdoHcy hydrolase [26]. This compound inhibited specifically P388 leukemia cells [26]. On the other hand, its hydrated derivative **3** was totally inactive. In comparison to adenine derivative **4** and phosphonates **6** and **7**, 6-chloropurine-containing butenolides **1a**–**c** displayed more pronounced inhibition on the majority of the examined malignant tumour cell lines [26]. We hypothesised that the biological activity of this class of compounds has its origin in the reactivity of their C_4=C_5 toward biological nucleophiles (i.e. cysteine residues of functional proteins). In addition, the lack of activity of phosphonobutenolide **10** (Fig. 4) [26] revealed that a purine or pyrimidine moiety is essential for the recognition by the target enzyme.

There is a variety of nucleophilic sites within proteins [27] where covalent adduct formation with electrophilic C_4=C_5 in **1**, **4**, **6**, and **7** may occur. These sites include imidazole in histidine, carboxylic acid in aspartic and glutamic acids, hydroxy in serine and threonine, thioether in methionine, indole nitrogen in tryptophan, amino group in lysine, and thiol in cysteine [28]. The extent of reaction of the C_4=C_5 at each of these sites will depend both on the mechanism of the interaction and on the three-dimensional protein structure. Among the aforementioned aminoacids, cysteine and GSH precursor [16], *N*-acetyl-L-cysteine, showed high affinity for the adduct formation with purine-containing butenolides **1** and **4**. Moreover, these derivatives inactivated RDPR through a non-specific alkylation process [29].

A new series of covalent mechanism-based inhibitors of AdoHcy hydrolase is recently reported [30]. Attack by amino acid functionalities of the active site of the enzyme to 5′-*S*-propionyl-5′-thioadenosine intermediate was accounted for the inhibitory property of 5′-*S*-allenyl-5′-thioadenosine and 5′-*S*-propynyl-5′-thioadenosine toward AdoHcy hydrolase [30]. Accordingly, we synthesised thioanhydride **8** as a potential substrate for AdoHcy hydrolase. Unlike adenine-containing butenolide **4**, its phosphonothioanhydride derivative **8** did not exhibit inhibitory property toward the enzyme. On the other hand, thioanhydride **8** was very reactive toward L-cysteine and *N*-acetyl-L-cysteine and rapidly inactivated RDPR via an acylation process that was found to be accompanied by the liberation of adenine-containing thiophosphonobutenolide **9**. In order to understand the role of the adenine base in **8** (see Fig. 2), phosphonothioanhydride **12** (see Fig. 4) was synthesised and found not to be a substrate for RDPR; yet it was reactive toward L-cysteine or *N*-acetyl-L-cysteine. As such, the pronounced activity of adenine-containing phosphonothioanhydride **8** against murine leukemias (L1210 and P388), breast carcinoma (MCF7), and human T-lymphoblasts (Molt4/C8 and CEM/0) could be due to its reaction with cysteine residues of RDPR.

Herein we report the inhibitory property of **1**, **2**–**4**, **6**–**8**, and **12** towards *Escherichia coli* RDPR as well as the reactions of **1a**–**c**, **2**–**4**, **6**–**8**, and **12** with model biological nucleophiles, L-cysteine or *N*-acetyl-L-cysteine. The reaction rates of RDPR inhibitors **1**, **4**, and **6**–**8** with L-cysteine are correlated with their activity against malignant tumour cell lines. Among all the newly synthesised compounds, the most efficient RDPR inhibitor, adenine-containing phosphonothioanhydride **8**, exhibited the ability to induce apoptosis in MCF7 cells (Fig. 5). The results of these experiments provide a more detailed view on the mode of action of these new

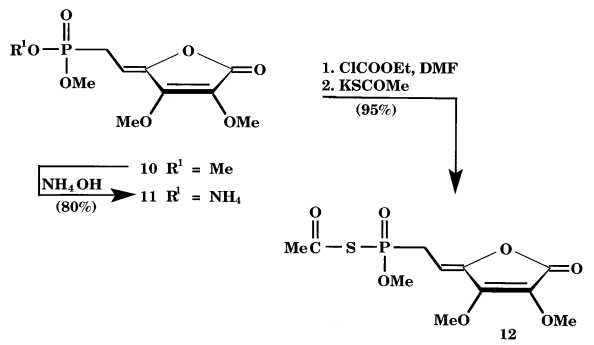

Fig. 4. Synthesis of phosphonobutenolide **11** and phosphonothioanhydride **12**.

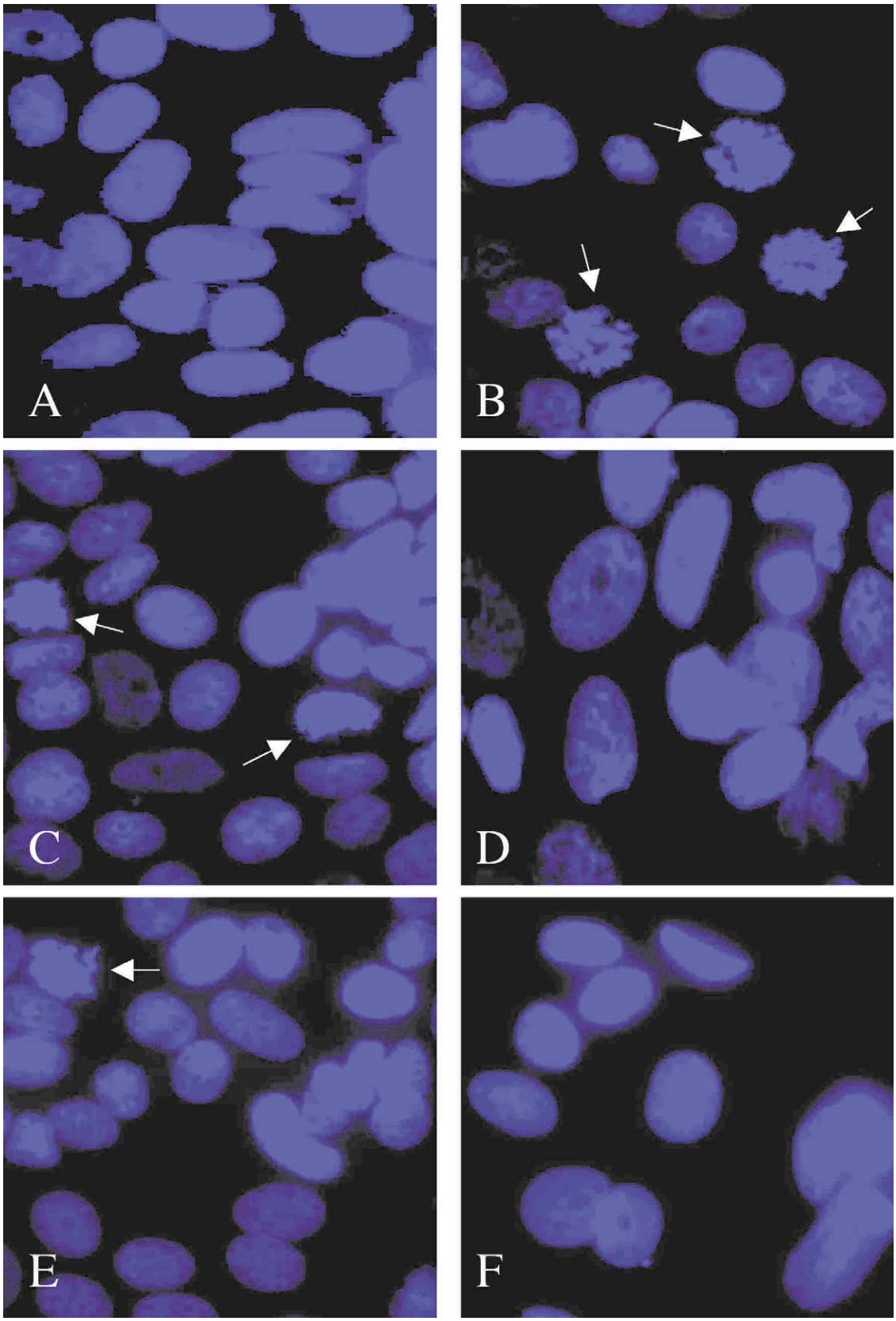

Fig. 5. Drug-induced apoptosis in MCF7 cells. (A) Untreated cells. (B) Cells treated with 0.76 μM adenine-containing phosphonothioanhydride (**8**). (C) Treatment with **8** (0.76 μM) + GSH (0.76 μM). (D) Treatment with **8** (0.76 μM) + GSH (7.6 μM). (E) Treatment with **8** (0.76 μM) + DTT (0.76 μM). (F) Treatment with **8** (0.76 μM) + DTT (7.6 μM). Apoptotic nuclei are indicated by white arrows.

class of compounds. Adduct **5** (Fig. 1) and thiophosphonate **9** along with *N*,*S*-diacetylcysteine (Fig. 2), resulting from the individual reactions of **4** and **8** with *N*-acetyl-L-cysteine, were also isolated, respectively.

2. Chemistry

*2.1. Reactions of purine-substituted butenolides **1a–c**, **2–4**, **6–8**, and thiophosphonoanhydride **12** with model biological nucleophiles*

6-Chloropurine-containing dimethylphosphono-γ-(*Z*)-ethylidene-2,3-dimethoxybutenolide (**6**) [26] was reacted with NH_4OH in CH_3CN at reflux to produce the corresponding adenine monoammonium phosphonate salt **7**, as a mixture of two diastereoisomers, in 70% yield (Fig. 2). Sequential treatment of **7** with $ClCO_2Et$–DMF and KSCOMe gave adenine-containing phosphonothioanhydride **8** in 85% yield. Similarly, dimethylphosphono-γ-(*Z*)-ethylidene-2,3-dimethoxybutenolide (**10**) [26] was treated with NH_4OH in CH_3CN to afford monoammonium phosphonate salt **11** in 80% yield (Fig. 4). Its reaction with $ClCO_2Et$–DMF, followed by treatment with KSCOMe produced phosphonothioanhydride **12** in 95% yield. The reactivity of butenolides **1a–c**, **2–4**, **6–8**, and **12** toward L-cysteine, a model biological nucleophile, was studied spectrophotometrically [3]. Iodoacetate was used as a reference compound ($K_2 = 1500$ l mol^{-1} min^{-1}) [3b]. To L-cysteine (10^{-4} M) in phosphate buffer (67.0 mM, pH 7.40), prepared in a 1.0-cm quartz cell (3.80 mL), was added, individually, **1a–c**, **2–4**, **6–8**, and **12** (10^{-4} M) in tetrahydrofuran (THF) (40.0 μL) at 25 °C. After an appropriate reaction time, the sulfhydryl content was measured by quenching the reaction with an excess of a THF solution of 2,2′-dipyridine disulfide, which reacts with unreacted L-cysteine to give 2-thiopyridone (343 nm, ε 7780). 6-Chloropurine-containing γ-(*Z*)-ethylidene-2,3-dialkoxybutenolides **1a–c** were reacted with L-cysteine within 7.0 min. Their second order rate constants were found to be 6500, 6200, and 6400 l mol^{-1} min^{-1}, respectively. Adenine-containing γ-(*Z*)-ethylidene-2,3-dimethoxybutenolide **4** reacted with L-cysteine within 15.0 min. Its K_2 was found to be 5095 l mol^{-1} min^{-1}. The reaction of 6-chloropurine derivative of 4-hydroxybutenolide **2** with L-cysteine was complete within 97.0 min and its rate constant (K_2) was 120 l mol^{-1} min^{-1}. Phosphonates **6** or **7** reacted with L-cysteine after 40.0 min with $K_2 = 1200$ and 1150 l mol^{-1} min^{-1}, respectively. Adenine-containing 4-hydroxybutenolide **3** did not react with L-cysteine even after 24 h. Consequently, the C_4=C_5 in **1a–c** readily undergoes nucleophilic addition with L-cysteine. The C_4=C_5 in **6** or **7** is sterically hindered by an adjacent phosphonate functionality. Thus, it is not very reactive toward L-cysteine relative to **1a–c** and **4**. On the other hand, the C_2=C_3 in **2** or **3** is not reactive toward L-cysteine. The rate constant ($K_2 = 120$ l mol^{-1} min^{-1}) observed with **2** is due to the replacement of chlorine atom therein with L-cysteine. On the other hand, the reaction of thioanhydrides **8** or **12** with L-cysteine ($K_2 = 23\,700$ and 20 960 l mol^{-1} min^{-1}, respectively) was found to be complete after 3 and 5 min, respectively.

To identify the site of reactivity of butenolides toward biological nucleophiles, adenine-containing γ-(*Z*)-ethylidene-2,3-dimethoxybutenolide (**4**) [26] and its corresponding phosphonothioanhydride derivative **8** were reacted with an equimolar amount of *N*-acetyl-L-cysteine. Compound **4** gave adduct **5** (Fig. 1 73% yield) via a reverse Michael-type addition mechanism (Fig. 3) [26], and phosphonothioanhydride **8** produced thiophosphonate **9** (90% yield) along with *N*,*S*-diacetylcysteine (Fig. 2).

3. Biological results

3.1. Inhibition of E. coli RDPR

Using the methodology previously described by Stubbe et al. [29], compounds **1a–c**, **2–4**, **6–8**, and **12** were evaluated for inhibitory property against *E. coli* RDPR (EC 1.17.4.1). The remaining enzyme activity was assayed as described by Steeper and Steuart [31]. The *E. coli* RDPR (1.35 μM) was significantly inactivated upon incubation with adenine-containing phosphonothioanhydride derivative **8** by concentration-dependent process. The maximal inactivation at 5, 10, 25, 50, and 100 μM concentrations used was 100% of the original enzyme activity within 15 s. No change in the remaining enzyme activity was detected over 15 s–2 h. Such rapid inactivation did not allow the determination of time-dependent inactivatory property of phosphonothioanhydride **8** towards RDPR. Chromatography of the inactivated enzyme on a Sephadex G-50 column resulted in no recovery of the RDPR activity. Since compound **8** readily reacts with sulfhydryls, the irreversible inhibition of RDPR is due to acylation of the cysteine residues of the protein, as evidenced by detection of adenine-containing thiophosphonate **9** by HPLC analysis. At a ten-fold higher concentration of thioredoxin (TR) (120 μM), the inhibitory property of phosphonothioanhydride **8** did not change significantly, but when a high concentration of glutathione (GSH, 50 μM) or dithiothreitol (DTT, 50 μM) was added to the test media, RDPR was protected from inactivation by **8**. As such, the sulfhydryl groups of these substances were acylated by thioanhydride **8** prior to those of the enzyme.

Table 1
Inhibition of *E. coli* RDPR with **1a**–**c**, **2**–**4**, **6**–**8**, and **12**

Compound	Enzyme activity remaining (%) [a,b]			
	1.0 μM	10 μM	100 μM	1000 μM
1a	>99	89.75	59.47	9.94
1b	>99	90.12	58.96	11.07
1c	>99	92.03	55.34	10.20
2	>99	>99	>99	>99
3	>99	>99	>99	>99
4	>99	94.23	67.23	17.16
6	>99	>99	80.54	30.45
7	>99	>99	89.06	35.10
8	6.98	<1.0	<1.0	0.00
12	>99	>99	>99	>99

[a] RDPR(1.35 μM) was incubated with varying concentrations of compounds at 37 °C for 20 min [29], and the remaining enzyme activity for the conversion of CDP to dCDP was determined as described [31].
[b] Data are the average of duplicate determinations.

On the other hand, with purine-containing butenolides **1a**–**c**, **4**, **6**, and **7**, the maximal inactivation of RDPR at the highest concentration used (1000 μM) was ca. 90% of the original enzyme activity with **1a**–**c**, 83% with **4**, 69% with **6**, and 65% with **7** after 20 min (Table 1). Hydrated derivatives **2** and **3** were found not to be inhibitors of RDPR. We believe that the weak inhibition of RDPR by **1a**–**c**, **4**, **6**, and **7** could be attributed to a non-specific reverse Michael-type attack on the conjugated butenolides by a thiol group from TR and not the thioredoxin reductase (TRR). In fact, an equimolar concentration of DTT protected RDPR against inactivation by **1a**–**c**, **4**, **6**, and **7**.

Butenolide-containing phosphonothioanhydride **12** was also evaluated for the inhibitory property against RDPR. It was found not to be an inhibitor of the enzyme even at concentration as high as 2000 μM. While compound **12** was reactive toward GSH or DTT, it was not reactive against TR.

3.1.1. Activity against malignant tumour cell lines

The anticancer screening experiments for compounds **1a**–**c**, **2**–**12**, **4** + cysteine, **4** + GSH, **8** + cysteine, **8** + GSH, and 9-(β-D-arabinofuranosyl)cytosine (ara-C) were carried out in vitro against murine leukemias (L1210 and P388), breast carcinoma (MCF7), and human T-lymphoblasts (Molt4/C8 and CEM/0) cell lines. The activity is expressed as the concentration (μM) required to inhibit tumour cell proliferation by 50% (IC_{50}) [13]. Results are listed in Table 2.

3.1.2. Apoptotic morphology in MCF7 cells caused by adenine-containing thiophosphonoanhydride *8*

MCF7 cells were treated with IC_{50} of compound **8** (0.76 μM), two combinations of **8** and GSH (0.76 + 0.76 μM and 0.76 + 7.6 μM, respectively), and two combinations of **8** and DTT (0.76 + 0.76 μM and 0.76 + 7.6 μM, respectively). Chromatin staining of the cells with Hoechst 33342 revealed nucleus shrinkage or

Table 2
Inhibitory effects of butenolides **1a**–**c**, **2**–**12**, equimolar combinations of **4** with cysteine and GSH, equimolar combinations of **8** with cysteine and GSH, as well as ara-C on the growth of malignant tumour cell lines

Compound	IC_{50} (μM) [a]				
	L1210	P388	MCF7	Molt4/C8	CEM/0
ara-C	0.17	0.14	1.03	0.65	0.78
1a	4.52	8.12	16.87	5.97	4.71
1b	5.81	6.49	15.43	7.35	3.98
1c	6.03	7.42	14.21	6.16	5.04
2	>120	>120	>120	>120	>120
3	>120	>120	>120	>120	>120
4	93.35	2.67	>120	78.46	84.51
5	>120	>120	>120	>120	>120
6	16.25	14.31	90.11	12.47	96.05
7	>120	32.16	>120	95.21	59.71
8	1.03	0.28	0.76	1.74	0.98
9	>120	98.72	>120	>120	120
10	>120	>120	>120	>120	>120
11	>120	>120	>120	>120	>120
12	>120	110	93.67	>120	>120
4+cysteine	>120	48.54	>120	>120	>120
8+cysteine	78.60	69.87	85.07	90.24	79.89
4+GSH	99.68	19.18	>120	89.35	93.96
8+GSH	75.49	70.54	90.31	87.45	75.77

[a] The IC_{50} values were estimated from dose–response curves compiled from at least two independent experiments and represent the compound concentration required to inhibit tumour cell proliferation by 50%.

Table 3
Toxicity of butenolides **1a–c**, **2–9**, and ara-C on the growth of normal cell lines

Compound	CC_{50} (μM) [a]		
	HEL	MEF	Hef522
ara-C	1.98×10^{-3}	0.98×10^{-2}	2.14 $\times 10^{-2}$
1a	25.13	30.65	30.47
1b	29.84	34.09	27.86
1c	27.98	29.43	32.21
2	>120	>120	>120
3	>120	>120	>120
4	97.65	87.69	93.80
5	>120	>120	>120
6	110	99.64	>120
7	>120	120	>120
8	16.72	14.93	17.40
9	>120	120	>120

[a] Cytotoxic concentration (CC_{50}) required to reduce cell growth by 50%.

fragmentation after 4.0 h incubation with **8** (Fig. 5). This morphological change is associated with cell death occurring by a process called apoptosis. As shown in Fig. 5, adenine-containing thiophosphonoanhydride **8** did not induce apoptosis in MCF7 cells in the presence of high concentrations of GSH or DTT.

3.1.3. Toxicity on the growth of normal cell lines

Inhibition of the proliferation of human embryonic cell (HEL), murine embryo fibroblasts (MEF), and normal fibroblasts (Hef522) by compounds **1a–c**, **2–9**, and ara-C were carried out according to an established procedure [13]. Toxicity of the tested compounds is expressed as the cytotoxic concentration required to reduce normal cell growth by 50% (CC_{50}). Results are summarised in Table 3.

4. Discussion

Purine-containing butenolides **1a–c**, **2–4**, and **6–8** were allowed to react with L-cysteine. Rates of the reactions of 6-chloropurine derivatives **1a–c** with L-cysteine were faster ($K_2 = 6200–6500$ l mol^{-1} min^{-1}) than that of the adenine derivative **4** ($K_2 = 5095$ l mol^{-1} min^{-1}). As a result, in comparison to **4**, butenolides **1a–c** were found to possess broader spectrum of anticancer activity. The rate constant for **2** was 120 l mol^{-1} min^{-1}, and **3** was not reactive toward L-cysteine. Our results indicate that the C_4=C_5 is essential for biological activity. Hydrated analogues **2** and **3** are not reactive enough toward L-cysteine. Consequently, they did not show activity against tumour cell lines. Adduct **5** was also found not to be active against murine leukemias (L1210 and P388), breast carcinoma (MCF7), and human T-lymphoblasts (Molt4/C8 and CEM/0) cell lines. On the other hand, purine derivatives of phosphono-γ-(*Z*)-ethylidene-2,3-dimethoxybutenolides (**6**) ($K_2 = 1200$ l mol^{-1} min^{-1}) and **7** ($K_2 = 1150$ l mol^{-1} min^{-1}) exhibited weak biological activity. The C_4=C_5 in **6** or **7** is sterically hindered by an adjacent phosphonate functionality at the C-6 position. Thus in comparison to **1a–c** and **4**, trap of L-cysteine by the C_4=C_5 in **6** or **7** would be much slower. Similar to phosphonates **6** and **7**, thiophosphonate **9** also did not exhibit significant anticancer activity. On the other hand, adenine-containing phosphonothioanhydride **8** exhibited profound reactivity toward L-cysteine ($K_2 = 23\,700$ l mol^{-1} min^{-1}), showed excellent inhibitory property against RDPR (Table 1), and displayed notable activity against malignant tumour cell lines (Table 2). Unlike adenine-containing phosphonothioanhydride **8**, phosphonothioanhydride **12** ($K_2 = 20\,960$ l mol^{-1} min^{-1} toward L-cysteine) was found not to be an inhibitor for RDPR. Thus, it did not exhibit activity against malignant tumour cell lines. Results from biological screening experiments indicate that the cytotoxicity of adenine-containing phosphonothioanhydride **8** profoundly reduced in the presence of cysteine or GSH; yet butenolide **4** was found to be more sensitive to the addition of cysteine rather than GSH (see Table 2). After treatment of MCF7 cells with compound **8**, chromatin staining revealed apoptotic nuclei. On the other hand, in the presence of high concentrations of nucleophiles such as GSH or DTT apoptotic morphology inside MCF7 cells was not observed. Indeed, the ability of compound **8**, in the presence or absence of the nucleophiles (Table 2), to induce apoptosis in MCF7 cells (see Fig. 5) correlates well with its anticancer activities. In comparison to the reference compound, ara-C, the tested compounds did not exhibit significant toxicity against normal cell lines (Table 3). Moreover, malignant tumour cells were found to be more susceptible to the inhibition of biological nucleophiles, crucial to their metabolic pathways in cell division, than are the normal cells (see Tables 1–3). Nonetheless, butenolides **1a–c**, **4**, and **6–9**, possessing C_4=C_5, were more toxic toward HEL, MEF, and Hef522 than **2**, **3**, and **5** (Table 3). Summing up the results, the view that reaction of a suitable system with sulfhydryl groups of biologically important proteins would play a significant role in the mechanisms by which these compounds exert their biological activities, is proven.

5. Conclusion

Adenine-containing phosphono-γ-(*Z*)-ethylidene-2,3-dimethoxybutenolide (**7**) and the respective phospho-

nothioanhydride **8**, as well as the butenolide-containing phosphonothioanhydride **12** were synthesised by chemical methods. Compounds **1a**–**c**, **2**–**4**, and **6**–**8** were allowed to react with L-cysteine. Rates of reactions of **1a**–**c** and **4** with L-cysteine were fast ($K_2 = 5095–6500$ l mol^{-1} min^{-1}). Thus, butenolides **1a**–**c** and **4** are biologically active. The rate constants for **2** were 120 l mol^{-1} min^{-1}, for **6** and **7** were 1200 and 1150 l mol^{-1} min^{-1}, respectively, and **3** was not reactive toward L-cysteine. Our results indicate that the C_4=C_5 is essential for biological activity. Hydrated analogues **2** and **3** are not reactive toward L-cysteine. Consequently, they did not show activity against malignant tumour cell lines. In addition, the *N*-acetyl-L-cysteine adduct product **5** was found not to be active against the examined malignant tumour cell lines, as expected. Phosphonates **6** and **7** as well as the thiophosphonate **9** possess weak biological activity. The C_4=C_5 in **6**, **7**, or **9** is sterically hindered by an adjacent phosphonate or thiophosphonate functionality at the C-6 position. As a result, the trap of L-cysteine by their C_4=C_5 would be much slower compare with **1a**–**c** and **4**. On the other hand, adenine-containing phosphonothioanhydride **8** exhibited profound activity against murine leukemias (L1210 and P388), breast carcinoma (MCF7), and human T-lymphoblasts (Molt4/C8 and CEM/0) cell lines. Compound **8** was very reactive toward L-cysteine or GSH precursor, *N*-acetyl-L-cysteine. This reactivity plays a significant role in the mechanism by which adenine-containing phosphonothioanhydride **8** directed its cytotoxicity toward tumour cell lines. Indeed, acylation of RDPR by compound **8** resulted in inactivation of the enzyme, observation of apoptosis inside MCF7 cells, and exhibition of biological activity against malignant tumour cell lines. On the other hand, phosphonothioanhydride **12**, lacking an adenine moiety, was found not to be an inhibitor for RDPR. Consequently, it did not show any anticancer activity. All compounds were found to be less toxic than ara-C against HEL, MEF, and normal fibroblasts (Hef522). Finally, normal cell lines were found to be less sensitive to this new class of compounds than malignant tumour cell lines.

6. Experimental

6.1. Chemistry

Reagents purchased from Fluka Chemical Co. (Switzerland). Solvents, including dry ether and THF, were obtained by distillation from the sodium ketyl of benzophenone under nitrogen. Other solvents were distilled and then stored over molecular sieves 4 Å. Absolute methanol and ethanol were purchased from Merck (Germany) and used as received. R1 ($\varepsilon_{280\ nm} = 189\,000$ M^{-1} cm^{-1}) and R2 ($\varepsilon_{280\ nm} = 130\,500$ M^{-1} cm^{-1}) were prepared as described 29b. *E. coli* TR was isolated from SK3981 [32] and TRR was isolated from K91/pMR14 [33]. ATP, NADPH, and Hoechst 33342 were obtained from Sigma (St. Louis, USA). DTT was purchased from Aldrich (USA).

Melting points (m.p.) were obtained with a Büchi 510 m.p. apparatus. Infrared (IR) spectra were recorded on a Beckman IR-8 spectrophotometer. The wavenumbers reported are referenced to the 1601 cm^{-1} absorption of polystyrene. Proton NMR spectra were obtained on a Varian XL-300 (300 MHz) Spectrometer. Dimethylsulfoxide-d_6 and D_2O were used as solvent; Me_4Si (δ 0.00 ppm) was used as an internal standard. All NMR chemical shifts are reported as δ values in parts per million (ppm) and coupling constants (J) are given in hertz (Hz). The splitting pattern abbreviations are as follows: s, singlet; d, doublet; t, triplet; m, unresolved multiplet due to the field strength of the instrument; and dd, doublet of doublets. UV–vis spectroscopy was carried out using a Shimadzu 160 spectrophotometer. Mass spectra were carried out on a VG 70-250 S mass spectrometer. Microanalyses were performed on a Perkin–Elmer 240-B microanalyser.

Purification refers to gravity column chromatography on a DEAE Sephadex or Merck Silica Gel 60 (particle size 230–400 mesh). Analytical TLC was performed on precoated plates purchased from Merck (Silica Gel 60 F_{254}, Germany). Compounds were visualised by use of UV light.

*6.1.1. Reactions of L-cysteine with purine-substituted butenolides **1a**–**c**, **2**–**4**, **6**–**8**, and phosphonothioanhydride **12***

An established procedure [3] was used for determination of the second-order rate constants at 25 °C for the reactions of **1a**–**c**, **2**–**4**, **6**–**8**, and **12** with L-cysteine.

*6.1.2. 4-N-Acetyl-L-cysteinyl-4-[2-(adenin-9-yl)ethyl]-2,3-dimethoxy-Δα,β-butenolide (distereoisomeric mixture **5**)*

To a solution of **4** (0.30 g, 0.99 mmol) in 50% aqueous (aq.) THF (15.0 mL, pH 7.4) was added *N*-acetyl-L-cysteine (0.22 g, 1.3 mmol). The mixture was stirred at 25 °C for 2.0 h. The solution was concentrated under reduced pressure, and the residue was purified by use of column chromatography (silica gel, EtOAc–MeOH 1:1 as eluant) to afford **5** (0.34 g, 0.73 mmol) in 73% yield: m.p. 184–187 °C (dec.); R_f (EtOAc–MeOH 3:1) 0.18; IR (NUJOL) ν 2600–3410 (NH_2, NH, CO_2H, $C_{8'}H$, $C_{2'}H$), 1774 (C=O), 1680 (C=O), 1668 (C=C) cm^{-1}; UV (EtOH) λ_{max} 260 (log ε 4.12); ^{1}H-NMR (DMSO-d_6–D_2O) δ 2.29 (br s, 3H, CH_3), 2.27–2.41 (m, 2H, C_5H_2), 2.72 (d, 2H, $J = 5.91$ Hz, SCH_2), 3.72 (br s, 3H, C_2OCH_3), 3.78 (t, $J = 5.91$ Hz, 1H, CHN), 4.01 (br s, 3H, C_3OCH_3), 4.45 (m, 2H, C_6H_2), 8.40, 8.68 (2 br s, 2H, $C_{2'}H + C_{8'}H$); CIMS m/z

467 [$M^+ + 1$]. Anal. Calc. for $C_{18}H_{22}N_6O_7S$: C, 46.35; H, 4.75; N, 18.02; S, 6.87. Found: C, 46.24; H, 4.80; N, 18.21; S, 6.75%.

6.1.3. (Z)-4-[2-Ammonium methylphosphono-2-(adenine-9-yl)ethylidene]-2,3-dimethoxy-Δα,β-butenolide (distereoisomeric mixture 7)

To a solution of **6** (4.12 g, 9.60 mmol) in CH_3CN (50 mL) was added concentrated NH_4OH solution (100 mL). The solution was refluxed for 6 h. The solvents were evaporated, and the residue was crystallised from EtOH to give **7** (2.69 g, 6.72 mmol) in 70% yield: m.p. 211–214 °C; R_f (EtOAc–MeOH 3:1) 0.10; IR (KBr) ν 2450–3600 (NH_4, NH_2), 3065 ($C_{8'}H$), 3054 ($C_{2'}H$), 2945 (C_5H), 1780 (C=O), 1695 (C=C), 1221 (P=O) cm^{-1}; UV (EtOH) λ_{max} 212 (log ε 4.60), λ_{max} 260 (log ε 4.25); ^{1}H-NMR (DMSO-d_6–D_2O) δ 3.71 (d, $J = 11.53$ Hz, 3H, $P(OCH_3)$), 3.76 (d, $J = 11.50$ Hz, 3H, $P(OCH_3)$ of other isomer), 3.80 (br s, 3H, C_2OCH_3), 4.24 (br s, 3H, C_3OCH_3), 6.14 (dd, $J = 16.01$, 31.78 Hz, 1H, PCH), 6.23 (dd, $J = 16.23$, 32.15 Hz, 1H, PCH of other isomer), 6.96–7.23 (m, 1H, =CH), 7.65, 7.83, 8.32, 8.41 (4 s, 2H, $C_{2'}H + C_{8'}H$ of isomers); ^{13}C-NMR (DMSO-d_6) δ 51.87, 52.01 ($POCH_3$), 59.98, 60.01 (2-OCH_3), 60.20, 60.46 (3-OCH_3), 82.16, 83.14 (C_6), 98.79, 99.87 (C_5), 126.13, 127.51 (C_4), 132.98, 133.04 ($C_{5'}$), 141.11, 142.20 (C_2), 144.62, 145.31 ($C_{2'}$), 147.98, 148.45 ($C_{4'}$), 149.79, 150.12 (C_3), 155.86, 156.41 ($C_{6'}$), 156.95, 157.02 ($C_{8'}$), 167.89, 167.96 (C=O); ^{31}P-NMR (acetone-d_6) δ 16.37, 16.45. Anal. Calc. for $C_{14}H_{19}N_6O_7P$: C, 40.58; H, 4.62; N, 20.28; P, 7.48. Found: C, 40.46; H, 4.59; N, 20.31; P, 7.60%.

6.1.4. (Z)-4-[2-Methyl-2-acetylthiophosphono-2-(adenine-9-yl)ethylidene]-2,3-dimethoxy-Δα,β-butenolide (distereoisomeric mixture 8)

To a solution of **7** (4.14 g, 9.99 mmol) in dry DMF (30 mL) was added $ClCO_2Et$ (1.50 g, 13.8 mmol) at 0 °C. After stirring for 15 min, $KSCOCH_3$ (1.20 g, 10.5 mmol) was added and the reaction mixture stirred at 25 °C for 1 h. The solvents were evaporated under reduced pressure, and the residue was purified by use of column chromatography (neutral silica gel, dry EtOAc as eluant) to afford **8** (3.87 g, 8.50 mmol) in 85% yield: m.p. 118–120 °C; R_f (EtOAc) 0.32; IR (NUJOL) ν 3143–3250 (NH_2), 3105 ($C_{8'}H$), 3071 ($C_{2'}H$), 2984 (C_5H), 1826 (SC=O), 1778 (C=O), 1690 (C=C), 1286 (P=O) cm^{-1}; UV (EtOH) λ_{max} 219 (log ε 4.54), λ_{max} 262 (log ε 4.18); ^{1}H-NMR (DMSO-d_6) δ 2.18 (br s, 3H, CH_3), 3.68 (d, $J = 12.0$ Hz, 3H, $P(OCH_3)$), 3.70 (d, $J = 12.0$ Hz, 3H, $P(OCH_3)$ of other isomer), 3.85 (br s, 3H, C_2OCH_3), 4.30 (br s, 3H, C_3OCH_3), 6.03–6.22 (m, 1H, PCH), 6.87–7.15 (m, 1H, =CH), 7.26 (br s, 2H, NH_2), 7.76, 8.35 (2 br s, 2H, $C_{2'}H + C_{8'}H$); ^{13}C-NMR (DMSO-d_6) δ 51.99, 52.30 ($POCH_3$), 60.02, 60.05 (2-OCH_3), 60.25, 60.34 (3-OCH_3), 79.65, 79.67 (CH_3), 83.23, 83.32 (C_6), 98.29, 99.17 (C_5), 128.63, 129.41 (C_4), 130.79, 132.11 ($C_{5'}$), 142.40, 142.86 (C_2), 146.10, 146.25 ($C_{2'}$), 148.12, 148.88 ($C_{4'}$), 148.19, 149.70 (C_3), 155.19, 156.08 ($C_{6'}$), 157.17, 157.31 ($C_{8'}$), 169.12, 169.90 (C=O), 180.43, 180.45 (SC=O); ^{31}P-NMR (acetone-d_6) δ 17.70, 17.76. Anal. Calc. for $C_{16}H_{18}N_5O_7PS$: C, 42.19; H, 3.98; N, 15.38; P, 6.81; S, 7.04. Found: C, 42.38; H, 4.19; N, 15.57; P, 7.04, S, 7.28%.

6.1.5. (Z)-4-[2-Methylthiophosphono-2-(adenine-9-yl)-ethylidene]-2,3-dimethoxy-Δα,β-butenolide (distereoisomeric mixture 9)

To a solution of **8** (2.28 g, 5.01 mmol) in THF (100 mL) was added a solution of *N*-acetyl-L-cysteine (0.82 g, 5.02 mmol) in H_2O (10 mL). The solution was stirred at 25 °C for 1 h. The solvents were evaporated under reduced pressure, and the residue was washed with EtOAc to afford a solid. Filtration and evaporation of the filtrate gave *N*,*S*-diacetylcysteine. Crystallisation of the solid from MeOH gave **9** (1.86 g, 4.50 mmol) in 90% yield: m.p. 174–176 °C; R_f (EtOAc–MeOH 3:1) 0.17; IR (KBr) ν 3100–3245 (OH, NH_2), 3098 ($C_{8'}H$), 3065 ($C_{2'}H$), 2978 (C_5H), 1783 (C=O), 1690 (C=C), 1398 (P=S) cm^{-1}; UV (EtOH) λ_{max} 212 (log ε 4.50), λ_{max} 259 (log ε 4.19); ^{1}H-NMR (DMSO-d_6–D_2O) δ 3.52 (d, $J =$ 12.0 Hz, 3H, $P(OCH_3)$), 3.59 (d, $J = 12.0$ Hz, 3H, $P(OCH_3)$ of other isomer), 3.77 (br s, 3H, C_2OCH_3), 4.18 (br s, 3H, C_3OCH_3), 5.87 (dd, $J = 16.0$, 32.2 Hz, 1H, PCH), 5.99 (dd, $J = 16.0$, 32.2 Hz, 1H, PCH of other isomer), 7.01–7.18 (m, 1H, =CH), 7.57, 7.90, 8.23 8.39 (4 s, 2H, $C_{2'}H + C_{8'}H$ of isomers); ^{13}C-NMR (DMSO-d_6) δ 47.98, 48.34 ($POCH_3$), 59.73, 60.10 (2-OCH_3), 60.45, 60.76 (3-OCH_3), 83.17, 83.95 (C_6), 98.00, 98.54 (C_5), 126.43, 127.05 (C_4), 133.12, 133.87 ($C_{5'}$), 142.09, 142.87 (C_2), 144.80, 145.43 ($C_{2'}$), 147.78, 148.11 ($C_{4'}$), 149.98, 150.04 (C_3), 156.07, 156.88 ($C_{6'}$), 157.15, 157.27 ($C_{8'}$), 166.97, 167.21 (C=O); ^{31}P-NMR (acetone-d_6) δ 18.70, 19.03. Anal. Calc. for $C_{14}H_{16}N_5O_6PS$: C, 40.68; H, 3.90; N, 16.94; P, 7.50; S, 7.75. Found: C, 40.66; H, 3.73; N, 17.05; P, 7.62; S, 7.83%.

6.1.6. (Z)-4-[2-Ammonium methylphosphonoethylidene]-2,3-dimethoxy-Δα,β-butenolide (11)

Compound **11** (80% yield) was prepared from **10** in the same manner that was described for the preparation of **7** from **6**. M.p. 95–96 °C; R_f (EtOAc) 0.19; IR (CH_2Cl_2) ν 2980–3560 (NH_4), 2876 (C_5H), 1778 (C=O), 1660 (C=C), 1249 (P=O) cm^{-1}; UV (EtOH) λ_{max} 215 (log ε 5.20); ^{1}H-NMR (DMSO-d_6–D_2O) δ 2.77 (dd, $J = 10.22$, 22.31 Hz, 2H, PCH_2), 3.71 (d, $J = 12.01$ Hz, 3H, $P(OCH_3)$), 3.79 (s, 3H, C_2OCH_3), 4.12 (s, 3H, C_3OCH_3), 5.64 (dt, $J = 7.24$, 12.42 Hz, 1H, =CH); ^{13}C-NMR (DMSO-d_6) δ 31.21 (C_6), 39.90 ($POCH_3$), 60.31 (2-OCH_3), 69.57 (3-OCH_3), 101.89 (C_5), 125.83 (C_4), 144.01 (C_2), 148.07 (C_3), 160.43 (C=O); ^{31}P-NMR (acetone-d_6) δ 17.38.

*6.1.7. (Z)-4-[2-Methyl-2-acetylthiophosphono-ethylidene]-2,3-dimethoxy-Δα,β-butenolide (**12**)*

Compound **12** (95% yield) was synthesised, as a foam, from **11** by the same method, which was described for the synthesis of **8** from **7**. R_f (EtOAc) 0.69; IR (CH_2Cl_2) ν 2908 (C_5H), 1830 (SC=O), 1780 (C=O), 1686 (C=C), 1288 (P=O) cm^{-1}; UV (EtOH) λ_{max} 218 (log ε 5.02); ^{1}H-NMR ($CDCl_3$) δ 2.21 (s, 3H, CH_3), 2.79 (dd, $J = 11.83$, 23.15 Hz, 2H, PCH_2), 3.70 (d, $J = 12.03$ Hz, 3H, $P(OCH_3)$), 3.82 (s, 3H, C_2OCH_3), 4.26 (s, 3H, C_3OCH_3), 5.76 (dt, $J = 7.64$, 11.85 Hz, 1H, =CH); ^{13}C-NMR ($CDCl_3$) δ 32.09 (C_6), 41.53 ($POCH_3$), 61.03 (2-OCH_3), 65.77 (3-OCH_3), 80.12 (CH_3), 102.48 (C_5), 127.10 (C_4), 143.49 (C_2), 148.75 (C_3), 167.23 (C=O), 179.86 (SC=O); ^{31}P-NMR (acetone-d_6) δ 18.26.

6.2. Biology

*6.2.1. Evaluation of the effectiveness of compounds **1a–c**, **2–4**, **6–8**, and **12** against RDPR*

E. coli RDPR (EC 1.17.4.1) was isolated and used as described [29]. Briefly, incubation of HEPES (50.0 mM, pH 7.60), $MgSO_4$ (15.0 mM), EDTA (1.0 mM), ATP (1.60 mM), NADPH (0.50 mM), TR (12.0 μM), TRR (0.79 μM), R1 subunit (12.8 μM), and R2 subunit (12.8 μM) along with variable concentrations of the compounds **1a–c**, **2–4**, **6–8**, and **12** at 37 °C for 20 min gave data shown in Table 1 (adenine-containing thiophosphonoanhydride **8** also afforded the same result only after 15 s incubation with RDPR). The remaining enzyme activity for the conversion of CDP to dCDP was determined by the method of Steeper and Steuart [31]. Control experiments were performed with no inhibitor in the incubation mixture.

6.2.2. Anticancer test procedure in vitro

Murine leukemias (L1210 and P388), breast carcinoma (MCF7), and human T-lymphoblasts (Molt4/C8 and CEM/0) cell lines were cultured in DMEM supplemented with 10% FBS, 2.0 mM glutamine, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C [13]. Under this condition, the generation time for L1210, P388, MCF7, Molt4/C8 and CEM/0 cells was about 13, 12, 17, 18, and 21 h, respectively. Compounds **1a–c**, **2–9**, **4** + cysteine, **4** + GSH, **8** + cysteine, **8** + GSH, and ara-C, at various concentrations, were added to L1210, P388, MCF7, Molt4/C8 and CEM/0 cells (200 cells per mL) in their exponential phase of growth. The cell numbers of the control cultures, as well as that of the cultures supplemented with the test compounds, were determined after 24, 48, and 72 h of growth. The IC_{50} values (Table 2) were estimated from dose–response curves compiled from two independent experiments and represent the compound concentration (μM) required to inhibit proliferation of the respective malignant tumour cell lines by 50% after 72 h incubation.

6.2.3. Observation of apoptotic morphology in MCF7 cells by a fluorescence microscope

Cells were grown on coverslips and remained untreated or were incubated with adenine-containing phosphonothioanhydride **8** (0.76 μM), **8** (0.76 μM) + GSH (0.76 μM), **8** (0.76 μM) + GSH (7.6 μM), **8** (0.76 μM) + DTT (0.76 μM), and **8** (0.76 μM) + DTT (7.6 μM) for 4.0 h. The coverslips were air-dried and fixed with freshly prepared 3.7% paraformaldehyde in phosphate-buffered saline (PBS) for 10.0 min. Residual aldehyde was quenched with 0.10 M glycine in PBS for 5.0 min. Cells were permeabilised for 2.0 min at room temperature (r.t.) with 0.5% Triton X-100 in PBS and blocked for 14.0 h at 4.0 °C with normal goat serum in PBS containing 0.2% bovine serum albumin (BSA) and 50.0 mM NH_4Cl (1:1000 μL μL^{-1}). Nuclei were labelled with Hoechst 33342 in PBS (1.0 μg mL^{-1}) for 15.0 min in the dark at r.t. [34]. The coverslips were mounted on glass slides and viewed under a Zeiss fluorescence microscope. The apoptotic morphology of the breast carcinoma MCF7 cells in the presence of compound **8** or different combinations of phosphonothioanhydride **8** with GSH or DTT is shown in Fig. 5.

6.2.4. Toxicity test procedure in vitro

Anticancer test procedure [13] was used to estimate the toxicity of **1a–c**, **2–9**, and ara-C against HEL, MEF, and Hef522 cell lines. The CC_{50} values (Table 3) were estimated from dose–response curves compiled from two independent experiments and represent the compound concentration (μM) required to inhibit the growth of the respective normal cell lines by 50% after 72 h incubation.

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