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ScienceDirect
European Journal of Medicinal Chemistry 44 (2009) 541–550
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
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Original article

Novel derivatives of 6-mercaptopurine: Synthesis, characterization and antiproliferative activities of *S*-allylthio-mercaptopurines

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Received 25 November 2007; received in revised form 26 March 2008; accepted 28 March 2008
Available online 4 April 2008

Abstract

Biologically active *S*-allylthio derivatives of 6-mercaptopurine (6-MP) and 6-mercaptopurine riboside (6-MPR) were synthesized. The products, *S*-allylthio-6-mercaptopurine (SA-6MP) and *S*-allylthio-6-mercaptopurine riboside (SA-6MPR) were characterized. The antiproliferative activity of the new prodrugs was tested on human leukemia and monolayer cell lines, and compared to that of their parent reactants. The new prodrugs acted by a concentration-dependent mechanism. They inhibited cell proliferation and induced-apoptosis more efficiently than the parent molecules. Leukemia cell lines were more sensitive to the new prodrugs than monolayer cell lines. Higher hydrophobicity of the derivatives improves their penetration into cells, where upon reaction with glutathione, *S*-allylthioglutathione (GSSA) is formed, and 6-MP or 6-MPR is released for further processing.

Keywords: 6-Mercaptopurine; *S*-Allylthio-6-mercaptopurine; Prodrug; Allicin; Antiproliferative effect; Apoptosis; Human tumor cell lines

1. Introduction

6-MP and 6-MPR are cytotoxic prodrugs that interfere with nucleic acid synthesis by either direct substitution of deoxythioGTP, thereby causing further modifications and mismatches upon replication, or by inhibition of de novo purine biosynthesis (recently reviewed [1]). They are commonly used, in combination with other drugs, in the treatment of leukemia or in its remission maintenance programs. 6-MP (first synthesized by Elion et al. [2]) has been one of the most effective antineoplastic prodrugs for the last 45 years [3]. It is widely used as an antileukemic agent in the treatment of childhood acute lymphoblastic leukemia (ALL), [4,5]. It also exerts immunosuppressive effects and is used in the treatment of inflammatory diseases such as Crohn's disease and ulcerative colitis [6]. Although various analogs of mercaptopurine have been devised, they suffer major therapeutic disadvantages, particularly dose limiting toxicity, which we here pursued to rectify by minimizing drug dosage [7].

Abbreviations: 6-MP, 6-Mercaptopurine; 6-MPR, 6-Mercaptopurine riboside; ALL, Acute lymphoblastic leukemia; B-CLL, Human B chronic lymphocytic leukemia cells; CSSA, *S*-Allylthiocysteine; CPSSA, *S*-Allylthiocaptopril; GSSA, *S*-Allylthioglutathione; HGPRT, Hypoxanthine-guanine phosphoribosyl transferase; MeMP, Methyl-mercaptopurine; MeTIMP, Methyl-thioinosine 5′-monophosphate; NTB, 2-Nitro 5-thio-benzoic acid; PBMC, Peripheral blood mononuclear cells; PI, Propidium iodide; PMS, Phenazine methosulfate; SA-6MP, *S*-Allylthio-6-mercaptopurine (also known as 6-allyldithiopurine or *S*-allyldisulfanyl-7H-purine); SA-6MPR, *S*-Allylthio-6-mercaptopurine riboside (also known as 2-(6-Allyldisulfanyl-purin-9-yl)-5-hydroxymethyl-tetrahydro-furan-3,4-diol); TPMT, Thiopurine methyltransferase; XTT, 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide.

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doi:10.1016/j.ejmech.2008.03.027

Thiopurines are prodrugs that are transformed enzymatically by three competitive enzymatic pathways: the first, xanthine oxidase, catalyzes the oxidation of 6-MP to the biologically inactive metabolite, thiouric acid. The second, hypoxanthine-guanine phosphoribosyl transferase (HGPRT) catalyzes the formation of 6-thioinosine monophosphate. Upon being further processed by cellular enzymes it is converted to thioguanine nucleotides that may be incorporated by polymerases directly into the DNA. The third, thiopurine methyltransferase (TPMT) catalyzes *S*-methylation of 6-MP to 6-methyl-mercaptopurine (MeMP) and *S*-methylation of 6-thioinosine monophosphate to *S*-methyl-thioinosine 5′-monophosphate (MeTIMP). The latter is a potent inhibitor of phosphorybosilpyrophosphate amidotransferase, the first step of de novo purine biosynthesis, thereby causing purine depletion [1,3,8,9].

Allicin, the biologically active compound from garlic, is produced upon crushing the garlic clove, thus exposing the enzyme alliinase (alliin lyase; EC 4.4.1.4) to its substrate, alliin (*S*-allyl-L-cysteine sulfoxide) [10] (Scheme 1).

Allicin is known to confer many health beneficial effects, amongst which are its anti-microbial, anti-fungal and anti-parasitic activities [11], anti-hypertensive activity [12], remedial effects on cardiovascular risk factors [13–15], anti-inflammatory activity [16] and anti-cancer activities [11,17–20].

Allicin is a short-lived compound, which rapidly reacts with free thiol groups [21–23] (Scheme 2). Previously, we showed that allicin derivatives, such as *S*-allylthiocysteine (CSSA) [21], *S*-allylthioglutathione (GSSA) [22,23], and *S*-allylthiocaptopril (CPSSA) [24] possess antioxidant and SH-modifying activities similar to those of allicin, albeit milder. We showed that a 16 h allicin treatment of, N-87 cells and CB2 (a Chinese hamster ovary cell line) caused inhibition of DNA synthesis and cell proliferation in a dose-response manner [19]. We also showed that allicin-induced apoptosis in B-CLL cells [20], HL60 and U937 cell lines [25]. We, therefore, hoped that the combination of the antiproliferative properties of allicin with those of 6-MP and 6-MPR, by chemical conjugation, would improve their anti-cancer and anti-inflammatory performance.

In the present work we describe the synthesis and the cytotoxic properties of two new prodrugs, *S*-allylthio-6-mercaptopurine (SA-6MP) and *S*-allylthio-6-mercaptopurine riboside (SA-6MPR). The effects of these prodrugs on the viability and proliferation of several human tumor cell lines were determined.

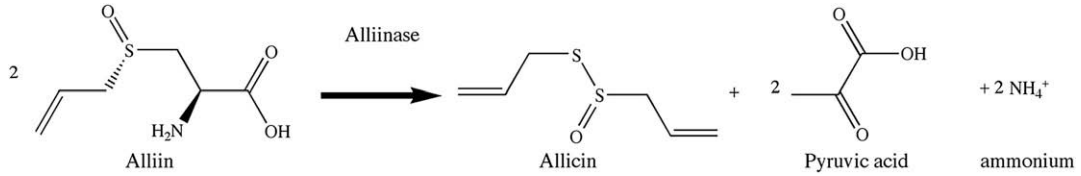
2. Materials and methods

2.1. Materials and general methods

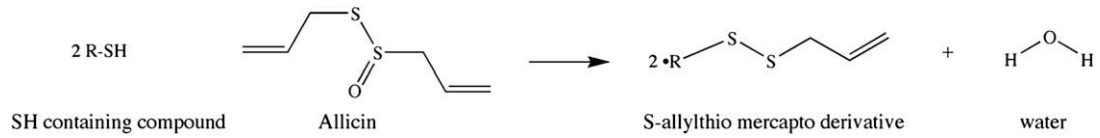
2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2*H*-tetrazolium hydroxide (XTT), 6-mercaptopurine, 6-mercaptopurine riboside, deuterochloroform ($CDCl_3$), phenazine methosulfate (PMS) and propidium iodide (PI) were obtained from Sigma (St. Louis, MO). [Methyl-^{3}H] thymidine was purchased from Amersham (UK). Alliin was synthesized as described [10]. Allicin was produced by applying synthetic alliin on an immobilized alliinase column [26]. The concentration was determined by HPLC as previously described [27]. 2-Nitro 5-mercaptobenzoic acid (NTB) was prepared as described [28]. Mass spectra were recorded on a Micromass Platform LCZ 4000 Mass Spectrometer Instrument, using ESI-Electro Spray Ionization Mode, at the following conditions: Samples were directly infused at 5 μl min^{-1}, maintaining the nitrogen flow at 360 l h^{-1}; the capillary used was 4.16 kV; the cone voltage was 43 V, and the extractor voltage was 4 V; the source block temperature was kept at 100 °C and the desolvation temperature was kept at 150 °C; LM RES 14.4; HM RES 14.4; and Ion Energy 0.5. NMR experiments were performed on a Bruker Avance-500 spectrometer. SA-6MP and SA-6MPR were dissolved in $CDCl_3$ at about 5–10 mM. Their complete assignments were determined using a combination of 1D (^{1}H, ^{13}C, DEPT) and 2D (gs-COSY, gs-HSQC) NMR experiments. HPLC analyses of 6-MP derivatives were done on a LiChrosorb RP-18 (7 μm) column, using methanol (60%) in water containing 0.01% trifluoroacetic acid, at a flow rate of 0.55 ml min^{-1}, and their absorbance was detected at 210 nm. The concentration of the pure *S*-allylthio-6-mercaptopurine derivatives was also determined with NTB [28] using ϵ_M 14,150 M^{-1} cm^{-1} at 412 nm.

2.2. Synthesis of S-allylthio-6MP (SA-6MP)

A solution of 6-MP (1 mmol) in ethanol (100 ml) was added at room temperature to allicin (0.55 mmol) in aqueous solution (55 ml). The pH was adjusted to 8.0–8.4 using solid $NaHCO_3$ to 0.025 M (final concentration). The reaction rate was monitored by HPLC analysis until 6-MP was no longer detected (about 10 h). Ethanol was partially removed by rotaevaporation and the slightly turbid solution was stored at 4 °C. The product, SA-6MP, which crystallized, was collected by filtration, washed with cold water and dried. A second harvest was done after removal of ethanol from the filtrate and storage



Scheme 1. Allicin production in the crushed garlic clove.



Scheme 2. Allicin reaction with free thiols.

at 4 °C for precipitation. The overall yield was 80%. Crystallization was obtained after dissolving the precipitate in ethanol and adding water.

2.3. Synthesis and isolation of S-allylthio-6MP riboside (SA-6MPR)

A solution of 6-MPR (0.6 mmol), in 0.04 M phosphate buffer, pH 7.2 (40 ml), was added at room temperature to a solution of allicin (0.35 mmol), in 50% ethanol (10 ml). The reaction proceeded for 4 h and was stored at 4 °C. The reaction rate was monitored by HPLC analysis. The product, a white precipitate, was harvested by filtration. A second harvest was done after removal of ethanol and storage at 4 °C. The overall yield was 85%. Re-crystallization was done as above.

2.4. Biological studies

2.4.1. Cell culture, cell viability and apoptosis assay

The following cell lines were used: N-87, a human gastric adenocarcinoma cell line; Hela HtTA-1 cells, a human cervix carcinoma cell line, clone HtTA-1 [29] and MDR HT-29, a human colon adenocarcinoma cell line. These cells were grown in monolayers, using Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics and 10% heat-inactivated fetal calf serum (FCS); All the other cells were grown in suspension: among them were established cell lines such as HL60, a human leukocyte promyelocytic leukemia; U937, human myelomonocytic cells; MOLT-4, a T-lymphoblastic cell line derived from acute lymphoblastic leukemia; Jurkat, a human T-cell, lymphoblast-like cell, Daudi, a B-lymphoblastoid cell line derived from Burkitt lymphoma. B-CLL, peripheral blood mononuclear cells (PBMC) were obtained from heparinized whole blood drawn from patients at Rai stage IV with their written consent. Blood cells were subjected to Ficoll density gradient centrifugation and the mononuclear cells were diluted to the desired concentration. The cells in suspensions were maintained in RPMI-1640 supplemented with 2 mM L-glutamine, antibiotics and 10% (v/v) heat-inactivated fetal calf serum (FCS).

Cell proliferation was determined by the XTT viability assay in 96-well plates, based on the reduction of tetrazolium salt to soluble formazan compounds by living cells. Cells (10,000–15,000 cell/well) were seeded in a 96-well plate. After 16 h incubation with various concentrations of 6-MP, 6-MPR and their *S*-allylthio-derivatives, 50 μl of XTT/PMS mixture (50 μM PMS, 0.1% XTT in medium) was added onto the cells. After an incubation period of 3–4 h at 37 °C the absorbance of the samples was measured in an ELISA Reader at 450 nm. SDS (1%, 10 μl/well) was added to reference wells before adding the XTT/PMS solution.

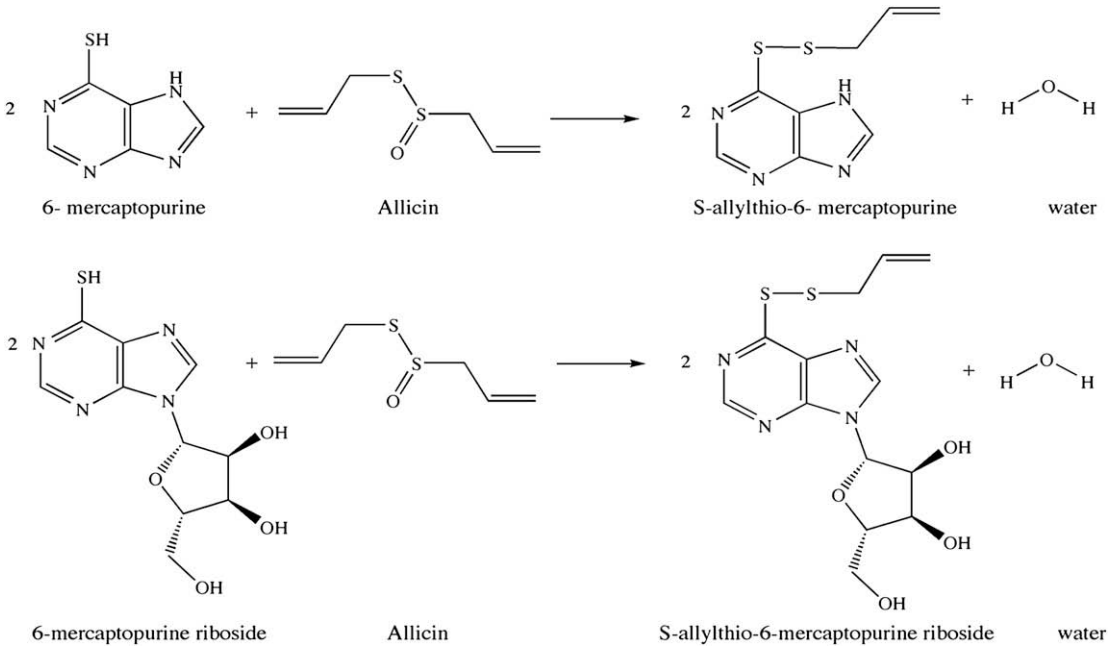
The effects of 6-MP derivatives on DNA synthesis were assessed by [methyl-^{3}H] thymidine incorporation into DNA. All the experiments performed with cells were carried out at least in triplicates. Adhesive cells (N-87, Hela HtTA-1, and MDR HT-29) were seeded at 10,000 cell/well (96-well plate) or 60,000 cells/well (24-well plate). Cells in suspension (B-CLL, Daudi, HL60, Jurkat, MOLT-4 and U937 cells) were seeded at 15,000 cells/well (96-wells plate) or 100,000 cells/well (24-well plate). Adhesive cells were grown at 37 °C for 6 h after seeding, before treatment. For the assessment of [methyl-^{3}H] thymidine incorporation, cells were treated with various concentrations of 6-MP derivatives at 37 °C for 16 h in the presence of [methyl-^{3}H] thymidine (0.8–1.0 μCi/well). Then, plates were frozen (−20 °C, 1 h). Adhesive cells were trypsinized before harvesting. Cells in suspension were directly harvested after thawing out the frozen cells.

Apoptosis analysis in B-CLL cells treated with 6-MP derivatives at different concentrations (16 h, at 37 °C) was done by FACS analysis. B-CLL cells were incubated with FITC-CD19 anti-human antibodies (Becton Dickinson, NJ, USA) for 20 min at 4 °C. After washing off the unbound antibodies, samples were incubated with 5 μl annexin-CY5 (Pharmingen, San Diego, CA, USA) in 10 mM HEPES pH 7.4 buffer containing 140 mM NaCl and 2.5 mM $CaCl_2$, (HBS) for 10 min at room temperature. Subsequently, unbound annexin was washed out and the samples were analyzed using FACScan analyzer (Becton Dickinson, NJ, USA). The lymphocytes were counted and gated according to their size in forward and side scatters.

Cell death was monitored by trypan blue dye exclusion test or propidium iodide (PI) incorporation. Treated cells were incubated with PI (2 μg ml^{-1}) for 20 min at 37 °C, washed with HBS and examined by fluorescence microscopy or analyzed by flow cytometry using fluorescence-activated cell sorting (Becton Dickinson FACScan Instrument using CellQuest software (BD Bioscience, San Jose, CA). Monolayer cells were trypsinized and washed with HBS before FACS analysis.

2.5. Statistical analysis

The results of viability and proliferation were expressed as mean values ± SD ($n = 3$–6). For each cell line the results were analyzed using two-way analysis of variance (ANOVA) followed by Bonferroni's posttest for the factors of the drugs used and their various concentrations, considering $p < 0.05$ as significant. IC_{50} values (mean ± SEM) were obtained from the linear range of the viability curve versus drug concentration (XTT assay).



Scheme 3. Allicin reaction with 6-MP and 6-MPR.

3. Results

3.1. Chemistry

Synthesis of *S*-allylthio-6-mercaptopurine (SA-6MP) and *S*-allylthio-6-mercaptopurine riboside (SA-6MPR) (Scheme 3), was confirmed by mass spectroscopy analysis (electrospray ionization, ESI) and NMR. SA-6MP is an off white crystal, (molecular weight 224; decomposed at 152–154 °C), showing a maximum absorbance in ethanol at 283 nm, E^{M}_{283} was 13,780 $M^{-1}\,cm^{-1}$. ESI-MS: *m/z* (%) = $[M+H]^+ = 225.2$ (40); DMSO = 79 (100). SA-6MPR appeared as white crystals, (ESI-MS: molecular weight 356; mp 105–107 °C), its maximum absorbance in ethanol at 284 nm E^{M}_{284} was 14,240 $M^{-1}\,cm^{-1}$. HPLC retention times for SA-6MP and SA-6MPR were 8.7 and 7.3 min, respectively (Fig. 1). NMR analysis is shown in Table 1. The *C* log *P* values (hydrophobicity partition coefficient) were: 6-MP: 0.823; SA-6MP: 1.344; 6-MPR: −1.191; SA-6MPR: 0.90.

3.2. Biological results

The antiproliferative effect of 6-MP and SA-6MP on various cell lines was assessed by determining [^{3}H] thymidine incorporation into the DNA. 6-MP and SA-6MP at 0–200 μM were applied to Daudi, Hela and N-87 cells cultured in 96-well plates in the presence of [^{3}H] thymidine for 16 h at 37 °C. In all the cell lines tested treatment resulted in a dose-dependent inhibition of cell proliferation. SA-6MP inhibited DNA synthesis at a much higher efficacy than 6-MP. The sensitivity to the prodrug is cell type dependent. Thus, in SA-6MP-treated Daudi cells, 50% inhibition was observed at about 20 μM, compared to 100 μM in N-87 cells and 110 μM in Hela cells. 6-MP caused no inhibition of proliferation, at the same concentrations (Fig. 2).

In parallel, the trypan blue dye exclusion test was used to assess cell death. Using cell death as a parameter, 6-MP and SA-6MP were also shown to be concentration and cell type

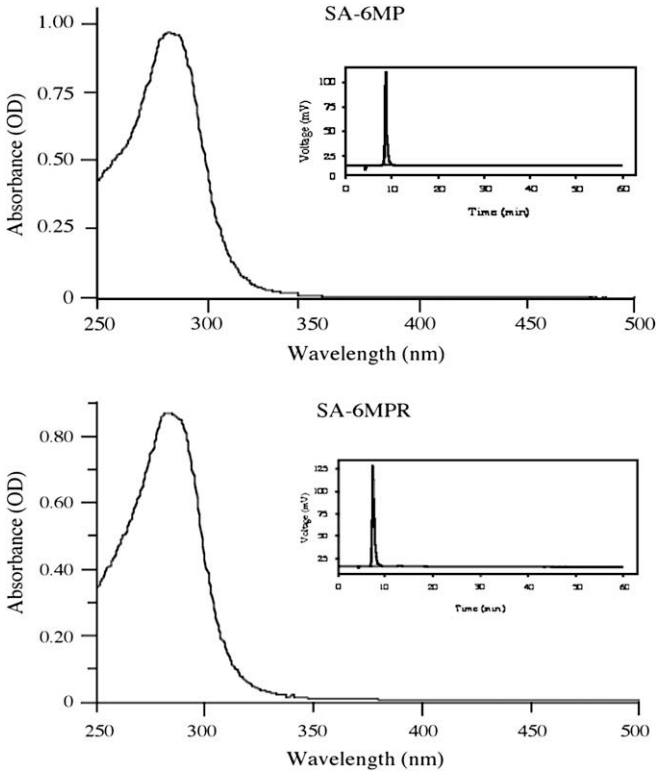


Fig. 1. Spectrum analysis in ethanol, of SA-6MP (upper part) and SA-6MPR (lower part). The inset showed the HPLC elution pattern of SA-6MP (upper) and SA-6MPR (lower). Absorbance was monitored at 210 nm.

Table 1
^{1}H and ^{13}C NMR chemical shifts of SA-6MP and SA-6MPR in $CDCl_3$

No.	SA-6MP		SA-6MPR	
	H (ppm)	C (ppm)	H (ppm)	C (ppm)
1	12.19 (s)			
2		131.10		132.53
3		160.46		161.40
5	8.96 (s)	152.11	8.78 (s)	151.60
7		149.36		147.29
9	8.30 (s)	141.47	8.10 (s)	143.64
12	3.61 (d)	41.76	3.56 (d)	41.65
13	5.91 (m)	132.00	5.87 (m)	131.84
14	5.14 (m)	119.63	5.12 (m)	119.80
15			5.88 (d)	91.5
17			4.39 (s)	87.72
18			3.90 (dd)	63.09
19			4.55 (d)	72.39
20			5.12 (m)	73.74

dependent. Monolayer cell lines, such as MDR HT-29 and Hela cells were almost non-sensitive to 6-MP and SA-6MP treatment for 16 h at 100 μM. However, MDR-HT-29 and Hela cells, treated with 150 μM SA-6MP showed a reduced viability, 40 and 65%, respectively, compared to non-treated cells. Treatment of Hela cells with 6-MP at 200 μM resulted in slightly reduced viability (75%). In Daudi cells treated with SA-6MP at 50 μM, the residual viability was about 20% after 16 h, whereas 6-MP at concentrations higher than 100 μM showed no significant effect on cell viability. B-CLL cells were almost insensitive to 6-MP treatment (100–200 μM, 16 h). SA-6MP at concentrations higher than 150 μM brought down the residual viability to 75% (Fig. 3).

In order to determine the toxic effect of 6-MP and SA-6MP on multidrug resistant cell lines, MDR HT-29 cells were treated for 16 h with 150 μM of either drug for 16 h, and then stained with PI. Phase microscopy results showed that the epithelial-like growth was inhibited in cells treated with SA-6MP (Fig. 4, upper right), whereas no inhibition was observed for 6-MP treatment. Fluorescence microscopy of cells stained with PI indicated that the inhibition resulted in cell death (Fig. 4, lower right).

The slight decrease in viability of B-CLL human peripheral blood mononuclear cells (PBMC) upon incubation in the presence of 6-MP or SA-6MP was further investigated. B-CLL cells were treated with various concentrations of the drugs and were subjected to flow cytometry analysis in order to monitor cells undergoing apoptosis. Sorting showed that there was no significant increase in the percent of apoptotic cells treated for 16 h with 6-MP at the range between 0 and 150 μM. When B-CLL cells were treated with SA-6MP, the percent of apoptotic cells at 50 and 100 μM increased to 38 and 95%, respectively (Fig. 5).

Apoptosis (FACS analysis with annexin) and cell death (trypan blue test) induced in B-CLL by 6-MP and SA-6MP did not occur simultaneously (shown in Fig. 6).

The inhibitory effects of 6-MP, 6-MPR, SA-6MP, and SA-6MPR on Daudi leukemia cell and on monolayer N-87 cells

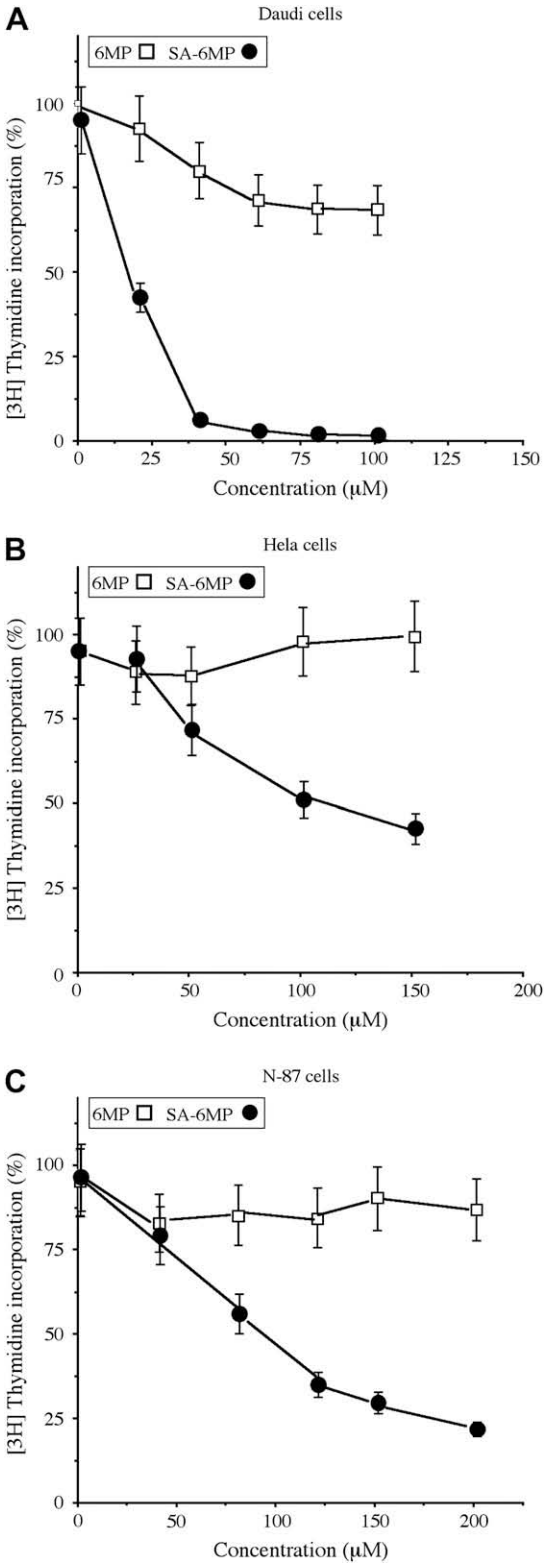


Fig. 2. Dependence of cell proliferation on 6-MP and SA-6MP concentration, as determined by [^{3}H] thymidine incorporation in Daudi (A), Hela (B) and N-87 (C). Non-treated cells were used as control (100%). Cells were treated for 16 h at 37 °C. The values presented are the mean ± SEM.

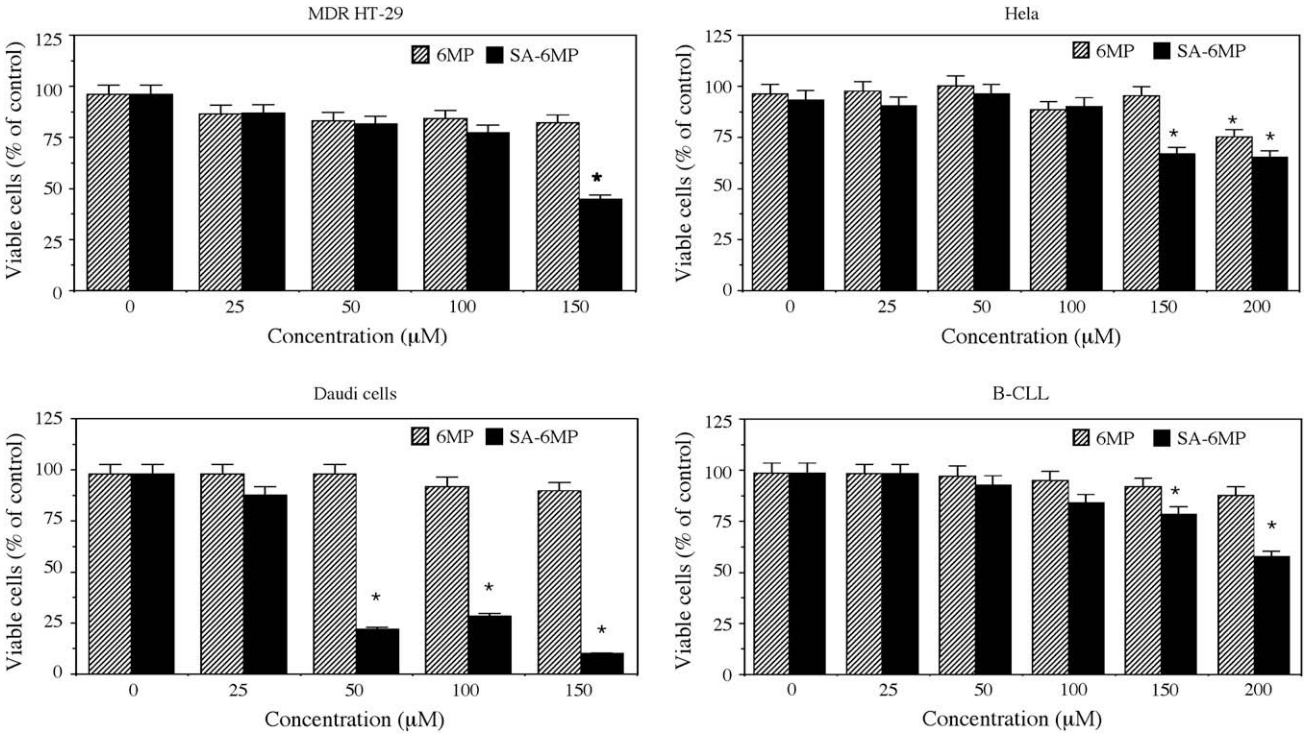


Fig. 3. The lethal effect of 6-MP and SA-6MP on cells. MDR-HT-29, Hela, Daudi and B-CLL cells were incubated with the prodrugs for 16 h incubation at 37 °C and stained with trypan blue and PI. Cells were counted after the trypan blue exclusion test and the percentage of viable cells was calculated. Alternatively, the percentage of viable PI stained cells was determined by FACS analysis. The values presented are the mean ± SD. Viability values that are significantly different ($p < 0.05$) from non-treated cells (*).

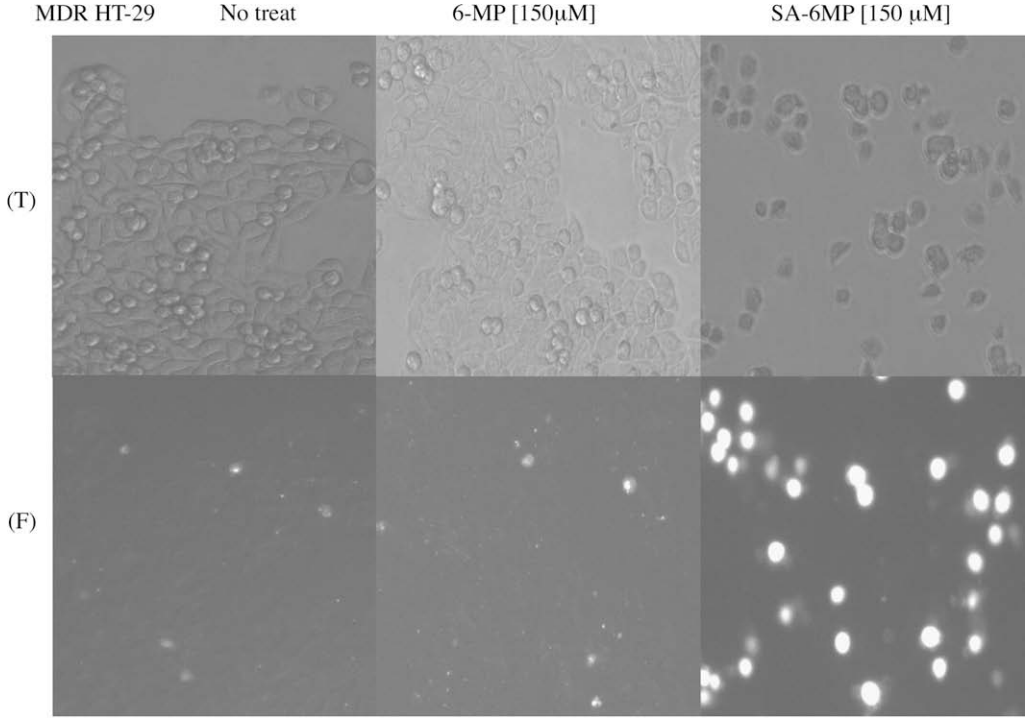


Fig. 4. Cell death assessment using PI staining. MDR HT-29 cells were cultured in the presence of 6-MP or SA-6MP (150 μM) for 16 h at 37 °C and stained with PI. Images of treated cells were observed by phase-contrast microscopy to determine normal growth patterns (T, upper panel) and fluorescent images of the treated cells indicated dead cells stained with propidium iodide, (F, lower panel).

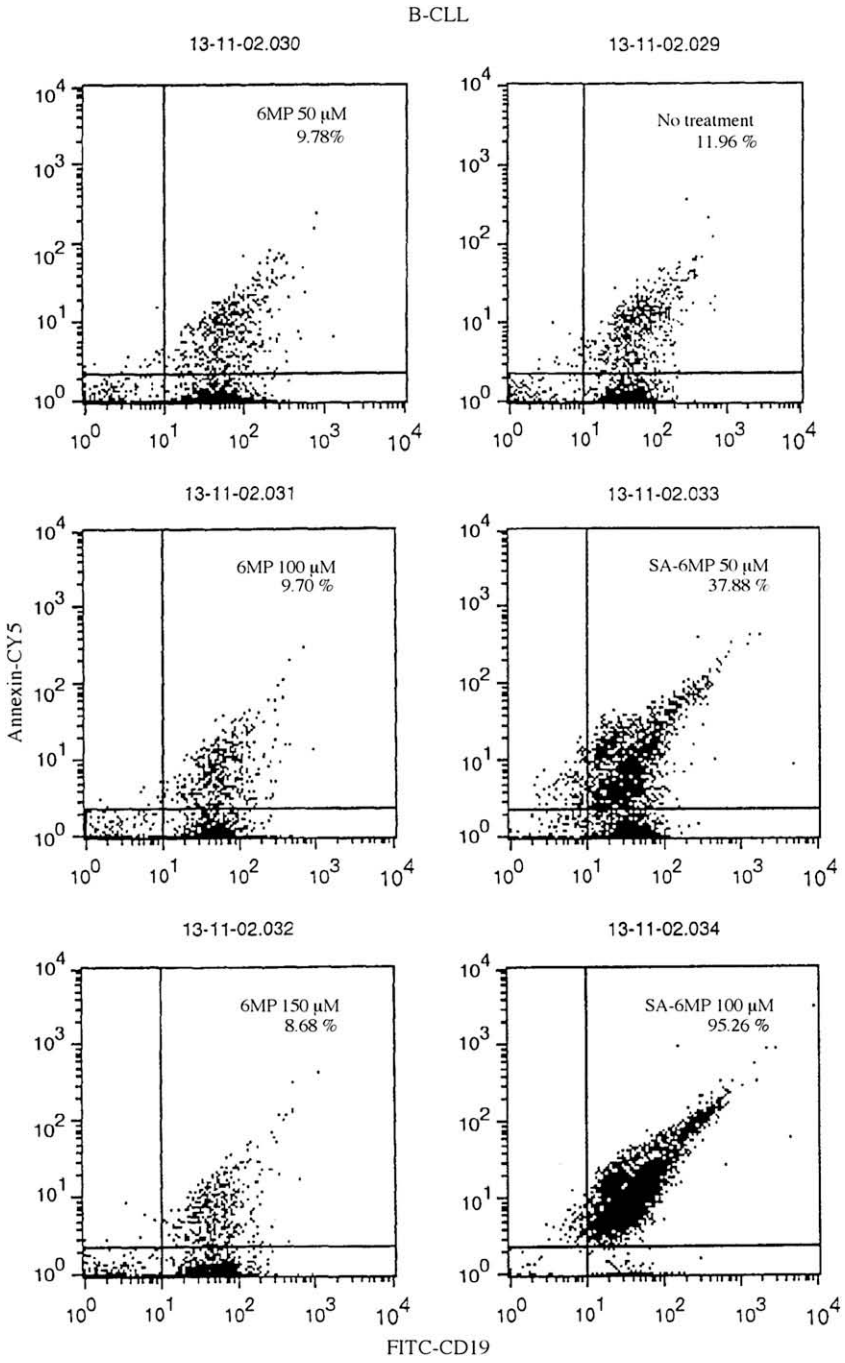


Fig. 5. FACS analysis of B-CLL cells treated with various concentrations of 6-MP and SA-6MP for 16 h at 37 °C. Cells were stained with annexin-CY5 and analyzed by FACS. The percentage of apoptotic cells at various drug concentrations is superimposed in the upper right part of each analysis.

were compared. Daudi cells and N-87 cells grown in the presence of 6-MP or 6-MPR (0–100 μM) showed no significant loss of cell proliferation. A slightly decreased proliferation was observed for N-87 cells at 150 μM (6-MP ∼ 80%; 6-MPR ∼75%, $p < 0.05$). SA-6MP, however, had a very potent antiproliferative effect on Daudi cells, reducing their proliferation to 15–30% at 50 μM. In N-87 cells treated with the same concentration, the residual proliferation was 50–60%. Treatment with SA-6MPR showed similar results. The residual proliferation of Daudi cells treated with SA-6MPR at 50 or 100 μM, was 60 and 25%, respectively, whereas in N-87 treated with SA-6MPR at 50 or 100 μM, it was about 70 and 55%, respectively. There was a complete loss of proliferation in N-87 cells treated with SA-6MPR at 150 μM (Fig. 7).

The concentration effects of 6-MP, 6-MPR SA-6MP and SA-6MPR on cell proliferation (IC_{50} values) were used to assess the efficacy of the drugs in various cell lines (Table 2). The new derivatives were found to be much more effective in inhibiting proliferation than the parent drugs. SA-6MP was a better agent than SA-6MPR. Among the leukemia cell

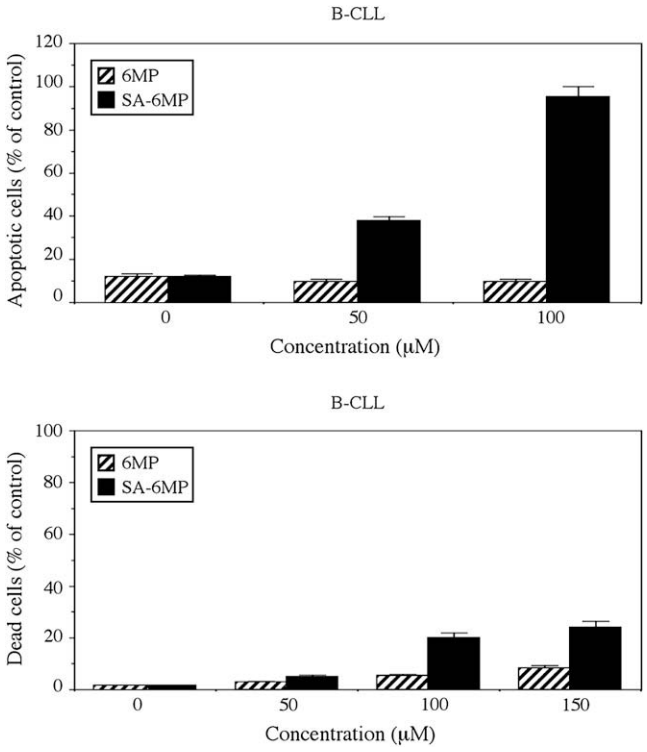


Fig. 6. Apoptotic B-CLL cells treated with 6-MP and SA-6MP (upper panel). Viability of cells treated with the new derivatives, between 0 and 150 μM, for 16 h at 37 °C was determined by trypan blue exclusion (lower panel).

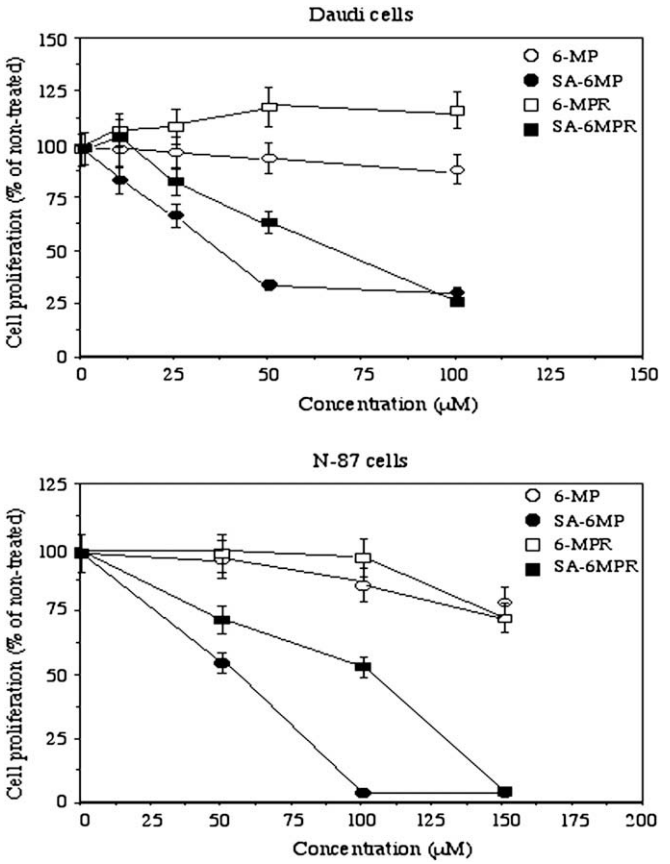


Fig. 7. The effects of 6-MP, 6-MPR, SA-6MP and SA-6MPR on Daudi and N-87 cell proliferation. Cells were incubated with the prodrugs for 16 h at 37 °C, and the antiproliferative effect was assessed using the XTT assay. The values presented are the mean ± SEM.

lines treated, the most sensitive was MOLT-4 cell line. It is noteworthy that, MOLT-4 and Jurkat cells, both T-cell leukemia cell lines, were more sensitive to 6-MPR than to 6-MP, compared to the other cell lines tested.

The sensitivity of the leukemia cell lines, Daudi, HL60 and U937 to treatment of either of the new prodrugs was similar. B-CLL cells from human peripheral blood mononuclear cells (PBMC) were highly resistant to the treatment. Nevertheless, the FACS results indicating apoptotic processes in progress (Fig. 5), suggest that longer incubation times may result in cell death.

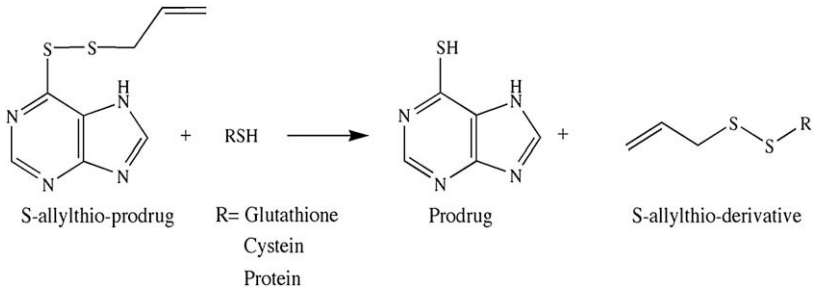
The monolayer cell lines tested were less sensitive to the novel prodrug than cells in suspension. In our experiments, the calculated IC_{50} of 6-MP for various cells was more than 200 μM under a 16 h exposure to the various drugs. This might seem a rather high concentration, as compared with other results reported in the nM range. Sugiyama et al. [30] showed

Table 2
Antiproliferative concentrations of various 6-MP derivatives on cancer cell lines

Cell line	6-MP (μM)	SA-6MP (μM)	6-MPR (μM)	SA-6MPR (μM)	Allicin (μM)
Daudi	>200	38.3 ± 3.9	[b]	70.8 ± 7.8	35.3 ± 4.2
HL60	[b]	42.0 ± 3.8	[c]	86.1 ± 9.5	5.5 ± 0.6
U937	>200	46.7 ± 4.8	>200	112.6 ± 10.9	19.7 ± 2.1
MOLT-4	50.5 ± 6.1	8.9 ± 1.2	24.8 ± 2.5	16.9 ± 2.3	[d]
Jurkat	>200	54.7 ± 6.3	99.0 ± 11.9	69.1 ± 7.5	[d]
B-CLL[a]	>200	129.1 ± 14.2	[d]	[d]	[d]
Hela	>200	114.4 ± 12.0	[c]	>200	103.5 ± 10.2
MDR HT-29	>200	126.3 ± 19.1	[b]	>200	83.8 ± 7.8
N-87	[b]	70.5 ± 8.5	[b]	120.0 ± 15.6	16.8 ± 3.3

Data are presented as IC_{50}, (mean ± SE) for 6-MP derivatives-treated cells for 16 h.
Cells in suspension (Daudi, HL60, U937, MOLT-4, Jurkat, B-CLL) were tested at 0–100 μM. Monolayer cells (Hela HtTA-1, MDR HT-29, N-87) were tested at 0–200 μM.

[a] Cell viability was determined by trypan blue dye exclusion assay.
[b] No change in viability.
[c] Viability of treated cells increase (+20%) of non-treated.
[d] Not determined.



Scheme 4. Proposed mechanism for the reaction of SA-6MP with free thiols in the cells.

in vitro effect of 6-MP on T-cell mitogen-induced blastogenesis of human peripheral blood mononuclear cells (PBMCs). The authors report values of IC_{50} after 4 days of treatment for azathioprine (AZ) and 6-MP, of 230.4 ± 231.3 and 149.5 ± 124.9 nM, respectively. As the treated cells in their work were different from those we used, and so was the period of exposure, it is impossible to compare their results with ours. However, when B-CLL cells were treated with allicin for 48 h (Fig. 1, [20]), the calculated IC_{50} for allicin induced-apoptosis in B-CLL, was 20 nM, whereas exposure of allicin for only 16 h resulted in IC_{50} which was approximately 1000 times higher (Table 2).

4. Discussion

In this study, two new 6-MP analogs, *S*-allylthio-6-MP (SA-6MP) and *S*-allylthio-6-MPR (SA-6MPR) were synthesized and characterized. The biological effects of these new prodrugs on several cancer cell lines were assessed. The IC_{50} values obtained from the XTT assay represent the susceptibility of cells to SA-6MP and SA-6MPR. While the various types of leukemia cells showed a high sensitivity to the new drugs, adhesive cell lines were less sensitive.

The effects of the *S*-allylthio derivatives on cell viability and proliferation were compared to those of the parent reactants. The biological effects of the new compounds were concentration dependent. Both, SA-6MP and SA-6MPR were found to exert more potent deleterious effects than those of the original prodrugs on all the cell lines tested. There was only a slight difference between the antiproliferative activities of SA-6MP and SA-6MPR, in favor of SA-6MP, in most cell lines. However, MOLT-4 and Jurkat cells were more sensitive to 6-MPR than to 6-MP, compared to the other cell lines tested As it was shown, by Fotoohi et al. [31], that 6-MP-resistant MOLT-4 cells exhibited enhanced sensitivity to methylmercaptopurine riboside (meMPR), a possible explanation for the ''inverted'' sensitivity may dwell in 6-MP resistance mechanisms. The resistant cells exhibited significant reduction in levels of mRNA encoding several proteins involved in the de novo purine synthesis, as well as in levels of ribonucleoside triphosphates, as compared to non-resistant cells. The authors suggest the existence of a distinct transport route for meMPR and the bypass of that of 6-MP.

It might have been expected that the combined activity of allicin and 6-mercaptopurines would increase the antiproliferative potential of the new derivatives (as compared with each parental component) but it did not exceed the antiproliferative activity of allicin. This can be explained by the dual potency of the allicin molecule. It did, however, improve the antiproliferative properties of 6-MP and 6-MPR.

The increased potency of the *S*-allylthio-derivatives (as compared to the parent prodrugs) can be attributed to three mechanisms of action

(a) The combined properties of both moieties, i.e. the mercaptopurine, a nucleotide analog that interferes with nucleic acid synthesis, and the allylmercapto residue (derived from allicin), that causes depletion of reduced glutathione and other essential free SH groups in the cell, thereby leading to apoptosis [25]. Both effects were shown to be exerted by the new prodrugs; inhibition of DNA synthesis in Daudi, Hela and N-87 cells, and an increased number of apoptotic B-CLL cells.
(b) Higher hydrophobicity of the new prodrugs enables better penetration into the cells, as compared with the parent molecules. Consequently, larger amounts of 6-MP or 6-MPR are released from the intracellular reaction between glutathione and the allylthio-prodrugs (paper in preparation).
(c) 6-MP and 6-MPR have a free SH, which upon oxidation, forms an inactive dimer connected by an S–S bridge (purine–*S*–*S*–purine) [32]. Nonactive 6-MP dimers were indeed found in the medium of 6-MP treated cell lines but not in the medium of SA-6MP treated cells. The fact that only a fraction of the thiopurine molecules occurs in its active form explains the need for a high prodrug concentration. The allylthio moiety of the new derivative protects the free SH from such oxidation. Only later, upon entry to the cellular reducing environment is the mixed disulfide SA-6MP cleaved, which renders higher efficiency at lower concentrations. The overall process inside the cells can be summarized in Scheme 4.

The highest antiproliferative activity of all the compounds tested was exerted by allicin [19,20,25]. Since targeted killing by allicin production in situ [19,20] is a complex procedure, and other means of administration suffer drawbacks, its combination with 6-MP and 6-MPR is the most feasible treatment at present.

Acknowledgement

We would like to thank Dr. Anna Gakamsky, Department of Biological Chemistry, Weizmann Institute of Science, for her help in the statistical analysis.

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