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S-allyl derivatives of 6-mercaptopurine are highly potent drugs against human B-CLL through synergism between 6-mercaptopurine and allicin

Talia Miron[a,*], Meir Wilchek[a], Lev Shvidel[b], Alain Berrebi[b], Fabian D. Arditti[a,b]

[a] Departments of Biological Chemistry, Weizmann Institute of Science, Rehovot, Israel
[b] Department of Hematology, Kaplan Medical Center, Rehovot, Israel

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ABSTRACT

S-allylthio-6-mercaptopurine and its ribose derivative were tested for anti-leukemic activity, using a human- mouse B-CLL model. The novel prodrugs contain two components, a purine analog, which interferes with DNA synthesis, and an S-allylthio, readily engaging in thiol-disulfide exchange reactions. The latter component targets the redox homeostasis which is more sensitive in leukemic cells, than in normal B-cells. Upon administration, the prodrug permeates cells, instantly reacts with free thiol, forming S-allyl mixed disulfides and releasing purine. Several cycles of thiol-disulfide exchange reactions occur, thus extending the duration of the prodrug effects.

The concerted action of 2 components, as compared with purine alone, boosted *in vitro* apoptotis in B-CLL cells from 10% to 38%, and decreased in *vivo* engraftment of B-CLL from 30% to 0.7%.

1. Introduction

The cytotoxic effects of allicin, extensively studied previously [1–3] led us to investigate the therapeutic potential of more promising derivatives of this molecule.

Allicin, the highly active compound found in the extract of freshly crushed garlic, is the product of the interaction of alliin (S-allyl-L-cysteine sulfoxide) with the enzyme alliinase (alliin lyase: EC 4.4.1.4). Allicin shows anticancer properties in a dose-dependent manner in various *in vitro* systems. Antiproliferative effects, cell cycle arrest [G_2/M] and induction of apoptosis were tested in mammalian cancer cell lines, such as human mammary cancer cells, (MCF-7), Ishikawa endometrial cancer cells, HT-29 cells originating from a human colon carcinoma [1], gastric cancer SGC-7901 cells [4], HL60 human promyelocytic leukemia-derived cells and U937 human myelomonocytic cells [5].

In situ generation of allicin and the consecutive killing of tumor cells in mice were previously obtained by targeting conjugates of alliinase and monoclonal antibodies against specific tumor markers to the respective cells [2,3]. After binding of the enzyme conjugate to tumor cells, the substrate, alliin was administrated intraperitoneally (IP) and allicin produced on the cell surface exerted its cytotoxic activity on the cells.

The short half-life of allicin and the fast rate of its clearance from the blood, established a belief that it might not be applicable for *in vivo* use. However, its wide range of activity suggests the formation of intermediates that exert therapeutic effects in various tissues [1,6,7]. It has been shown that the allylmercapto residue retains the disulfide exchange properties of its parental molecule, allicin, particularly when it is conjugated to an aromatic residue [8].

6-Mercaptopurine (6-MP) and its various derivatives are SH-containing purine analogs, manifesting cytotoxic and immunosuppressive properties. They are therefore widely used in the treatment of hematological malignancies, chronic inflammatory diseases and transplantation [reviewed [9–11]. 6-MP derivatives inhibit the proliferation of T and B lymphocytes, consequently leading to reduced numbers of cytotoxic T cells and plasma cells. The different antimetabolite, antineoplastic and immunosuppressive activities of thiopurines are due to their metabolic conversion by several enzymes such as;

Abbreviations: 6-MP, 6-mercaptopurine; 6-MPR, 6-mercaptopurine riboside; B-CLL, human B chronic lymphocytic leukemia; CLL, chronic lymphocytic leukemia; GGCSγ, -glutamyl cysteine synthase; GSH, reduced glutathione; HGPRT, hypoxanthine–guanine phosphoribosyl transferase; IP, intraperitoneal injection; MMPR, methyl-mercaptopurine ribonucleotide; PBMC, peripheral blood mononuclear cells; PEITCβ, -phenylethyl isothiocyanate; PI, propidium iodide; SA-6MP, S-allylthio-6-mercaptopurine; SA-6MPR, S-allylthio-6-mercaptopurine riboside; TIMP, thioininosine monophosphate; TPMT, thiopurine methyltransferase.

* Corresponding author at: Weizmann Institute of Science, 76100 Rehovot, Herzel Street 230, Israel. Tel.: +972 8 9343627; fax: +972 8 9468256.
E-mail address: Talia.Miron@weizmann.ac.il (T. Miron).

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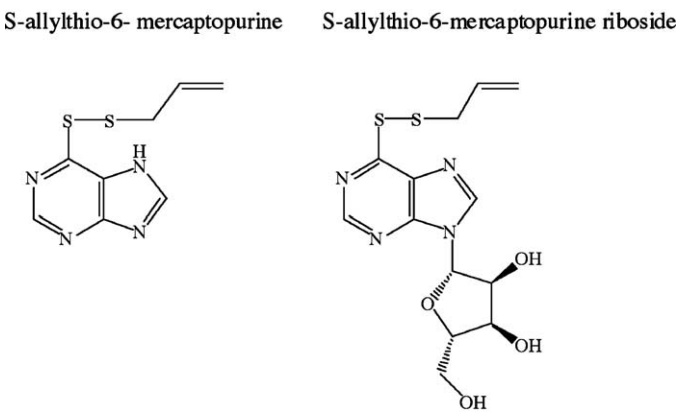

Fig. 1. The chemical structure of S-allylthio-6-mercaptopurine (SA-6MP) and S-allylthio-6-mercaptopurine riboside (SA-6MPR).

thiopurine-*S*-methyltransferase (TPMT), that catalyses the methylation of 6MP to 6-methyl-mercaptopurine ribonucleotide (MMPR); hypoxanthine–guanine-phosphoribosyltransferase (HGPRT) that converts 6-MP to 6-thioguanine nucleotides and 6-thioininosine monophosphate (TIMP). Metabolite accumulation of the first two enzymes results in the inhibition of nucleic acid biosynthesis, and incorporation to DNA during synthesis [12,13]. Xanthine oxidase catalyses the conversion of 6-MP to thiourate, the accumulation of which has not been found to cause any damage.

The bioavailability of thiopurines is very low due to their cellular inaccessibility. Additionally, the metabolic conversion of these prodrugs by three different enzymes, results not only in a great variability of activity as mentioned above but also in their low bioavailability.

The propensity of allicin to react with SH-containing molecules and form S-allyl mixed disulfide, inspired the synthesis of allicin chimeras with 6-MP and its derivative, 6-MP-riboside (6-MPR). The resulting molecules, S-allyl-6-MP and S-allyl-6-MPR (Fig. 1) are comprised of two different cytotoxic moieties, thus enhancing the anticancer and antiiflammatory potency of the purine analogs. Additionally, the novel prodrugs have a much better cell permeability than either of the parent compounds 6-MP and 6-MPR, which improves their performance.

Moreover, while allicin has a short shelf life, the novel allyl derivatives of purine analogs are stable powders, a fact that facilitates storage and marketing.

The aim of this study was to evaluate the cytotoxic potential of the two novel chimeric prodrugs, S-allyl-6-MP and S-allyl-6-MPR, by using primary human B-cell chronic lymphocytic leukemia (B-CLL) cells obtained from the peripheral blood of patients at Binet stage C. In contrast to the increased proliferation of malignant cells that characterizes most malignant diseases, B-CLL is characterized by a progressive accumulation of B cells due to a failure in their apoptosis machinery. The accumulation of these cells in the bone marrow and peripheral blood is accompanied by abnormal hematopoiesis and the generation of autoantibodies by the aberrant B cells. Isolated primary B-CLL cells, however, have a short life span (3–6 weeks), therefore an *in vivo* model may not last more than 3 weeks [3]. The effect of the aforementioned prodrugs was examined both *in vitro* and *in vivo.*

2. Materials and methods

2.1. Chemicals

DMSO, 6-mercaptopurine (6-MP), 6-mercaptopurine riboside (6-MPR) and propidium iodide (PI) were purchased from Sigma (St. Louis, MO). S-allyl thio-6-MP (SA-6MP) and S-allyl thio-6-MPR (SA-6MPR) were synthesized according to Miron et al. [14], RPMI-1640 medium, fetal bovine serum (FBS), glutamine (100×), and Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS × 10) were obtained from Biological Industries (Beth Haemek, Israel).

PBS × 1 containing tri sodium citrate 1% was prepared and stored at 4 °C.

2.2. Preparation of B-CLL cells

B-CLL, peripheral blood mononuclear cells (PBMC) were obtained from heparinized whole blood drawn from CLL patients at Binet stage C (human B chronic lymphocytic leukemia, B-CLL) with their written consent. Blood cells were subjected to Ficoll–Hypaque density gradient centrifugation and the mononuclear cells were diluted to the desired concentration in PBS. Cells were washed with PBS by centrifugation and were resuspended in PBS to a final concentration of 150×10^6 cells ml^{-1}.

2.3. In vitro studies

B-CLL cells were seeded at 1.5×10^4 cells per well (96-well plates) or 5×10^5 cells per well (24-well plates) and were treated with the 6-MP derivatives at different concentrations, at 37 °C for 16 h or 48 h. The cells were cultured in RPMI-1640 supplemented with 2 mM L-glutamine, antibiotics and 10% FBS.

Cell death was monitored by PI staining (2 μg ml^{-1}) for 30 min. After staining, cells were washed with PBS, and observed under fluorescence and phase microscopes. Photomicrographs were taken at 20× magnification.

Apoptosis was examined in B-CLL cells treated with 6-MP derivatives at different concentration (16 h, at 37 °C) by FACS analysis. Treated B-CLL cells were incubated with Annexin-CY5 (PharMingen, San Diego, CA) in Annexin buffer for 10 min at room temperature. Subsequently, unbound Annexin was washed out and samples were analyzed by using FACScan analyzer (Becton-Dickinson, NJ, USA.

2.4. In vivo studies

The Weizmann Institutional Animal Care and Use Committee approved all the protocols and animal studies. Mice CB-17/ICR-SCID Beige (six to eight mice/group), 9 weeks old, females were from Harlan laboratories Ltd., (Jerusalem, Israel). Mice were irradiated with 215 Rads 24 h prior to delivery of the human cells. Irradiation was carried out with Gammacel II 40 Exactor (MDS Nordion, Canada). Primary human B-CLL cells were injected by the intraperitoneal route (IP) at 150×10^6 cells in 1 ml PBS per mouse. Mice were treated with prodrugs 1 day after B-CLL injection, for 10 successive days. Treatment was based on the equivalent of 1–2 times of the maximum human daily 6-MP dose [15]. Prodrugs were administered IP in 200 μl PBS containing 1% DMSO. Stock solutions 100× of the various derivates were prepared in DMSO. Each day, a fresh dilution was prepared with PBS. Vehicle (transplanted mice) and control (non-transplanted mice) were injected with 1% DMSO in PBS. Administration of the prodrugs was done at 11 μmols/kg (6-MP 1.9 mg/kg, SA-6MP 2.5 mg/kg, and SA-6MPR 3.8 mg/kg). On day 15 after the onset of the experiment, 6 ml of citrate–PBS were injected IP. Mice were killed by dislocation, and the peritoneal washes were collected, cells were collected by centrifugation, and analyzed by FACS for content of human B-CLL cells (CD5, CD19, CD45 antigen presence), and their apoptotic state (Annexin).

For the detection of apoptosis, cells were incubated with FITC-CD45 anti-human antibodies (Becton Dickinson, NJ, USA) for 20 min at 4 °C. After washing off the unbound antibodies, samples were incubated with Annexin-CY5 (5 mmol/L HEPES pH 7.4 buffer, containing 140 mM NaCl and 2.5 mM $CaCl_2$) for 10 min at room temperature. Subsequently, unbound Annexin was washed out and FACS analysis of the cells was performed. The lymphocytes were counted and gated according to their size in forward and side scatters.

2.5. Statistic analysis

Results representing cell viability, degree of apoptosis and presence of human markers were expressed as mean values and their SD or SE. Comparison of values was assessed using Student's *t*-test and was compared to the vehicle group. Differences were considered statistically significant for values of $p < 0.05$.

3. Results

3.1. In vitro effect of the S-allyl-6-MP derivatives on B-CLL cells

Based on previous *in vitro* studies of the 6-MP derivatives' effects at a range of concentrations, on various cells lines [14], cell death upon treatment with 6-MP derivatives was tested (Fig. 1), B-CLL cells were cultured with prodrugs at a final concentration of 50 μM for 48 h. Treatment of human B-CLL with the novel 6-MP derivatives resulted in higher cell death rates, than those induced by the parent molecules as shown in Fig. 2 (A and B) (Table 1).

The ability of the novel conjugates to induce apoptosis in B-CLL, *in vitro*, was done at a lower concentration of the prodrug (SA-6MP,