Leukemia (2001) 15, 1633–1640

www.nature.com/leu

Sulfasalazine, a potent suppressor of lymphoma growth by inhibition of the x_c^- cystine transporter: a new action for an old drug

PW Gout[1], AR Buckley[2], CR Simms[1] and N Bruchovsky[1]

[1]*Department of Cancer Endocrinology, BC Cancer Agency, Vancouver, BC, Canada; and* [2]*College of Pharmacy, University of Cincinnati Medical Center, Cincinnati, OH, USA*

Although cyst(e)ine is nutritionally a non-essential amino acid, lymphoid cells cannot synthesize it, rendering their growth dependent on uptake of cyst(e)ine from their micro-environment. Accordingly, we previously suggested that the x_c^- plasma membrane cystine transporter provided a target for lymphoid cancer therapy. Its inhibition could lead to cyst(e)ine deficiency in lymphoma cells via reduction of both their cystine uptake and cysteine supply by somatic cells. In this study, using rat Nb2 lymphoma cultures, drugs were screened for growth arrest based on x_c^- inhibition. Sulfasalazine was fortuitously found to be a novel, potent inhibitor of the x_c^- transporter. It showed high rat lymphoma growth-inhibitory and lytic activity *in vitro* (IC_{50} = 0.16 mM), based specifically on inhibition of x_c^--mediated cystine uptake, in contrast to its colonic metabolites, sulfapyridine and 5-aminosalicylic acid. Sulfasalazine was even more effective against human non-Hodgkin's lymphoma (DoHH2) cultures. In rats (n = 13), sulfasalazine (i.p.) markedly inhibited growth of well-developed, rapidly growing rat Nb2 lymphoma transplants without apparent side-effects. Reduced, macrophage-mediated supply of cysteine was probably involved. In five rats, 90–100% tumor growth suppression, relative to controls, was obtained. The x_c^- cystine transporter represents a novel target for sulfasalazine-like drugs with high potential for application in therapy of lymphoblastic and other malignancies dependent on extracellular cyst(e)ine. *Leukemia* (2001) **15,** 1633–1640.
Keywords: sulfasalazine; x_c^- cystine transporter; Nb2 lymphoma; anticancer agent

Introduction

In past decades, significant advances have been made in the treatment of lymphomas and leukemias, primarily as a result of combination chemotherapy. A cure for these malignancies, however, has not yet been accomplished.[1] To identify new therapeutic targets and potentially useful drugs for treatment of these diseases, we developed an experimental *in vitro* and *in vivo* model for lymphoblastic cancers as they progress toward greater malignancy.[2] The model consists of a rat pre-T 'Nb2 lymphoma' cell line at an early stage of malignant progression and sublines representing different stages of advanced progression, including growth factor autonomy and increased metastatic ability.[2,3] In view of the origin and cytokine dependence of the parental line, the Nb2 lymphoma cell lines may serve as a model for lymphoblastic non-Hodgkin's lymphoma (NHL) and childhood acute lymphoblastic leukemia (ALL).[1,2]

Our research focused on a specific growth and viability requirement of lymphoid cells. L-cystine, or its reduced form, L-cysteine, is nutritionally a non-essential amino acid since it can be generated in the body from L-methionine. However, mammalian cells of lymphoid origin, including human leukemic cells, are unable to synthesize cyst(e)ine from methionine, rendering the cells critically dependent on uptake of the amino acid from their micro-environment.[4] Nb2 lymphoma cell lines are also dependent on extracellular cyst(e)ine for growth.[5] Lymphoid cells typically exhibit a low uptake capability for cystine as distinct from cysteine which they take up readily.[6] In culture medium, cystine is the predominant form of the amino acid due to rapid oxidation of cysteine.[7] Consequently, lymphoid cell cultures generally require addition of a cystine uptake enhancer such as 2-mercaptoethanol (2-ME) or elevated levels of cystine in the medium.[5,7] Alternatively, the cultures can be maintained using feeder layers of macrophages which provide the lymphoid cells with cysteine.[8] This phenomenon is based on the ability of macrophages to take up extracellular cystine via a plasma membrane cystine transporter (x_c^-), reduce the amino acid intracellularly and secrete it as cysteine.[8,9] This mechanism is apparently also used by macrophages *in vivo* to supply lymphocytes with cysteine to induce their activity.[10,11]

Recently, we showed that the malignant progression of the Nb2 lymphoma was associated with expression by Nb2-SFJCD1 subline cells of an active x_c^- cystine transporter, as used by macrophages. This acquired property enhanced the growth autonomy of these subline cells, enabling them to take up extracellular cystine in contrast to other Nb2 cell lines.[5] The studies led to the suggestion that the x_c^- cystine transporter provided a potentially useful target for therapy of lymphoblastic malignancies. Its inhibition *in vivo* would not only inhibit cystine uptake by lymphoma cells expressing an x_c^- cystine transporter, but also reduce cystine uptake by macrophages and hence interfere with the maintenance of cysteine levels in the circulation.[5] The latter effect could lead to a marked decrease in plasma cysteine levels since cysteine has a short half-life.[7] It was envisioned that a short-term inhibition of the x_c^- cystine transporter, as induced by a drug, could promote elimination of relatively fast growing lymphoma cells by cysteine starvation without major side-effects to the host.

In the present study, a number of clinically useful drugs was screened for inhibition of the x_c^- cystine transporter. It was found that sulfasalazine, an immunosuppressant, is a novel and potent inhibitor of this transporter. *In vitro*, sulfasalazine at therapeutic levels markedly inhibited lymphoma cell replication, in contrast to its colonic metabolites sulfapyridine and 5-aminosalicylic acid, an effect based specifically on inhibition of x_c^--mediated cystine uptake. Intraperitoneal administration of sulfasalazine to rats led to a high reduction in growth of lymphoma transplants without major toxicity to the hosts, indicating that this drug has a high potential for application in therapy of lymphoblastic malignancies, as outlined in a preliminary account.[12]

Correspondence: PW Gout, Department of Cancer Endocrinology, BC Cancer Agency, 600 West 10th Avenue, Vancouver, BC, Canada V5Z 4E6; Fax: (604) 877–6011
Received 9 February 2001; accepted 25 May 2001

Materials and methods

Materials

All drugs, compounds, solvents or culture media were obtained from Sigma-Aldrich Canada Ltd, Oakville, ON, Canada, unless otherwise indicated.

Cell cultures

Nb2-SFJCD1 cells were cultured as suspensions in Fischer's medium (FM) supplemented with 10% horse gelding serum (lactogen-deficient; one batch, ICN Biomedicals, Aurora, OH, USA), penicillin (50 U/ml) and streptomycin (50 μg/ml), as previously described.[2,5] FM is cysteine-free but contains cystine at 84 μM. The supplemented culture medium supports proliferation of Nb2-SFJCD1 cells in absence of cystine uptake enhancers such as 2-ME by virtue of their x_c^- transporter.[5] Nb2-U17 cells were cultured in FM, supplemented with 10% fetal bovine serum (Gibco-BRL, Burlington, ON, Canada), antibiotics and 2-ME (60 μM).[2,5] DoHH2 lymphoma cells[13] were cultured in FM, or RPMI-1640 (Stem Cell Technologies, Vancouver, BC, Canada), supplemented with 5% horse serum, 5% fetal bovine serum and antibiotics.

In vitro drug testing

Drugs were tested for culture growth-inhibitory properties as solutions in culture medium with the pH adjusted to 7.5–7.7. Indomethacin, piroxicam, ibuprofen and aspirin were solubilized in dimethylsulfoxide and tested as solutions containing 0.2% of the vehicle, a concentration which did not interfere with culture growth. Solubilization of sulfasalazine and sulfapyridine involved use of 0.1 N NaOH and adjustment of the pH using 1 N HCl, which was added slowly to the solutions swirled by a magnetic stirrer to avoid precipitation of the compounds. Drug and vehicle solutions were prepared and assayed under subdued light conditions. For the assays, Nb2-SFJCD1 cells from log phase cultures (doubling time = 13 h) were centrifuged (3.5 min at 350 *g*) and resuspended in fresh FM containing 10.0% horse serum and antibiotics. Aliquots (1.80 ml) were distributed in 12-well tissue culture plates (Linbro, Flow Laboratories, Mississauga, ON, Canada) and preincubated for 2–5 h at 37°C in a water-saturated 5% CO_2/air atmosphere. Solutions of the drugs at a range of concentrations (200 μl portions; in triplicate) were then added to the cultures for a 45 h incubation ($C_{in} = 1.0 \times 10^5$ cells/ml). Cell populations were determined using an electronic counter (Coulter Electronics, Hialeah, FL, USA). Culture growth inhibitions were calculated from the cell number increases found at hour 45 in drug-treated cultures and their controls. Percent inhibition = $100 - (\text{increase}_{\text{drug-treated}}/\text{increase}_{\text{control}}) \times 100$. Assays using Nb2-U17 cells were carried out with their maintenance medium (doubling time = 20 h); C_{in} = *ca.* 1.5×10^5 cells/ml. Assays using DoHH2 cells were performed with FM, or RPMI-1640, containing fetal bovine serum (5.0%), horse serum (5.0%) and antibiotics (doubling time = 22 and 19 h, respectively); C_{in} = *ca.* 1.5×10^5 cells/ml.

Cellular uptake of ^{35}S-L-cystine

Cultures of Nb2-SFJCD1 log phase cells suspended in Ca- and Mg-free Hanks' balanced salt solution containing 10% horse serum and 10 mM Hepes (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]), pH 7.2 (*ca.* 10^7 cells/ml) were preincubated with drugs for *ca.* 20 min. They were then further incubated for 15 min with ^{35}S-L-cystine (Amersham Canada, Oakville, ON, Canada; 10.7 μCi/ml, 57 μM) and cell-associated radioactivity processed as previously described.[5] Briefly, ice-cooled 200 μl aliquots of cell suspension were layered on to 200 μl aliquots of a mixture of mineral oil and di-n-butyl phtalate (10:90, v:v) in Eppendorf centrifuge tubes and, following centrifugation and aspiration of the supernatants, the tips of the centrifuge tubes were cut off and the cell pellets solubilized for counting in a liquid scintillation counter.[5]

Northern blot analysis

Total RNA was isolated from 2×10^7 log phase cells using RNAzol-B (Tel-Test, Friendswood, TX, USA) and quantified spectrophotometrically. The RNA was denatured in formaldehyde and fractionated on 1% agarose gels and then transferred to GeneScreen Plus (DuPont, Wilmington, DE, USA). Equal loading per lane was verified by ethidium bromide staining of 18S and 28S ribosomal RNA, which was visualized and photographed under UV illumination. Analysis was carried out using macrophage-derived 4F2hc and xCT cDNA probes (kindly provided by Dr S Bannai, University of Tsukuba, Ibaraki, Japan), labeled with ^{32}P. Labeling, hybridization and washing procedures were conducted as previously described.[14]

Testing of sulfasalazine efficacy in vivo

Rats were used from a colony of Noble rats maintained by random litter-mate breeding in micro-isolator cages in the BC Cancer Research Centre.[2] Tolerated dosages of sulfasalazine were established using non-tumor-bearing animals. For drug efficacy studies, approximately equal portions of minced Nb2-U17 tumor tissue, developed in a rat following subcutaneous (s.c.) injection of Nb2-U17 cells, were injected by trocar s.c. in the nape of the neck of groups of male rats (one injection per rat), that had been lightly anesthetized with isoflurane (Abbott Laboratories, Montreal, Canada).[2] When the single tumors reached a measurable size (*ca.* 1 cm^3), therapy of the animals (350–400 g) was started using intraperitoneal (i.p.) injections at a site remote from the transplant at approximately 12 h intervals. Fresh sulfasalazine solutions were prepared every day in 0.1 N NaOH subsequently adjusted with 1 N HCl to a pH of about 8. Phosphate-buffered saline was used for controls. Drug preparation and administration were carried out under subdued light conditions. Food and water were provided *ad libitum*. Tumor size, body weight and general health of the rats were monitored daily. Tumor size was measured using calipers and expressed in grams using the formula: $\pi/6 \times \text{length} \times \text{width} \times \text{height}$ in cm.[15] Animals were killed by carbondioxide asphyxiation as required by the protocol or as soon as they showed signs of stress. Tissue sectioning and histologic analysis were carried out by Criterion Service Laboratory, Vancouver, BC, Canada (pathologist: Dr Jean leRiche, BC Cancer Agency). Animal care and experiments were carried out in accordance with the guidelines of the Canadian Council on Animal Care.

Results

Screening of non-steroidal anti-inflammatory drugs (NSAIDs) for x_c^- inhibition

A literature search indicated that certain NSAIDs inhibited the x_c^- cystine transporter[16] with a potency roughly similar to their prostaglandin synthesis (cyclooxygenase-1) inhibitory activity.[17] Moreover, some NSAIDs exhibit anticancer activity.[18] A study was therefore initiated to determine whether such drugs could arrest growth of Nb2 lymphoma cell cultures by inhibition of x_c^--mediated cystine uptake. Nb2-SFJCD1 subline cultures were used in which cell proliferation critically depended on uptake of cystine via the x_c^- transporter.[5] A variety of clinically useful NSAIDs, spanning a range of anti-inflammatory potency, was tested, including indomethacin, a potent inhibitor of prostaglandin synthesis, and sodium salicylate, a much weaker NSAID.[17] The drugs were assayed at a range of concentrations spanning their 'therapeutic serum/plasma levels', as reported for patients undergoing treatment for inflammatory diseases. It was found that all the NSAIDs tested were able to abrogate population growth in a concentration-dependent fashion. Table 1A shows the IC_{50}s obtained for selected NSAIDs, presented in order of decreasing anti-cyclooxygenase activity[17] *vs* their therapeutic levels in the circulation.[19–22] In general, the growth-inhibiting potency of the drugs declined with decreasing anti-cyclooxygenase activity. However, the IC_{50}s of indomethacin, piroxicam and ibuprofen markedly exceeded their therapeutic levels, in contrast to the IC_{50}s of the salicylates, aspirin (acetylsalicylic acid) and sodium salicylate, which were in the same range as their therapeutic levels. This suggested that, of the NSAIDs studied, only the salicylates had therapeutic potential. However, in no case could NSAID-induced growth arrest be substantially reduced by supplying the cells with cysteine via addition of 2-ME (*ca.* 60 μM), a thiol which allows cellular uptake of cystine via the leucine transporter[23] (data not shown). (This procedure had previously been used to overcome highly specific, monosodium glutamate-induced inhibition of the x_c^- transporter.[5]) Taken together, the results indicate that the observed culture growth-inhibitory activities of the NSAIDs were not based on major inhibition of the x_c^- cystine transporter, but involved different mechanisms of action.

Table 1 Growth-inhibitory activities of NSAIDs and sulfasalazine in Nb2-SFJCD1 cultures (IC_{50}s)[a] *vs* their reported therapeutic levels in human serum/plasma

Drug	*IC_{50} (mM) average ± s.d.*	*Therapeutic drug levels (mM)*[b]
A		
Indomethacin	0.15 ± 0.01	0.005–0.02[19]
Piroxicam	0.30 ± 0.02	0.015–0.03[20]
Ibuprofen	0.43 ± 0.01	0.05–0.1[21]
Acetylsalicylic acid	3.09 ± 0.28	1–3[22]
Salicylate, sodium	1.82 ± 0.12	1–3[22]
B		
Sulfasalazine	0.16 ± 0.01	0.08–0.2[28]

[a]Cultures of Nb2-SFJCD1 rat lymphoma cells in Fischer's medium, containing 10% horse serum and antibiotics, were incubated for 45 h with a drug at a range of concentrations; growth inhibitions were calculated from cell number increases relative to controls.
[b]As reported in the literature for patients/controls undergoing treatment.

Sulfasalazine

Following the lack of success in identifying a specific x_c^- inhibitor, further research focused initially on the salicylates. With regard to sodium salicylate, it may be noted that its lymphoma growth-inhibitory activity was apparently not based on inhibition of cyclooxygenase (COX-1) activity. Thus, the growth-inhibitory potency of salicylate was significantly greater than that of aspirin, by a factor of 1.7 (Table 1A), whereas aspirin has a >20-fold higher anti-COX-1 activity than salicylate, as reported.[17] With regard to aspirin it appears likely that its lymphoma growth-inhibitory activity was mediated by salicylate, a major metabolite of aspirin.[22] Salicylates are known to inhibit activation of NFkappaB, a transcription factor important in regulation of inflammatory responses.[24] On the off-chance that their lymphoma growth-inhibitory activities were based on inhibition of NFkappaB activation, we extended our studies to include sulfasalazine, a salicylate derivative reported to be a potent inhibitor of NFkappaB activation.[25] Sulfasalazine was first synthesized in the 1940s as a combination of sulfapyridine, an antibiotic, and 5-aminosalicylic acid, an anti-inflammatory agent, linked by an azo bridge. It is commonly used to treat chronic inflammatory diseases such as inflammatory bowel (Crohn's) disease and rheumatoid arthritis.[25] Sulfasalazine is usually administered orally and approximately 70% of the drug is degraded by colonic bacteria via azo cleavage to sulfapyridine and 5-aminosalicylic acid, compounds with different modes of action. In Crohn's disease, 5-aminosalicylic acid and sulfasalazine appear to have equivalent activity, each superior to that of sulfapyridine, whereas, in rheumatoid arthritis, sulfapyridine is the active moiety.[25] The mechanism of action of sulfasalazine *per se* is still unclear, but the drug has been demonstrated to inhibit a number of immunological processes, including interleukin-2 synthesis and lymphocyte proliferation, as well as interleukin-1 production by monocytes.[26] In addition to its inhibitory effect on NFkappaB activation,[25] sulfasalazine has been reported to inhibit glutathione-S-transferases;[27] it is not classified as an NSAID.[26] In studying sulfasalazine, we found that it potently inhibited Nb2-SFJCD1 culture growth, exhibiting an IC_{50} of 0.16 mM (Table 1B). This raised the possibility that the drug had potential as a chemotherapeutic agent, since sulfasalazine levels in plasma from children as high as 70.7 μg/ml (0.18 mM) have been reported.[28]

Effects of sulfasalazine and its colonic metabolites on lymphoma culture growth

The effect of sulfasalazine on growth of Nb2-SFJCD1 cultures is shown in Figure 1a. At 0.3 mM it induced severe cell lysis commencing after approximately 24 h and resulting in near total cell death; microscopic observation showed that cell numbers at hour 45 essentially represented lysing cells. However, even after 17 h of incubation with sulfasalazine at such a lytic concentration, cells fully recovered following resuspension in drug-free medium (data not shown). This suggests that continuous exposure to the drug is required for complete elimination of the cells.

A comparison of growth-inhibitory activities of sulfasalazine and its colonic metabolites (Figure 1b) showed that whereas 0.1–0.3 mM sulfasalazine was effective, both sulfapyridine and 5-aminosalicylic acid were without any inhibitory activity in this concentration range which exceeded their therapeutic

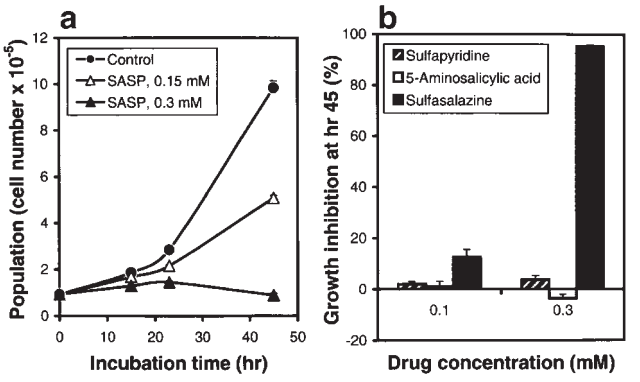

Figure 1 (a) Effect of sulfasalazine (SASP) on the growth of Nb2-SFJCD1 lymphoma cell cultures. Points represent means ± s.d.; the error bars are in most cases covered by the symbols. Results are representative of two experiments. (b) Growth-inhibitory activity in Nb2-SFJCD1 cell cultures of sulfasalazine as distinct from its components, sulfapyridine and 5-aminosalicylic acid. Columns and error bars represent means ± s.d. Results are representative of three experiments.

plasma levels (0.08–0.2 mM[29] and *ca.* 0.01 mM[30] for sulfapyridine and 5-aminosalicylic acid, respectively). These results suggest that the observed growth-inhibitory activity of sulfasalazine reflected action of the intact molecule and was not based on a metabolite such as sulfapyridine, responsible for the activity of sulfasalazine in rheumatoid arthritis.[25]

Inhibitory effect of sulfasalazine on growth of human NHL cell cultures

Sulfasalazine also markedly inhibited proliferation of the B cell type, non-Hodgkin's DoHH2 lymphoma cell line.[13] As shown in Figure 2a, these human lymphoblastic cells were much more sensitive to sulfasalazine than the rat Nb2-SFJCD1 cells when compared under similar conditions, ie suspended in Fischer's medium. Severe lysis of DoHH2 cells was observed at concentrations as low as 0.15 mM sulfasalazine.

Sulfasalazine action based on inhibition of the x_c^- cystine transporter

A comparison of the growth-inhibitory potencies of sulfasalazine in cultures of Nb2-SFJCD1 and Nb2-11 subline cells showed that the efficacy of the drug was substantially lower in the Nb2-11 cell cultures (data not shown). This unexpected finding raised the possibility that the growth-inhibitory activity of sulfasalazine could be overcome by 2-ME, a growth requirement of Nb2-11 cultures as distinct from Nb2-SFJCD1 cultures.[5] As shown in Figure 2b, the presence of 2-ME (60 μM) in Nb2-SFJCD1 cultures indeed reduced the very high growth inhibitions induced by 0.2 mM and 0.3 mM sulfasalazine by *ca.* 90% and 80%, respectively. Furthermore, increasing cystine levels in Fischer's medium from 84 μM to *ca.* 230 μM also reduced the efficacy of the drug, evidence that the action of 2-ME was based on enhanced uptake of cystine (data not shown). Consistent with the latter is the much lower growth inhibition by sulfasalazine in DoHH2 cultures suspended in RPMI-1640, containing 207 μM cystine, compared to DoHH2 cultures in Fischer's medium containing only 84 μM cystine (Figure 2a). In this regard, it is notable that the concentration of cystine in Fischer's medium approximates

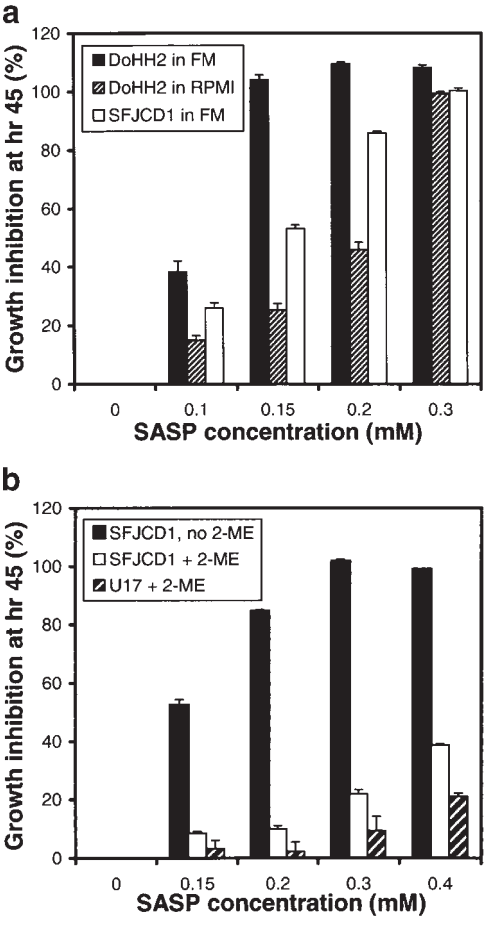

Figure 2 (a) Inhibitory effects of sulfasalazine (SASP) on growth of human DoHH2 and rat Nb2-SFJCD1 cell cultures in Fischer's medium (FM) containing 10% horse serum; reduced sensitivity of DoHH2 cells in RPMI-1640 as distinct from FM. Columns and error bars represent means ± s.d. Results are representative of three experiments. (b) Reduction by 2-ME (60 μM) of SASP-induced inhibition of Nb2-SFJCD1 culture growth; relative insensitivity of Nb2-U17 cells to SASP. Columns and error bars represent means ± s.d. Results are representative of three experiments.

the levels of cyst(e)ine in human plasma.[31] It may also be noted that sulfasalazine at relatively high concentrations (0.3 and 0.4 mM) had only minor inhibitory effects on the proliferation of parental Nb2-U17 cells propagated in the presence of 60 μM 2-ME (Figure 2b).

As previously reported, uptake of extracellular cystine by Nb2-SFJCD1 cells *in vitro* is primarily mediated by the x_c^- cystine transporter,[5] a cystine/glutamate antiporter system.[9] This had been demonstrated by an 85% inhibition of ^{35}S-L-cystine uptake induced by 10 mM monosodium glutamate in a highly specific and Na^+-independent fashion.[5] In the present study, 0.3 mM sulfasalazine inhibited uptake of ^{35}S-L-cystine by Nb2-SFJCD1 cells to the same extent as 10 mM monosodium glutamate (Figure 3). Sulfasalazine, however, did not appear to interfere with 2-ME-enhanced uptake of ^{35}S-L-cystine which presumably proceeds via the leucine transporter[23] (Figure 3).

Taken together, the results in Figures 2a, b and 3 show that the growth-inhibitory activity of sulfasalazine in the lymphoma cell cultures was based on a potent and highly specific inhibition of the x_c^- cystine transporter leading to intracellular depletion of cyst(e)ine. Furthermore, the action of the drug may be based on competitive inhibition, since the efficacy of

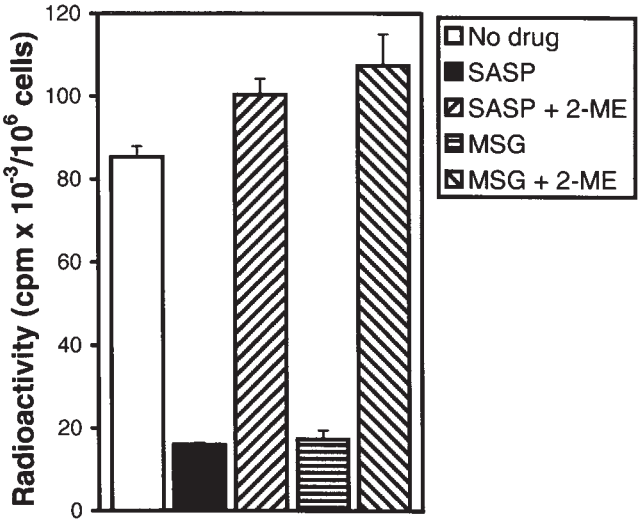

Figure 3 Uptake of ^{35}S-L-cystine by Nb2-SFJCD1 cells as affected by sulfasalazine (SASP) and monosodium glutamate (MSG), a specific inhibitor of the x_c^- cystine transporter.[5] Cultures were preincubated for *ca.* 20 min with medium (no drug), SASP (0.3 mM), 2-ME (60 μM) and MSG (10 mM) in the combinations indicated. The samples were then further incubated for 15 min with ^{35}S-L-cystine (10.7 μCi/ml, 57 μM) for determination of cell-associated radioactivity (Materials and methods). Columns and error bars represent means ± s.d.

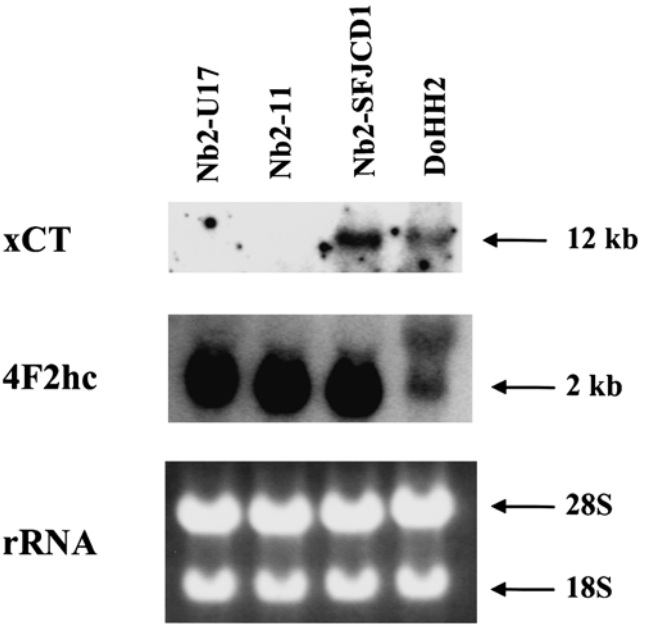

Figure 4 Expression of 4F2hc and xCT mRNA in rat Nb2 lymphoma and human DoHH2 non-Hodgkin's cells. Northern blot analysis of total RNA from log phase cells (20 μg/lane) was conducted using ^{32}P-labeled mouse, macrophage-derived complementary DNA probes. Equal loading of RNA was verified by ethidium bromide-stained 18S and 28S ribosomal RNA (rRNA).

the drug was reduced by increasing the levels of cystine in the medium.

Expression of the x_c^- cystine transporter in cell lines

Recently, the x_c^- cystine transporter was cloned by Sato and co-workers.[32] It was found to be a protein heterodimer consisting of 4F2hc, a common component of amino acid transporters, and xCT, which provides specificity for cystine.[32] Using macrophage-derived 4F2hc and xCT cDNA probes, we found that each of four examined lymphoma cell lines expressed 4F2hc mRNA. In contrast, the 12 kb xCT transcript was not found in Nb2-U17 or Nb2–11 cells, but only in Nb2-SFJCD1 and DoHH2 cells (Figure 4). The absence of an x_c^- cystine transporter in Nb2-U17 and Nb2-11 cells is consistent with their growth requirement for cystine uptake enhancement by 2-ME when cultured in Fischer's medium which contains relatively low cystine levels.[5] The expression of the transporter in Nb2-SFJCD1 and DoHH2 cells is in agreement with their ability to proliferate in such medium in the absence of cystine uptake enhancers.[5]

Suppression of tumor growth in rats by sulfasalazine

The effect of sulfasalazine on tumor growth was evaluated using Noble rats, each carrying a single, well-developed, rapidly growing, subcutaneous rat lymphoma transplant. Parental, cyst(e)ine-dependent Nb2-U17 tumors were utilized since they are non-metastatic, in contrast to Nb2-SFJCD1 tumors.[2,5] Use of non-metastatic tumors would avoid dissemination of cells from the tumor which would interfere with the use of tumor mass as a measure of drug-induced tumor growth inhibition. In preliminary experiments, oral administration of sulfasalazine had a minor tumor growth-arresting effect. This led to a more comprehensive study in which sulfasalazine was administered (1) intraperitoneally, to avoid its degradation in the gut and concomitant loss of growth-inhibitory activity, and (2) at 12 h intervals, in an attempt to maintain elevated levels of the drug in the circulation. As shown in Figure 5, tumor transplants in saline-treated control rats (n = 6) grew rapidly from 0.95 g (range 0.30–1.76 g) to 12.60 g (10.59–16.12 g) in 7 days, consistent with rapid growth of Nb2-U17 transplants observed in previous studies.[2] In contrast, tumor growth was substantially inhibited in the two groups of rats treated with sulfasalazine (total n = 13). Sulfasalazine at a dose of 200 mg/kg body weight, twice a day (n = 8) reduced tumor growth by nearly 80% on average as indicated by an increase in tumor mass from an average of 0.93 g (0.45–1.84 g) to only 3.43 g (1.18–6.59 g). Similar results were obtained with the higher sulfasalazine dosage. It is notable that sulfasalazine inhibited tumor growth in five of the 13 rats by 90–100% when compared with the average increase in tumor mass of the controls. It is not known if the lower tumor growth suppressions reflect temporary decreases in the levels of circulating sulfasalazine which could have been avoided by administering the drug at shorter intervals.

At necropsy there was no evidence of metastatic spread of the sulfasalazine-treated tumors to liver, spleen and kidney, target tissues for metastatic Nb2 lymphoma cell lines.[2] Histological analysis (n = 4) of these tissues confirmed this observation, indicating that the suppressive effect of sulfasalazine on the Nb2-U17 tumors was not the result of drug-induced dissemination of tumor cells from the transplant site, but reflected tumor growth inhibition. Importantly, at the dosages used, sulfasalazine caused no apparent toxicity to the host, as indicated by the good general health of the tumor-bearing rats (n = 13) during treatment and by the necropsy results. Control, non-tumor-bearing animals (n = 5) receiving an identical 7-day treatment with the drug and followed over a 3–6 month period, showed good health with only a temporary halt in

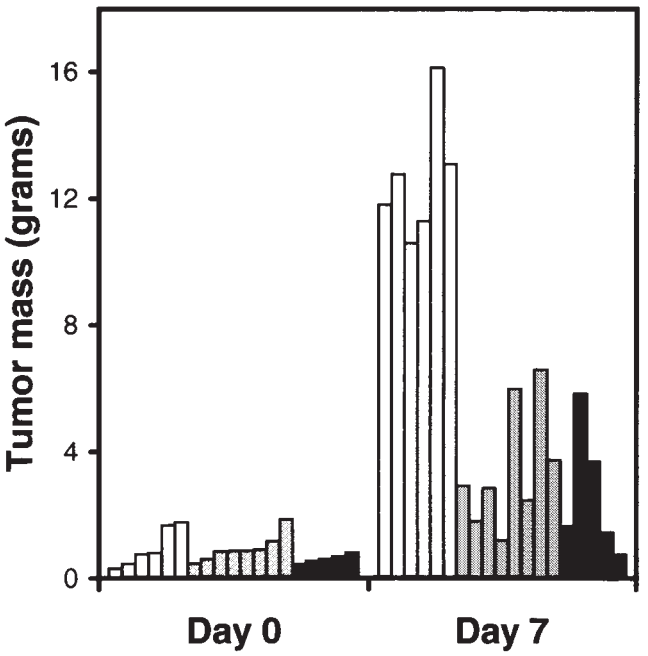

Figure 5 Effect of 7 days of treatment with sulfasalazine (SASP) on the growth of well-developed, rapidly growing, subcutaneous Nb2-U17 lymphoma transplants in Noble rats. Starting on day 0, groups of male rats (350–400 g), each carrying a single tumor in the nape of the neck (range 0.3–1.8 g), received i.p. injections, at 12 h intervals, of saline (controls, $n = 6$; open bars), SASP at 200 mg/kg body weight ($n = 8$; hatched bars) or SASP at 250 mg/kg body weight ($n = 5$; black bars). Tumor mass (g) was calculated from the length, width and height of the tumors in cm.[15] Each bar of the 'day 0' group, and the corresponding bar in the 'day 7' group, represent the mass of one particular tumor at the beginning and end of the treatment, respectively.

their weight gain; scar tissue at the sites of injections disappeared within a few weeks. Taken together, the results of these *in vivo* studies demonstrate that sulfasalazine was highly effective at inhibiting growth of transplanted Nb2-U17 lymphomas without significantly affecting the health of the hosts.

Discussion

The present study has identified, for the first time, that sulfasalazine is a potent inhibitor of the x_c^- cystine transporter. This fortuitously discovered property of sulfasalazine was demonstrated by its marked inhibition of the uptake of ^{35}S-L-cystine by Nb2-SFJCD1 cells, a process which is mediated by the x_c^- transporter[5] (Figure 3). The inhibition of the x_c^- transporter appears to be primarily responsible for the lymphoma growth-inhibitory activity of sulfasalazine *in vitro* (Figures 1a, b and 2a), since growth arrest could be largely prevented by addition of 2-ME which enhances cystine uptake via a different route, ie the leucine transporter[23] (Figure 2b). The *in vitro* growth-inhibitory activity of sulfasalazine is not based on its components, sulfapyridine or 5-aminosalicylic acid (Figure 1b), in contrast to its anti-inflammatory activity in rheumatoid arthritis and Crohn's disease, and in this regard resembles its inhibitory effect on NFkappaB activation.[25] It is notable that there is apparently no correlation between inhibition of NFkappaB activation, as induced by salicylates, and inhibition of cell proliferation.[24] Subsequent studies are needed to determine the relationship between the inhibitory effect of sulfasalazine on cystine uptake and its inhibition of NFkappaB activation.

It is not yet clear how sulfasalazine-induced cyst(e)ine starvation caused death of the lymphoma cells *in vitro*. However, it has been reported that incubation of human Jurkat T leukemic cells in cystine- and glutathione-free medium led to a build-up of intracellular peroxides and cell death by apoptosis within 18–24 h.[33] It is likely that sulfasalazine-induced cell lysis in Nb2-SFJCD1 and DoHH2 cultures (Figures 1a and 2a) developed along similar lines. The observed 24-h interval prior to the onset of lysis of Nb2-SFJCD1 cells incubated with 0.3 mM sulfasalazine (Figure 1a) is consistent with a gradual intracellular depletion of cyst(e)ine. The latter would naturally lead to a decrease in the intracellular levels of glutathione, a major free radical-scavenger with a short half-life which is generated from cysteine, leaving the cells less protected against oxidative stress.[33] The finding that continuous exposure to the drug is needed to induce cell lysis is also consistent with a starvation process. The reported inhibitory effect of sulfasalazine on glutathione-S-transferases, enzymes involved in protection of cells from oxidative stress,[27] together with depletion of glutathione,[33] would facilitate lysis of the tumor cells by metabolism-generated peroxides. Furthermore, inhibition by sulfasalazine of NFkappaB activation[25] would prevent cells from expressing anti-apoptotic genes as a protective response against injury.[34]

In analyzing the suppression by sulfasalazine of lymphoma growth in the animal, one has to take into account that, *in vivo*, the lymphoma cells are exposed not only to cystine, but also to cysteine which they can take up readily.[6,8] This implies that cyst(e)ine starvation of the lymphoma cells *in vivo* cannot be achieved by a direct sulfasalazine–tumor cell interaction leading to inhibition of cystine uptake, unless there is a concomitant reduction in the supply of plasma cysteine. In the case of the Nb2-U17 tumor transplants (Figure 5), the lymphoma cells did not possess an x_c^- cystine transporter (Figure 4). Furthermore, Nb2-U17 cell proliferation *in vitro* was not greatly affected by sulfasalazine, even at 0.4 mM, when 2-ME was present allowing pick up of extracellular cystine (Figure 2b). The latter condition closely resembles an *in vivo* situation, in which lymphoma cells have access to circulating cysteine. It appears therefore that the suppression of Nb2-U17 lymphoma growth by sulfasalazine (Figure 5) was not due to a direct drug–tumor cell interaction, but involved an indirect effect of the drug. As a potent inhibitor of the x_c^- cystine transporter, sulfasalazine probably interfered with secretion of cysteine by somatic cells (eg macrophages), described by Gmünder *et al*,[11] in which the generation of cysteine is dependent on x_c^--mediated uptake of cystine. The reduction in cysteine delivery to the tumor cells could have caused a decrease in their intracellular cyst(e)ine levels below a critical threshold leading to inhibition of growth. An inhibitory effect of sulfasalazine on somatic cell-mediated supply of cysteine could also underlie some of its immunosuppressive actions *in vivo*, including its inhibition of lymphocyte proliferation.[26]

The present study indicates that sulfasalazine, a drug commonly used for treatment of severe inflammatory diseases, could also be useful as an anticancer agent based on its inhibition of the x_c^- cystine transporter. The marked suppression by sulfasalazine of rat Nb2 pre-T cell lymphoma growth *in vivo* without major toxicity to the host (Figure 5), together with its efficacy against human DoHH2 B-cell lymphoma cells *in vitro* (Figure 2a), suggest that sulfasalazine has potential application in clinical treatment of cancers dependent on extracellular cyst(e)ine for growth and viability. Such malignancies

would include lymphoblastic leukemias and lymphomas (eg NHL) of both B and T cell origin. Since the oncolytic activity of sulfasalazine is apparently based on cyst(e)ine depletion within target cells, associated with loss of protection against oxidative stress, it appears advantageous to utilize sulfasalazine in combination with oxidative stress-causing drugs (eg alkylating agents). Furthermore, since the colonic metabolites of sulfasalazine have no growth-inhibitory activity (Figure 1b), the drug should not be administered via the GI tract.

Cancer treatment using sulfasalazine would resemble use of asparaginase, commonly applied in combination chemotherapy of acute lymphocytic leukemia, aimed at depletion of circulating asparagine, an essential amino acid for certain leukemic cells.[35] Attempts to develop therapy for leukemias on the basis of cyst(e)ine depletion have been reported.[36] Notably, a differential growth response between normal and certain leukemic cells was observed in culture. Whereas both types of cells required cyst(e)ine for growth, the normal cells could replicate in the absence of the amino acid if L-cystathionine, a precursor of L-cysteine, was supplied in the medium, whereas the leukemic cells could not. The latter was due to reduced levels in the leukemic cells of γ-cystathionase required for the generation of L-cysteine by cleavage of L-cystathionine.[36] These findings raise the possibility that administration of L-cystathionine during sulfasalazine therapy of γ-cystathionase-deficient cancers could have a protective effect on normal lymphoid tissue. Short-term exposure to cystathionine may be well tolerated since treatment with cystathionine has been used to protect rats from acute gastric mucosal injury[37] and extremely elevated levels of cystathionine have been observed in sera from patients with eg folate deficiency.[38]

Sulfasalazine may also be useful for treatment of malignancies other than lymphoblastic cancers. Human lymphocytic[4] and myelocytic[36] leukemia cells, and cells from solid cancers such as melanoma,[36] neuroblastoma,[36] and glioma[39] have been reported to be dependent on extracellular cyst(e)ine. Cystine deprivation *in vitro* has also led to death of lung fibroblasts[40] and preoligodendrocytes.[41]

In conclusion, the present study has demonstrated that the x_c^- cystine transporter provides a useful target for therapy of cancers which are dependent on extracellular cyst(e)ine for growth and viability. The finding that sulfasalazine is a powerful inhibitor of this transport system may lead to the development of a new class of chemotherapeutic, sulfasalazine-like compounds.

Acknowledgements

Dr CT Beer and Dr MC Robertson (BC Cancer Agency, Canada) are thanked for helpful discussions, and DJ Buckley (Cincinnati Medical Center, USA) for preparing the northern blots. Dr S Bannai (University of Tsukuba, Ibaraki, Japan) is thanked for providing the 4F2hc and xCT cDNA probes, and the Department of Advanced Therapeutics of the BC Cancer Agency for supplying DoHH2 cells. This study was supported by the BC Cancer Foundation, with core support from the BC Cancer Agency (PWG), and in part by NIH grant No. DK53452 and the Ohio Cancer Research Associates (ARB).

References

1 Poplack DG, Kun LE, Magrath IT, Pizzo PA. Leukemias and lymphomas of childhood. In: DeVita VT Jr, Hellman S, Rosenberg SA (eds). *Cancer: Principles and Practice of Oncology*, 4th edn. JB Lippincott: Philadelphia, 1993, pp 1792–1818.
2 Gout PW, Horsman DE, Fox K, de Jong G, Ma S, Bruchovsky N. The rat Nb2 lymphoma: a novel model for tumor progression. *Anticancer Res* 1994; **14**: 2485–2492.
3 Heppner GH, Miller FR. The cellular basis of tumor progression. *Int Rev Cytol* 1998; **177**: 1–56.
4 Iglehart JD, York RM, Modest AP, Lazarus H, Livingston DM. Cystine requirement of continuous human lymphoid cell lines of normal and leukemic origin. *J Biol Chem* 1977; **252**: 7184–7191.
5 Gout PW, Kang YJ, Buckley DJ, Bruchovsky N, Buckley AR. Increased cystine uptake capability associated with malignant progression of Nb2 lymphoma cells. *Leukemia* 1997; **11**: 1329–1337.
6 Gmünder H, Eck H-P, Dröge W. Low membrane transport activity for cystine in resting and mitogenically stimulated human lymphocyte preparations and human T cell clones. *Eur J Biochem* 1991; **201**: 113–117.
7 Broome JD, Jeng MW. Promotion of replication in lymphoid cells by specific thiols and disulfides *in vitro*. *J Exp Med* 1973; **138**: 574–592.
8 Ishii T, Hishinuma I, Bannai S, Sugita Y. Mechanism of growth promotion of mouse lymphoma L1210 cells *in vitro* by feeder layer or 2-mercaptoethanol. *J Cell Physiol* 1981; **107**: 283–293.
9 Bannai S. Exchange of cystine and glutamate across plasma membrane of human fibroblasts. *J Biol Chem* 1986; **261**: 2256–2263.
10 Watanabe H, Bannai S. Induction of cystine transport activity in mouse peritoneal macrophages. *J Exp Med* 1987; **165**: 628–640.
11 Gmünder H, Eck H-P, Benninghoff B, Roth S, Dröge W. Macrophages regulate intracellular glutathione levels of lymphocytes. Evidence for an immunoregulatory role of cysteine. *Cell Immunol* 1990; **129**: 32–46.
12 Gout PW, Buckley AR, Simms CR, Bruchovsky, N. Novel lymphoma growth-inhibitory activity of the immunosuppressant, sulfasalazine. Proc 11th Int Congress Endocrinology, Sydney, Australia, October 29–November 2, 2000, p 313, No. P873 (Abstr.).
13 Kluin-Nelemans HC, Limpens J, Meerabux J, Beverstock GC, Jansen JH, de Jong D, Kluin PM. A new non-Hodgkin's B-cell line (DoHH2) with a chromosomal translocation t(14;18)(q32;q21). *Leukemia* 1991; **5**: 221–224.
14 Krumenacker JS, Buckley DJ, Leff MA, McCormack JT, de Jong G, Gout PW, Reed JC, Miyashita T, Magnuson NS, Buckley AR. Prolactin-regulated apoptosis of Nb2 lymphoma cells: *pim-1*, *bcl-2* and *bax* expression. *Endocrine* 1998; **9**: 163–170.
15 Tomayko MM, Reynolds CP. Determination of subcutaneous tumor size in athymic (nude) mice. *Cancer Chemother Pharmacol* 1989; **24**: 148–154.
16 Bannai S, Kasuga H. Anti-inflammatory drug inhibition of transport of cystine and glutamate in cultured human fibroblasts. *Biochem Pharmacol* 1985; **34**: 1852–1854.
17 Riendeau D, Charleson S, Cromlish W, Mancini JA, Wong E, Guay J. Comparison of the cyclooxygenase-1 inhibitory properties of nonsteroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors, using sensitive microsomal and platelet assays. *Can J Physiol Pharmacol* 1997; **75**: 1088–1095.
18 Ding XZ, Tong WG, Adrian TE. Blockade of cyclooxygenase-2 inhibits proliferation and induces apoptosis in human pancreatic cancer cells. *Anticancer Res* 2000; **20**: 2625–2631.
19 Emori HW, Paulus H, Bluestone R, Champion GD, Pearson C. Indomethacin serum concentrations in man. *Ann Rheum Dis* 1976; **35**: 333–338.
20 Kurowski M, Dunky A. Transsynovial kinetics of piroxicam in patients with rheumatoid arthritis. *Eur J Clin Pharmacol* 1988; **34**: 401–406.
21 Slattery JT, Levy G. Effect of ibuprofen on protein binding of warfarin in human serum. *J Pharm Sci* 1977; **66**: 1060.
22 Insel PA. Analgesic-antipyretic and antiinflammatory agents and drugs employed in the treatment of gout. In: Hardman JG, Goodman Gilman A, Limbird LE (eds). *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, 9th edn. McGraw-Hill: New York, 1996, pp 617–657.
23 Ishii T, Bannai S, Sugita Y. Mechanism of growth stimulation of L1210 cells by 2-mercaptoethanol *in vitro*. Role of the mixed disulfide of 2-mercaptoethanol and cysteine. *J Biol Chem* 1981; **256**: 12387–12392.

24 Kopp E, Ghosh S. Inhibition of NF-kappa B by sodium salicylate and aspirin. *Science* 1994; **265**: 956–959.
25 Wahl C, Liptay S, Adler G, Schmid RM. Sulfasalazine, a potent and specific inhibitor of nuclear factor kappa B. *J Clin Invest* 1998; **101**: 1163–1174.
26 Hoult JR. Pharmacological and biochemical actions of sulphasalazine. *Drugs* 1986; **32** (Suppl. 1): 18–26.
27 Bach MK, Brashler JR, Johnson MA. Inhibition by sulfasalazine of LTC synthetase and of rat liver glutathione S-transferases. *Biochem Pharmacol* 1985; **34**: 2695–2704.
28 Guastavino E, Litwin NH, Heffes Nahmod L, Licastro R. Ulcerative colitis in children. Levels of salicylazosulfapyridine and sulfapyridine during treatment. *Acta Gastroenterol Latinoam* 1988; **18**: 107–113.
29 Rahav G, Zylber-Katz E, Rachmilewitz D, Levy M. Relationship between the acetylator phenotype, plasma sulfapyridine levels and adverse effects during treatment with salicylazosulfapyridine in patients with chronic bowel diseases. *Isr J Med Sci* 1990; **26**: 31–34.
30 Almer S, Norlander B, Strom M, Osterwald H. Steady-state pharmacokinetics of a new 4-gram 5-aminosalicylic acid retention enema in patients with ulcerative colitis in remission. *Scand J Gastroenterol* 1991; **26**: 327–335.
31 Chawla RK, Lewis FW, Kutner MH, Bate DM, Roy RGB, Rudman D. Plasma cysteine, cystine, and glutathione in cirrhosis. *Gastroenterology* 1984; **87**: 770–776.
32 Sato H, Tamba M, Ishii T, Bannai S. Cloning and expression of a plasma membrane cystine/glutamate exchange transporter composed of two distinct proteins. *J Biol Chem* 1999; **274**: 11455–11458.
33 Iwata S, Hori T, Sato N, Hirota K, Sasada T, Mitsui A, Hirakawa T, Yodoi J. Adult T cell leukemia (ATL)-derived factor/human thioredoxin prevents apoptosis of lymphoid cells induced by L-cystine and glutathione depletion. *J Immunol* 1997; **158**: 3108–3117.
34 Wang CY, Guttridge DC, Mayo MW, Baldwin Jr AS. NF-kappaB induces expression of the Bcl-2 homologue A1/Bfl-1 to preferentially suppress chemotherapy-induced apoptosis. *Mol Cell Biol* 1999; **19**: 5923–5929.
35 Keating MJ, Holmes R, Lerner S, Ho DH. L-Asparaginase and PEG asparaginase – past, present and future. *Leuk Lymphoma* 1993; **10**: 153–157.
36 Uren JR, Lazarus H. L-Cyst(e)ine requirements of malignant cells and progress toward depletion therapy. *Cancer Treat Rep* 1979; **63**: 1073–1079.
37 Wada K, Kamisaki Y, Kitano M, Nakamoto K, Itoh T. Protective effect of cystathionine on acute gastric mucosal injury induced by ischemia-reperfusion in rats. *Eur J Pharmacol* 1995; **294**: 377–382.
38 Stabler SP, Lindenbaum J, Savage DG, Allen RH. Elevation of serum cystathionine levels in patients with cobalamin and folate deficiency. *Blood* 1993; **81**: 3404–3413.
39 Kato S, Negishi K, Mawatari K, Kuo CH. A mechanism for glutamate toxicity in the C6 glioma cells involving inhibition of cystine uptake leading to glutathione depletion. *Neuroscience* 1992; **48**: 903–914.
40 Aoshiba K, Yasui S, Nishimura K, Nagai A. Thiol depletion induces apoptosis in cultured lung fibroblasts. *Am J Respir Cell Mol Biol* 1999; **21**: 54–64.
41 Back SA, Gan X, Li Y, Rosenberg PA, Volpe JJ. Maturation-dependent vulnerability of oligodendrocytes to oxidative stress-induced death caused by glutathione depletion. *J Neurosci* 1998; **18**: 6241–6253.