The activity of methylated and non-methylated selenium species in lymphoma cell lines and primary tumours

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Background: Diffuse large B-cell lymphoma patients with low serum selenium concentration at presentation have a lower response rate and overall survival than patients with higher serum selenium. The co-administration of selenium with conventional chemotherapy may be useful in these patients.

Patients and methods: We investigated the activity of two selenium species, methylseleninic acid (MSA) and selenodiglutathione (SDG) in a panel of human lymphoma cell lines and in a primary lymphoma culture system.

Results: Both compounds demonstrated cytostatic and cytotoxic activity with EC50 values in the range 1.0–10.2 μM. Cell death was associated with an increase in the sub-G1 (apoptotic) fraction by flow cytometry and was not preceded by any obvious cell cycle arrest. SDG, but not MSA, resulted in marked increases in intracellular ROS, particularly in CRL2261 and SUD4 cells in which the cytotoxic activity of SDG was partly, or completely, inhibited by N-acetyl cysteine, suggesting a dependence on ROS for activity in some cells. Both MSA and SDG showed a concentration dependent reduction in percentage viability after a 2-day exposure in primary lymphoma cultures, with EC50 values in the range 39–300 μM and 9–28 μM, respectively.

Conclusion: The selenium compounds MSA and SDG induce cell death in lymphoma cell lines and primary lymphoma cultures, which with SDG may be partly attributable to the generation of ROS.

Key words: lymphoma, methylseleninic acid, primary culture, selenium, selenodiglutathione

introduction

Selenoproteins play a critical role in a number of biochemical pathways, notably in cellular anti-oxidant systems such as glutathione peroxidase and thioredoxin reductase [1, 2]. Many studies have reported an association between selenium intake or serum selenium concentration and cancer incidence [3] or overall age adjusted cancer mortality [4]. Studies in animals have described the chemopreventive activity of selenium compounds (reviewed in [5]), while clinical trials have also reported the protective effect of selenium supplementation in the development of prostate, lung and colorectal cancer [6], with the benefit being clearest in prostate cancer [7].

Serum selenium concentration at diagnosis was recently reported to be independently predictive of both treatment response and long-term survival in patients with aggressive non-Hodgkin’s lymphoma [8]. In a group of 99 assessable patients, response to first treatment was 54% in the lowest serum quartile compared with 88% in the highest quartile, with a lower overall survival in patients with lower serum selenium.

Serum selenium remained predictive of outcome in a multivariate analysis that included clinical variables as cofactors, as also reported by Deffuant et al. [9] in cutaneous T-cell lymphoma patients.

One explanation for this effect may involve the role of selenium in apoptosis induction and may reflect decreased apoptosis in the presence of similar amounts of cytotoxic drug-induced DNA damage in selenium deplete, compared with selenium replete, cells. In a recently reported supplementation study in dogs, the amount of DNA damage in peripheral blood mononuclear cells was lower in supplemented animals, while the number of apoptotic cells in prostate epithelium was increased, again suggesting an alteration in the apoptotic threshold in selenium replete cells [10].

These data suggest a possible role for selenium in the treatment of established malignancy, most likely with DNA damaging agents. There are currently no data describing the activity of selenium compounds in human lymphomas.

Against the background of the clinical data above, a study of the effects of methylseleninic acid (MSA) and selenodiglutathione (SDG), representing methylated and non-methylated selenium species, respectively, in a lymphoma cell line panel and in a primary lymphoma culture system has been undertaken.
patients and methods

cell lines and culture conditions

Four non-Hodgkin’s lymphoma cell lines were used in these studies. DHL-4 was obtained from the Dana Farber Cancer Institute (gift from Dr Margaret Shipp) and DoHH2 [11], SUD4 [12] and CRL2261 from Cancer Research UK cell services. All lines were maintained in RPMI-1640 growth media with 10% fetal calf serum, 1% glutamine and 1% penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO₂.

MSA (PharamSea, Lubbock, TX, USA) was prepared in DMSO at a concentration of 80 mM and SDG (PharmaSe) in 0.05 M HCl at a concentration of 10 mM. These solutions were stored at −40°C for up to 3 months. The cytotoxic activity of these compounds was investigated after 3 day's continuous exposure, with cell number and viability determined by light microscopy following trypan blue staining. All data were expressed relative to control (untreated) cells and EC₅₀ concentrations derived using a sigmoidal dose-response model (Graphpad Prism, San Diego, CA, USA). The final DMSO concentration was <1% in all cultures. The data presented is the mean of a minimum of three experiments.

To investigate the possible influence of reactive oxygen species (ROS) on the activity of SDG and MSA, cell lines were treated at the EC₅₀ concentration of either drug for 3 days in the presence of 2.5 mM N-acetyl cysteine (NAC, Sigma Chemicals, Poole, Dorset, UK).

primary culture system

B-cell suspensions were prepared from ascitic fluid, peripheral blood or lymph nodes collected as part of normal clinical management from patients with histologically confirmed B-cell lymphoma or chronic lymphocytic leukaemia. All subjects gave informed consent and the study received approval from the institutional ethics review board. After disaggregation and/or density gradient separation, cells were plated at a density of 5 × 10⁵ cells/well into 96-well plates containing CHO cells transfected to express annexin conjugate, after which the sample was incubated for a further 30 min. ROS generation was then immediately determined by measuring the mean fluorescent intensity of 10 000 cells at 530 nM using a Becton Dickinson FACS Calibur flow cytometer and CellQuest software.

flow cytometry

To determine cell cycle distribution cells were washed in ice-cold nucleus buffer (0.15 M NaCl, 5 mM MgCl₂, 1 mM KH₂PO₄, 1 mM EGTA, 0.1 mM dithiothreitol, 10% glycerol in distilled water pH 6.5), cells (1 × 10⁶) re-suspended in 4 ml of permeabilising solution (0.35% Triton X-100, 0.1 mM PMSF in nucleus buffer) and mixed by rotation at 4°C for 20 min. Cells were then fixed in ice-cold methanol for 30 min and washed with ice-cold PBS before staining with 500 μl of PI stain (50 μg/ml propidium iodide and 50 μg/ml RNAse A in PBS). Acquisition of data was performed within 1 h. Ten thousand cells were analysed for each sample point using a Becton Dickinson FACS Calibur flow cytometer with CellQuest software, and the percentage of cells in the sub-G1 (apoptotic fraction), G1, S and G2/M phases were analysed using a cell cycle analysis program (WinMDI 2.4).

Apoptosis was confirmed by dual labelling using propidium iodide/annexin staining (Sigma, Poole Dorset, UK) of unfixed cells treated at the cytotoxic EC₅₀ concentration for that cell line. Briefly, 1 × 10⁵ cells were suspended in 500 μl of binding buffer (HEPES with NaOH, NaCl and CaCl₂) to which was added 5 μl annexin conjugate, after which the sample was incubated in the dark for 10 min. Propidium iodide (10 μl of 100 μg/ml was then added, and the sample immediately analysed on a Becton Dickinson FACS Calibur using CellQuest software.

To determine ROS generation, experiments were conducted at the cytotoxic EC₅₀ concentration of either MSA or SDG for each cell line. Cells were suspended in PBS (1 × 10⁵/ml) and incubated in 20 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA, Molecular Probes, OR, USA) for 15 min at 37°C in 5% CO₂. After the addition of MSA, SDG, 25 μM H₂O₂ (positive control) or 0.75% DMSO (negative control), cells were incubated for a further 30 min. ROS generation was then immediately determined by measuring the mean fluorescent intensity of 10 000 cells at 530 nM using a Becton Dickinson FACS Calibur flow cytometer and CellQuest software.

statistical analyses

Differences between treatments, or cell lines, were compared using an analysis of variance controlling for cell line and/or treatments. If this indicated significant differences within the data, pairs of treatments or cell lines were compared using a paired t-test. A P value of <0.05 was taken as the cut-off for statistical significance.

results

MSA and SDG are cytotoxic in lymphoma cell lines

Exposure to either MSA or SDG resulted in cytostasis and cytotoxicity in all lymphoma cell lines studied (Figure 1, Table 1). The EC₅₀ values for these effects were typically between 1 and 10 μM, with the exception of MSA in the DHL-4 cell line where the response was mainly cytostatic (EC₅₀ concentrations for cell number and % viability 1.7 μM and 166 μM, respectively).

Both MSA and SDG resulted in an increase in the apoptotic fraction with increasing concentration (as shown in Figure 2A for DoHH2 cells with MSA). Neither SDG nor MSA caused an apparent block in the cell cycle, but resulted in a reduction of cells from all phases as apoptosis increased with increasing selenium concentration. Similarly, the decreased sensitivity of DHL-4 cells to MSA was not associated with a cell cycle block (as shown in Figure 2B).

Three-day incubations at the cytotoxic EC₅₀ concentration of SDG or MSA increased both annexin and PI staining, resulting in cells moving from the lower left to lower right quadrant and then to the upper right quadrant, indicating increased annexin staining initially, followed by an increase in PI staining (as shown in Figure 3A for DHL4 cells and Figure 3B for SUD4 cells), indicative of cell death by apoptosis.

Cell lines varied in their ability to generate or inactivate ROS, as evidenced by the change induced by a 30-min exposure to 25 μM H₂O₂. This treatment did not result in a significant change in DCF-DA fluorescence in DoHH2 and DHL-4 cells compared with control cells (100%), but did increase ROS in CRL2261 and SUD4 cells (Figure 4A). In three of the four cell lines studied the cytotoxic EC₅₀ concentration of SDG generated significant increases in ROS compared with control cells, and greater than the H₂O₂ positive control. MSA at the EC₅₀ concentration resulted in little or no change in ROS in any cell line. The increase in ROS with SDG was most apparent at 30 min, had declined by 2 h and showed no difference at 24 h (data not shown).

To investigate whether ROS contributed to the cytotoxic activity of SDG or MSA, cells were incubated at the cytotoxic
EC50 concentration of either compound for 3 days in the presence or absence of 2.5 mM NAC (Figure 4B). NAC alone had no effect on cell viability. In the DoHH2 cell line, in which SDG did not generate ROS, the addition of NAC had no effect on the activity of SDG. However, in DHL4 and SUD4 cells, in which SDG resulted in significant increases in ROS, NAC reduced the cytotoxic activity of SDG, and almost abolished its activity in SUD4 cells in which the cytotoxic EC50 concentration of SDG resulted in a 450% increase in ROS over 30 min. NAC had no effect on the activity of MSA in any of the cell lines studied.

**SDG and MSA are active in primary lymphoma cultures**

The activity of a 48-h exposure to SDG or MSA was then investigated in primary B-cell malignancy samples from

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**Table 1.** EC50 values for cell number and percentage viability (95% confidence intervals, at least three separate experiments for each) for MSA and SDG in four lymphoma cell lines after a 3-day continuous exposure.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Mean EC50 concentration, µM (95% CI)</th>
<th>Cell number</th>
<th>% Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Methylseleninic acid</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUD4</td>
<td>6.5 (4.9–8.7)</td>
<td>10.2 (9.7–10.8)</td>
<td></td>
</tr>
<tr>
<td>DoHH2</td>
<td>1.0 (0.9–1.3)</td>
<td>1.6 (1.3–1.8)</td>
<td></td>
</tr>
<tr>
<td>CRL2261</td>
<td>4.7 (4.3–5.2)</td>
<td>8.5 (7.8–9.3)</td>
<td></td>
</tr>
<tr>
<td>DHL4</td>
<td>1.7 (1.3–1.9)</td>
<td>166 (141–196)</td>
<td></td>
</tr>
<tr>
<td><strong>Selenodiglutathione</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUD4</td>
<td>3.5 (3.3–3.8)</td>
<td>4.4 (4.2–4.5)</td>
<td></td>
</tr>
<tr>
<td>DoHH2</td>
<td>4.0 (3.1–5.2)</td>
<td>4.0 (3.8–4.2)</td>
<td></td>
</tr>
<tr>
<td>CRL2261</td>
<td>6.8 (6.1–7.7)</td>
<td>8.8 (8.3–9.4)</td>
<td></td>
</tr>
<tr>
<td>DHL4</td>
<td>4.9 (3.5–6.8)</td>
<td>6.5 (6.0–7.0)</td>
<td></td>
</tr>
</tbody>
</table>

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**Figure 1.** The effect (mean ± SD of at least three separate experiments) of MSA (A and B) and SDG (C and D) on proliferation (A, C) and viability (B, D) in four lymphoma cell lines.
patients with mantle cell lymphoma (No. 03 and No. 04), follicular lymphoma (No. 01), chronic lymphocytic leukemia (No. 02) and a normal B-cell sample from a healthy blood donor. All samples showed a concentration-dependent decrease in percentage viability with MSA and SDG (Figure 5A, B). Although these data are limited, cells appeared more sensitive to SDG (EC50 concentrations 9–28 μM) than MSA (EC50 values 39–300 μM). Cytotoxic EC50 concentrations in a normal B-cell sample were 109 μM and 1071 μM for SDG and MSA, respectively.

discussion

Following the observation that presentation serum selenium was an independent predictor of response to treatment and overall survival in patients with aggressive non-Hodgkin’s lymphoma, the activity of specific selenium compounds has now been studied in a panel of lymphoma cell lines and in a primary lymphoma culture system. Dietary forms of selenium, such as L-selenomethionine, require activation by β-lyase, typically in the liver, so have little in vitro activity [13]. Activated selenium species were therefore used in these studies, namely MSA, which is rapidly converted to the highly reactive methylselenol, and SDG (an active metabolite of the naturally occurring sodium selenite), which is activated further to hydrogen selenide with the generation of ROS in the process [14].

While the activity of these compounds has been reported in other cancer types, there are no data describing the activity of selenium compounds in human lymphomas. Both MSA and SDG induced cytostasis and cytotoxicity in the lymphoma cell line panel studied, in a concentration and time-dependent manner. The MSA cell number EC50 (1–7 μM) or percentage viability EC50 (2–10 μM; DHL-4 166 μM) is in the range reported by others using human cancer cell lines of epithelial origin [15–17], and the activity of SDG is in keeping with that found in primary cultures of oral carcinomas by Ghose et al. [18]. It is noteworthy that the concentration effect curves were much steeper for SDG than for MSA; most notably so in DoHH2 and SUD4 cells (sigmoid factor for DoHH2 and SUD4 cells –16.2 and –8.3, respectively, for cell number and –10.1 and –8.4, respectively, for percentage viability). This suggests a possible threshold concentration above which the effect increases dramatically.

Other reports have described the induction of G1 cell cycle arrest in response to MSA in cancer cell lines, and S phase arrest in response to selenite, an SDG precursor [15]. This was not apparent in our studies, with a decrease in all phases of the cell cycle concomitant with an increase in the sub-G1 (apoptotic) fraction.

As part of other studies we have determined that all of these lines contain p53 mutations, confirmed using a functional assay in yeast [19], which found no transcriptional p53 activity in SUD4, DHL-4 and CRL2261 cells, and reduced activity in DoHH2 cells that contain only one mutated p53 gene copy (Strauss et al., manuscript in preparation; Richard Camplejohn, personal communication). The activity of these selenium compounds is therefore not dependent on functional p53 activity, confirming previous reports that although SDG induces functional p53, this is in response to DNA damage and is not required for the induction of apoptosis [20].

The cell lines studied differed markedly in their ability to generate, or inactivate, ROS, with DoHH2 and DHL-4 cells showing no change in ROS in response to H2O2, but marked increases in CRL2261 and SUD4 cells. A similar result was seen with SDG, with no change in ROS in DoHH2 cells, a significant increase in DHL-4 cells and a more marked increase still in CRL2261 and SUD4 cells. MSA generated little or no increase in ROS in any cell line. The potential importance of ROS in the cytotoxic activity of MSA and SDG was investigated using NAC, which increases intracellular cysteine, resulting in increased glutathione (GSH) concentration [21]. NAC had no effect on the activity of MSA or SDG in DoHH2 cells, in which these compounds alone induced little or no change in ROS. In DHL-4 cells, in which SDG increased ROS, NAC significantly reduced SDG cytotoxicity, while in SUD4 cells that had shown the biggest increase in ROS in response to SDG, the addition of NAC almost abolished cytotoxic activity. NAC had no effect on the activity of MSA in any cell line studied. As MSA is activated to methylselenol in a reaction requiring GSH, increased intracellular GSH has been reported to increase the activity of MSA [21], an effect not seen in our studies. In contrast, GSH has a dual role in the activation of SDG in that it acts as a cofactor in the generation of ROS by SDG, but also acts as an antioxidant by mopping up the ROS produced [22]. GSH, or NAC, may
therefore reduce the activity of selenite, or SDG [22, 23]. These data clearly highlight the importance of ROS in the induction of apoptosis by SDG in some cell lines, but also suggest that this can be independent of ROS as the compound was active in DoHH2 cells in the absence of ROS generation, and in the presence of NAC.

These data also suggest mechanisms whereby SDG may be exerting its activity. ROS are potentially DNA damaging and other reports have described the induction of general [23] or specific [24] DNA damage with sodium selenite. Using a similar assay MSA did not generate DNA damage [25]. Selenite is also reported to cause topoisomerase II/DNA cleavable complex formation resulting in double-strand DNA breaks and apoptosis [23], albeit at concentrations higher than those used in our own studies. This activity resulted from the modification of important thiol groups in topoisomerase II by selenite generated ROS, an action reversed by GSH.

In addition to effects mediated by selenoproteins, both of these selenium species deplete intracellular GSH during their activation, so may make cells more sensitive to cytotoxic drugs that are inactivated by conjugation with GSH, such as cisplatin or doxorubicin.

The cytotoxic activity of MSA and SDG was also studied in primary lymphoma samples from patients who had received multiple previous lines of chemotherapy and in a normal B-cell sample. All primary samples showed a concentration dependent loss of viability after a 2-day exposure to MSA or SDG, with the potentially genotoxic compound SDG showing slightly greater potency than MSA (EC50 values 9–28 μM compared to 39–300 μM respectively). Although only one normal B-cell sample was studied it is noteworthy that the EC50 concentrations for both SDG and MSA were higher than for the lymphoma samples.

These studies were conducted to determine the activity of different selenium species as single agents, prior to examining the role of selenium in mediating chemosensitivity in vitro and in vivo in the lymphoma setting. The finding that SDG cytotoxicity is, at least in part, dependent on ROS generation further suggests that this compound may be directly genotoxic. SDG is not currently available in a form that can be administered to patients. In contrast other selenium species, most notably Se-Methylselenocysteine (MSC), are available for clinical use and in the case of MSC are rapidly converted by β-lyase to methylselenol [25], the same activated species as with MSA used in our studies. MSC has also shown impressive synergy with cytotoxic agents in a colorectal cancer mouse xenograft study, by both protecting normal tissue and by increasing the sensitivity of tumour tissue [26].

Figure 3. Dual labelling with PI (FL3-H) and annexin (FL1-H) in DHL4 (A) and SUD4 (B) cells treated with staurosporine (24 h), or the cytotoxic EC50 concentrations of SDG or MSA for 3 days (percentage of cells in each quadrant). Cell death with SDG and MSA is associated with increased signal from both labels, confirming cell death by apoptosis. Staurosporine is included as a positive control.
Further studies investigating the potential role of selenium compounds, particularly those activated rapidly to methylselenol, as an adjunct to established chemotherapy in the treatment of lymphomas, are clearly warranted.

acknowledgements

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references


