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In vitro cytoprotective activity of squalene on a bone marrow versus neuroblastoma model of cisplatin-induced toxicity: implications in cancer chemotherapy

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Abstract

The development of a non-toxic selective cytoprotective agent that preferentially protects normal tissues from chemotherapy toxicity, without protecting malignant tissues, is a major challenge in cancer chemotherapy research. The available cytoprotective agents are either toxic or lack selective cytoprotective activity. Here, we report the *in vitro* selective cytoprotective activity of squalene, an isoprenoid molecule with antioxidant properties. Normal human bone marrow (BM) derived colony-forming unit (CFU) growth was increased by squalene in a dose-dependent manner. Squalene (12.5–25 μM) treatment significantly protected the CFUs from cisplatin-induced toxicity; the protective effect was equivalent to reduced glutathione (GSH), a known cytoprotective agent. Squalene also increased the long-term survival of cisplatin-treated 4-week-old CFUs. Cisplatin-induced apoptosis of CFUs as measured by the TUNEL assay was reduced by squalene. To examine the squalene-induced protection of tumours, several neuroblastoma cell lines, including five *MYCN*-amplified cell lines, were grown in monolayers, as well as in anchorage-independent cultures, in the presence of squalene and cisplatin. Squalene did not protect the neuroblastoma (NBL) cell lines from cisplatin-induced toxicity. In addition, squalene did not protect the NBL cells from carboplatin, cyclophosphamide, etoposide and doxorubicin-induced toxicity. In conclusion, our results suggest that squalene has a selective *in vitro* cytoprotective effect on BM-derived haematopoietic stem cells that is equipotent to GSH.

Keywords: Squalene; Cytoprotection; Cisplatin toxicity; Apoptosis; Glutathione; Neuroblastoma

1. Introduction

One of the major limitations of cancer chemotherapy is the indiscriminate injury of normal tissue, leading to multiple organ toxicity and consequent dose limitation/treatment failure. Often the consequences of toxicity, such as myelosuppression, renal toxicity and neuropathy, have profound effects on adults and children with long-term remission that not only affects the therapy, but also the overall quality of life [1]. Several antioxidants have been tested and shown to protect normal tissues from the chemotherapeutic toxicity by scavenging free radicals. Amongst these antioxidant agents, amifostine, a thiol antioxidant, has cytoprotective action and was approved for the use against platinum-induced renal toxicity in ovarian carcinoma patients [2]. However, the clinical use of amifostine is associated with toxicity [3], and its efficacy in preventing acute and chronic toxicity remains doubtful, particularly in paediatric tumours [4]. We found that intravenous (i.v.) administration of amifostine was associated with significant toxicity, such as hypocalcaemia, anxiety, and hypotension [5]. Other cytoprotective agents such as sodium thiosulphate, mesna, and procainamide are not approved for wide clinical use due to a lack of efficacy and/or non-selective cytoprotection of tumour tissues against platinum and alkylating agents-induced toxicity [2]. Glutathione (GSH), an endogenous thiol antioxidant, is known to detoxify cisplatin metabolites [6,7]. We found that l-2-oxothiazolidine-4-carboxylate (OTZ),

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a GSH prodrug, is able to selectively modulate tumour GSH. It was observed that tumour GSH is depleted (chemopotentiation), while normal tissue GSH is elevated (chemoprotection). This phenomenon is known as the GSH paradox and it suggests that GSH enhancing agents may be used to differentially protect normal versus tumour tissues [8]. Despite these findings, the development of OTZ as a cytoprotective drug has not been possible because of its high toxicity. The direct clinical use of glutathione as a cytoprotective agent has been disappointing, likely because of the difficulty of achieving a therapeutic plasma level of GSH and its instability in plasma [7,9].

Therefore, there is a need for the development of novel agents, which maintain the fundamental characteristics of cytoprotective agents (these include protection of normal tissue from chemotherapy-induced toxicity, absence of tumour protection and absence of tumour growth stimulation). In addition, such agents must exhibit an excellent safety profile; good oral availability would be an asset.

Squalene is an isoprenoid compound having six isoprene units that possess antioxidant activities (Fig. 1); squalene is an intermediate of cholesterol metabolism, and is secreted in human sebum, where it may protect the skin from ultraviolet (UV) radiation [10]. Storm and colleagues [11] demonstrated the protective activity of squalene against radiation-induced injury in a mouse model. Several experimental models demonstrated the detoxifying activities of squalene against diverse chemicals such as hexachlorobiphenyl, hexachlorobenzene, arsenic, theophylline, phenobarbital and strychnine [12–14]. Squalene has also been found to have protective activity against several carcinogens, including azoxymethane-induced colon cancer [15] and nicotine-derived nitrosaminoketone - (NMK) induced lung carcinogenesis [16].

Squalene has shown differential activity in the growth and survival of normal versus tumour tissues. Hamilton and colleagues [17] found that Hjorth adjuvant (10% (w/v) squalene in water) increased the survival and proliferation of murine bone marrow (BM)-derived cells. Ikekawa and colleagues [18] found that squalene inhibited murine sarcoma growth and survival in a mouse model.

Such protective, anticarcinogenic and differential activities in normal versus tumour tissue suggest that squalene may have cytoprotective potential against chemotherapeutic agents. To address this potential, we investigated the protective activity of squalene in a BM versus neuroblastoma (NBL) model of cisplatin-induced toxicity, and compared it with the protective activity of GSH, a known cytoprotectant against cisplatin toxicity [7].

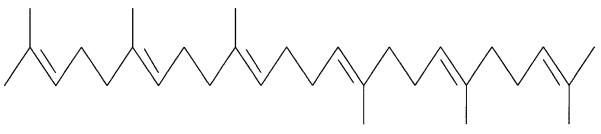

Fig. 1. Molecular structure of squalene. Squalene is an isoprenoid compound comprised of six isoprene units. The antioxidant property of squalene may be related to these isoprene units [10].

2. Materials and methods

2.1. Materials

Materials for the colony-forming unit (CFU) assay, including methylcellulose media (Methocult GF^{H4434}), were obtained from Stem Cell Inc., BC, Canada. Cisplatinum (II) diammine dichloride (cisplatin), reduced glutathione (GSH), etoposide, carboplatin and doxorubicin were obtained from Sigma; 4-hydroxy cyclophosphamide was a gift from Asta Medica, Germany. Squalene (Squalene iP6, derived from dogfish shark oil) was a gift from Isshogenki International, Hong Kong. Gas chromatography analysis performed in our laboratory established the purity of Squalene iP6 as more than 99.3% pure. The In Situ Cell Death Detection kit (fluorescein label) was obtained from Roche Molecular-Biochemical, Indianapolis, USA. Alamar blue reagent for cytotoxic assay was obtained from Biosource International, CA, USA. Soft-agarose (Sea Prep agarose) was obtained from Mandel Scientific Company, Guelph, Canada. Unless otherwise stated, tissue culture and other reagents/drugs were obtained from Sigma.

2.2. Cell culture

Fresh normal human BM specimens were obtained from normal healthy donors for BM transplantation after obtaining informed consent; normal light density BM mononuclear cells containing heamatopoitic progenitor cells (LD-BM cells) were separated by the Percoll method as described in Ref. [19]. Separated LD-BM cells were immediately used for the CFU assay as described below.

NBL cell lines, SKNSH, SKNBE-2, LAN-5, GOTO, IMR-32 and NBL-S were obtained from the American Type Culture Collection (ATCC), Manassas, USA; the NUB-7 cell line was developed in our laboratory [20]. GOTO [21] and NBL-S [22] cell lines were generously provided by Dr. Higuchio and Dr. S. Cohn, respectively. All NBL cell lines were maintained in alpha-MEM medium (GIBCO-BRL) supplemented with 20% fetal bovine serum (FBS) and antibiotics, in a humidified atmosphere of 5% CO_2 at 37 °C.

Cisplatin was dissolved in dimethyl sulphoxide (DMSO). Glutathione was prepared as a 10-mM stock solution in media just prior to the treatment of cells. Squalene was first prepared as a 20-mM stock in ethanol and then combined with bovine lipoprotein (1:1) before being added to media. Squalene was freshly prepared for each set of experiments.

2.2.1. CFU assay

This assay measures the functional capacity of the haematopoietic progenitor cells to form colonies; CFU granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM), burst forming unit-erythroid (BFU-E), and CFU granulocyte-macrophage (CFU-GM) are assessed using a standardised methylcellulose assay system [23]. Briefly, LD-BM cells were seeded in 35 mm dishes (50 000 cells in each dish) containing 1.1 ml of methylcellulose media (Methocult GF H4434, containing interleukin-3 (IL-3), stem cell factor (SCF), granulocyte macrophage-colony stimulating factor (GM-CSF) and erythropoietin) in the presence of test drugs. Culture dishes were incubated at 37 °C and 5% CO_2 and haematopoeitic colonies were counted after 14 days. Colonies were identified according to well-established morphological criteria [24] and scored in an inverted microscope using a gridded 60-mm dish. Results were expressed as percent of colony growth in control cultures; experiments were repeated four times. Colony composition was evaluated by pooling randomly selected colonies removed with a Pasteur pipette; cells were washed three times, cytocentrifuged on to glass slides, and stained with Wright-Giemsa (Sigma, MO, USA), and examined microscopically.

2.2.2. TUNEL assay for apoptosis

The terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay can detect apoptosis at the single-cell level by enzymatic labelling of DNA strand-breaks. The assay was performed using the In Situ Cell Death Detection kit (fluorescein label) as previously described in Ref. [25]. Briefly, 4-week-old CFU-GEMM colonies were randomly isolated with a Pasteur pipette; pooled cells (20 000 cells) were washed twice with phosphate-buffered solution (PBS) and then cytocentrifuged onto glass slides and fixed with 4% freshly prepared paraformaldehyde for 1 h at 25 °C. After washing with cold PBS, cells were permeabilised (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice and washed twice with cold PBS. The slides were stained with 50 μM of freshly prepared reaction mixture and incubated for 1 h at 37 °C in the dark. Slides were again washed twice using PBS and counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for 15 min and mounted in Prolong Antifade (Molecular Probes, Eugene, OR, USA) mounting media. The slides were examined under epifluorescence microscopy; apoptotic cells were viewed under a fluorescein isothiocyanate (FITC) filter and total DAPI-stained cells under an ultraviolet (UV) filter. Results were expressed as the percentage of apoptotic cells per field of DAPI-stained cells.

2.2.3. Alamar blue cytotoxicity assay

The effect of squalene on cisplatin-induced NBL cell toxicity was assessed by an Alamar blue cytotoxicity assay, which incorporates an oxidation-reduction (redox) indicator that both fluoresces and changes colour in response to chemical reduction, reflecting the extent of cell growth [26]. Briefly, NBL cells were grown in 24-multiwell plates (Costar) and treated with various doses of drugs, with and without squalene (0–25 μM). After 48 h of treatment, cells were washed with fresh media and Alamar blue solution was added to each well in an amount equal to 10% of the media volume. Cells were incubated for an additional 3 h and fluorescence readings were obtained using the Gemini Spectra MAX microplate reader (emission 540 nm, excitation 590 nm and cut-off 570 nm). Relative fluorescence values were normalised to the solvent control set at 100. All assays were run in triplicate, and EC_{50} values (the concentration of drug needed to reduce cell population by 50% *in vitro*) were obtained.

2.2.4. Clonogenic assay

In this assay, tumour cells are grown in the soft agarose gel system, and colonies were counted after 2 weeks of incubation [27]. We have used this assay to assess the effects of squalene against NBL growth. Briefly, 1×10^5 cells were added to medium plus agarose gel (1.5% bottom layer and 1% top layer of agarose) prepared in a 35 mm suspension culture dish in presence of squalene (0–25 μM) with serum supplementation. Culture dishes were incubated at 37 °C and 5% CO_2. After 2 weeks, colonies were counted under a phase-contrast microscope. The effect of squalene on cisplatin-induced NBL toxicity was also assessed by this assay.

2.2.5. Statistical analysis

The colony growth data for both the agarose and methylcellulose assays were normalised to the percentage of the control, and analysed by one-way ANOVA. Cisplatin-induced cytotoxicity results were analysed by non-linear regression (sigmoidal dose response) to obtain the EC_{50} value of cisplatin in both the NBL and BM colony growth assays. The apoptotic data was analysed by the Student *t*-test (unpaired). Graphpad prism software was used for the statistical analysis and graphs. Statistical significance was set at $P<0.05$.

3. Results

3.1. Effect of squalene on BM colony growth

In the CFU assay, squalene increased the number of haematopoietic colonies in a dose-dependent manner; colony stimulation was maximum at a dose range of 12.5–25 μM (Fig. 2a). CFU-GM exhibited maximal growth effect with a 40% increase in colony number at 12.5 μM of squalene ($P<0.02$); CFU-GEMM colony growth was not significantly increased. There were no

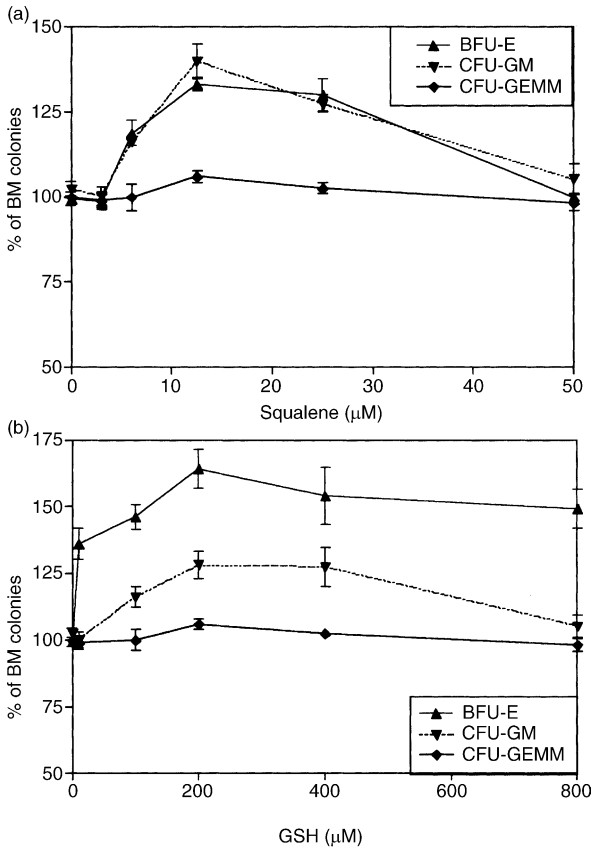

Fig. 2. Effect of squalene on bone marrow (BM) colony growth as measured by the colony forming unit (CFU) assay; (a) squalene (12.5 and 25 μM) increased colony forming unit-granulocyte-monocyte (CFU-GM) and blast forming unit-erythroblast (BFU-E) colony numbers significantly ($P<0.02$); (b) GSH (10–800 μM) used as a standard control also increased BFU-E and CFU-GM colony numbers ($P<0.001$). The data represent four independent experiments expressed as means±standard error of the means (SEM). Mean colony numbers in control cultures were: colony forming unit-granulocyte, erythrocyte, monocyte and megakarocyte (CFU-GEMM), 25±7; CFU-GM, 110±18; BFU-E, 126±21.

marked differences in the colony size between the control and squalene treatment groups. GSH treatment was used as a standard control. GSH stimulated a higher degree of BFU-E colony growth compared with squalene ($P<0.001$; Fig. 2b).

3.2. Effect of squalene on cisplatin-induced BM colony toxicity

We first evaluated the toxic effect of cisplatin on BM colony growth. Cisplatin reduced the colony number in a dose-dependent manner (Fig. 3a); 1–8 μM cisplatin doses were selected to study the effect of squalene on cisplatin-induced toxicity. Squalene (12.5–25 μM) demonstrated a significant protective effect against 1–2 μM cisplatin-induced toxicity. Squalene (25 μM) increased the colony growth by 62% compared with cisplatin 2 μM alone ($P=0.042$; Fig. 3c). At a higher dose of cisplatin (4 μM), 25 μM squalene increased the colony growth by 32% compared with cisplatin alone ($P=0.042$; Fig. 3d). Low doses of squalene (6–12.5 μM) failed to show significant protection against higher cisplatin doses. The protective effect was evident for all of the colony types, CFU-GEMM, CFU-GM and BFU-E (data not shown).

The protective effect of squalene was compared with GSH, a known cytoprotectant against cisplatin-induced toxicity [6]. The maximum protection was observed at 200 μM of GSH (Fig. 3e and f). At 8 μM of cisplatin, both squalene and GSH failed to show any significant protection of the BM colonies (data not shown).

To further compare the protective effect of squalene with GSH, LD-BM cells were treated with serial doses of cisplatin, with and without 25 μM squalene or 200 μM GSH, and dose response curves were obtained (Fig. 4). Non-linear regression analysis of the data revealed that squalene (25 μM) increased the cisplatin EC_{50} from 2.6 to 3.9 μM ($P=0.017$), and GSH (200 μM) increased the cisplatin EC_{50} from 2.6 to 4.1 μM ($P=0.042$), suggesting that 25 μM squalene has a similar degree of cytoprotective activity as 200 μM GSH. From the dose response curves, squalene and GSH-induced protection was seen within a cisplatin dose range of 0.1–4 μM. Both squalene and GSH maintained the stimulatory effect on BM colony growth at a cisplatin dose range of 0.1–2 μM.

3.3. Effect of squalene against the persistent toxicity of cisplatin

Platinum compounds have been found in tissues such as the bone marrow and kidney, even 6 months after the initial dose of cisplatin; the persistent toxic effect of deposited platinum may contribute to cumulative toxicity [28]. The *in vitro* testing of such persistent cisplatin toxicity has not yet been reported. Here, we have tried to assess the persistent cisplatin toxicity and the potential protective effect of squalene using the CFU assay. We hypothesised that accumulated platinum compounds in the methylcellulose media would increase the amount of apoptosis of individual cells of BM colonies, leading to a decreased survival of the BM colonies.

LD-BM cells were plated in methylcellulose media in the presence of 2 μM cisplatin, and CFU-GEMM colonies were counted at 2 and 4 weeks (CFU-GM and BFU-E colonies were not counted as they often degenerate at 3–4 weeks). We found that only 16% of the cisplatin-treated colonies survived compared with 82% of colonies in the control group after 4 weeks (Fig. 5a). To test for apoptosis, 4-week-old colonies were randomly isolated, washed in PBS and stained with the TUNEL reagent (described in the Materials and methods). Apoptosis increased by 5-folds in the 2 μM cisplatin-treated-treated cells relative to controls (Fig. 5b).

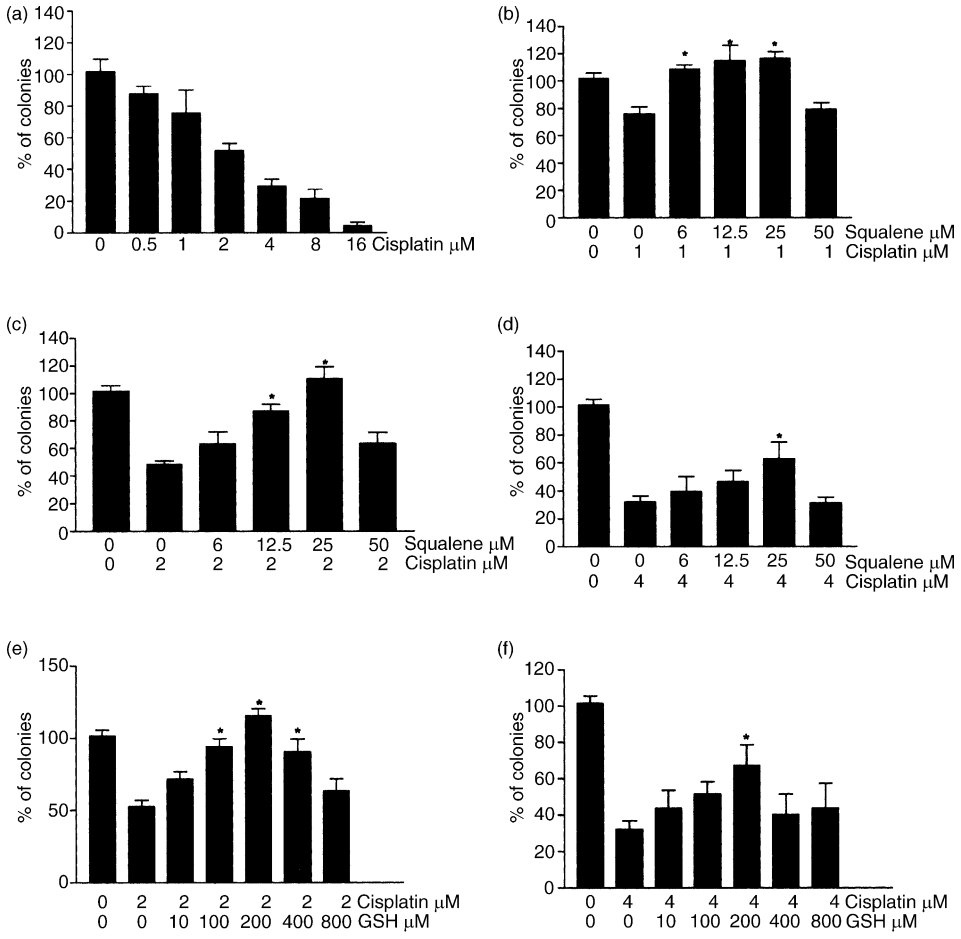

Fig. 3. Effect of squalene on cisplatin-induced bone marrow (BM) toxicity as measured by a colony-forming unit (CFU) assay: (a) cisplatin reduced BM colony growth in a dose-dependent manner; (b–d) the protective effect of squalene against 1–4 μM of cisplatin. A significant protective effect was seen with doses of 25 μM squalene against a cisplatin dose range of 1–4 μM. (e, f) glutathione (GSH) used as a standard control; 200 μM GSH resulted in the maximum protective activity. The data represent four independent experiments expressed as means±standard error of the mean (SEM). Mean colony numbers in the control cultures were: CFU-granulocyte, erythrocyte, monocyte and megakarocyte (CFU-GEMM), 15±2; CFU-granutocyte-monocyte (CFU-GM), 98±10; Blast-forming unit-erythroblast (BFU-E), 142±17; $*P<0.05$.

The addition of 25 μM squalene significantly decreased apoptosis by 2-folds ($P<0.05$) and increased survival of CFU-GEMM colonies by 2.9 folds compared with 2 μM cisplatin alone ($P<0.05$). GSH (200 μM) had a similar effect on the survival and apoptosis of CFU-GEMM colonies (Fig. 5a and b).

Microscopic observation of 4-week-old CFU-GEMM colonies (after Wright-Giemsa staining) revealed the presence of large numbers of foam cells (macrophages with lipid-filled vacuoles; Fig. 5c). The number of foam cells increased by 5-fold in the cisplatin-treated group relative to the control group; the addition of squalene and GSH reduced foam cell accumulation significantly (Fig. 5d).

Overall, the results demonstrated an increase in the number of foam cells, correlating with decreased colony survival and increased apoptosis in the cisplatin-treated group, suggesting the persistence of the toxic effect of cisplatin in the methylcellulose media. Both squalene and GSH were able to reduce apoptosis and foam cell accumulation, suggesting they have a protective activity against such cisplatin-induced persistent toxicity.

3.4. Effect of squalene on neuroblastoma (NBL) colony growth

It is very important that cytoprotective agents neither stimulate the growth of tumours nor protect them from the chemotherapeutic toxicity. We have found that squalene (6–25 μM) stimulated the growth of bone marrow colonies. Therefore, we examined the growth effect of squalene against several NBL cell lines of diverse characteristics; SKNBE-2, NUB-7, LAN-5, GOTO and IMR-32 were highly malignant *MYCN*-amplified cell lines, whereas SKNSH and NBL-S were non-*MYCN*-amplified cell lines [21,29].

The effect of squalene on the NBL colony growth was assessed by a clonogenic assay (see Material and methods), and the results are shown in Fig. 6. The growth of NUB-7 and SKNBE-2, LAN-5 and IMR-32 cell lines

remained unaffected; the growth of the GOTO cell line showed some degree of inhibition compared with the control, but the difference was not significant. The SKNSH and NBL-S cell lines showed a slight, yet significant, growth inhibition; 25 μM squalene inhibited SKNSH colony growth by 13% ($P<0.05$). To further evaluate the growth inhibitory activity, SKNSH cells were treated with 25 μM of squalene for 10 days in monolayer culture. Squalene-treated cells showed morphological changes such as the predominance of large flat cells in the culture suggestive of differentiation, while other cell lines failed to show such morphological changes (data not shown).

3.5. Effect of squalene on cisplatin-induced NBL toxicity

The effect of squalene against cisplatin-induced NBL toxicity was measured by an Alamar blue assay, and the EC_{50} of cisplatin, with and without various doses of squalene (3–25 μM), were obtained. The results of 12.5–25 μM squalene doses are shown in Table 1. Squalene slightly increased the cisplatin sensitivity in the SKNSH and SKNBE-2 cell lines. To further examine the effect of squalene against cisplatin-induced NBL cell toxicity, a clonogenic assay was performed as described in the Materials and methods. 25 μM squalene increased the toxicity of 4 μM cisplatin in the SKNSH cell line by 22% ($P<0.05$, Fig. 7b), whereas SKNBE-2 cells showed no significant potentiation or protective effect of squalene against cisplatin-induced toxicity (Fig. 7d). The other NBL cell lines also showed no significant changes in the cisplatin toxicity in the presence of squalene (data not shown).

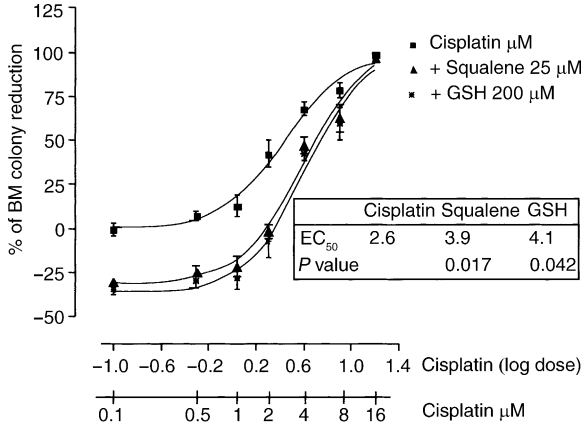

Fig. 4. Normal human light density fraction of bone marrow mononuclear cells containing haemopoietic progenitor cells (LD-BM cells) were plated in the methylcellulose media in the presence of serial doses of cisplatin (0–16 μM), with or without 25 μM squalene or 200 μM GSH. After 2 weeks, colonies were counted, data were converted to percent of colony reduction compared with control, and a non-linear regression analysis was done to obtain EC_{50} values. In the figure, the sigmoidal dose response curves show similar cytoprotective activity of squalene and glutathione (GSH) against cisplatin toxicity. The curves also showed that within a cisplatin dose range of 0.1–2 μM, the BM stimulatory effect of both squalene and GSH was maintained. Mean colony numbers in the control cultures were: colony-forming unit-granulocyte,monocyte and megakarocyte (CFU-GEMM), 18±5; CFU-granulocyte-monocyte (CFU-GM) 122±22.4; blast-forming unit-erythoblast (BFU-E), 109±20; Abbreviations: +squalene 25 μM: cisplatin plus squalene 25 μM; + GSH 200 μM: cisplatin plus GSH 200 μM.

3.6. Squalene did not protect NBL cells from other chemotherapeutic agents

Since cisplatin is often included in a multidrug chemotherapy regimen, it is necessary to determine if squalene may protect malignant tissues from other commonly used chemotherapeutic agents such as alkylating agents, and topoisomerase inhibitors. Two NBL cell lines, SKNBE-2 and SKNSH, were cultured in monolayers and treated with various doses of cyclophosphamide, etoposide, carboplatin and doxorubicin, with or without squalene (12.5 and 25 μM). An Alamar blue cytotoxicity assay was performed to obtain EC_{50} values. Squalene did not affect the EC_{50} of any of the agents (data not shown). In the clonogenic assay, 25 μM squalene appeared to potentiate the effect of etoposide toxicity in the SKNSH cell line; however, the result was not statistically significant (data not shown).

4. Discussion

Squalene is an isoprenoid compound having protective activities against certain chemicals and radiotherapy [10,11,15]. Here, we report that squalene (12.5–25 μM) increased the growth and protected BM colonies from cisplatin-induced toxicity without protecting NBL colonies *in vitro*.

We used GSH as a standard control and found that 100–200 μM GSH increased BFU-E colony growth. Previous investigators found maximum stimulation at 10–100 μM. This apparent discrepancy might be due to the use of a different methylcellulose media. We used methylcellulose media enriched with cytokines (SCF, IL-3, CSF-GM and erythropoietin), whereas previous investigators used leucocyte-conditioned media [30]. In our assay set-up, 25 μM squalene-induced protection resembled 200 μM GSH-induced protection, suggesting that squalene-induced protection is equipotent to GSH-induced protection in BM cell colonies. GSH is known to protect renal and neuronal cells from cisplatin-induced toxicity [7]. Thus, it remains to be seen whether squalene may have a similar effect as GSH in renal and neuronal tissues.

Earlier, Storm and colleagues [11] found that squalene protected the haematopoietic system from radiation injury in mice, and Hamilton and colleagues [17] observed that Hjorth adjuvant (10% squalene in water) increased the survival and proliferation of murine bone marrow-derived lymphocytes. Such a haematopoietic

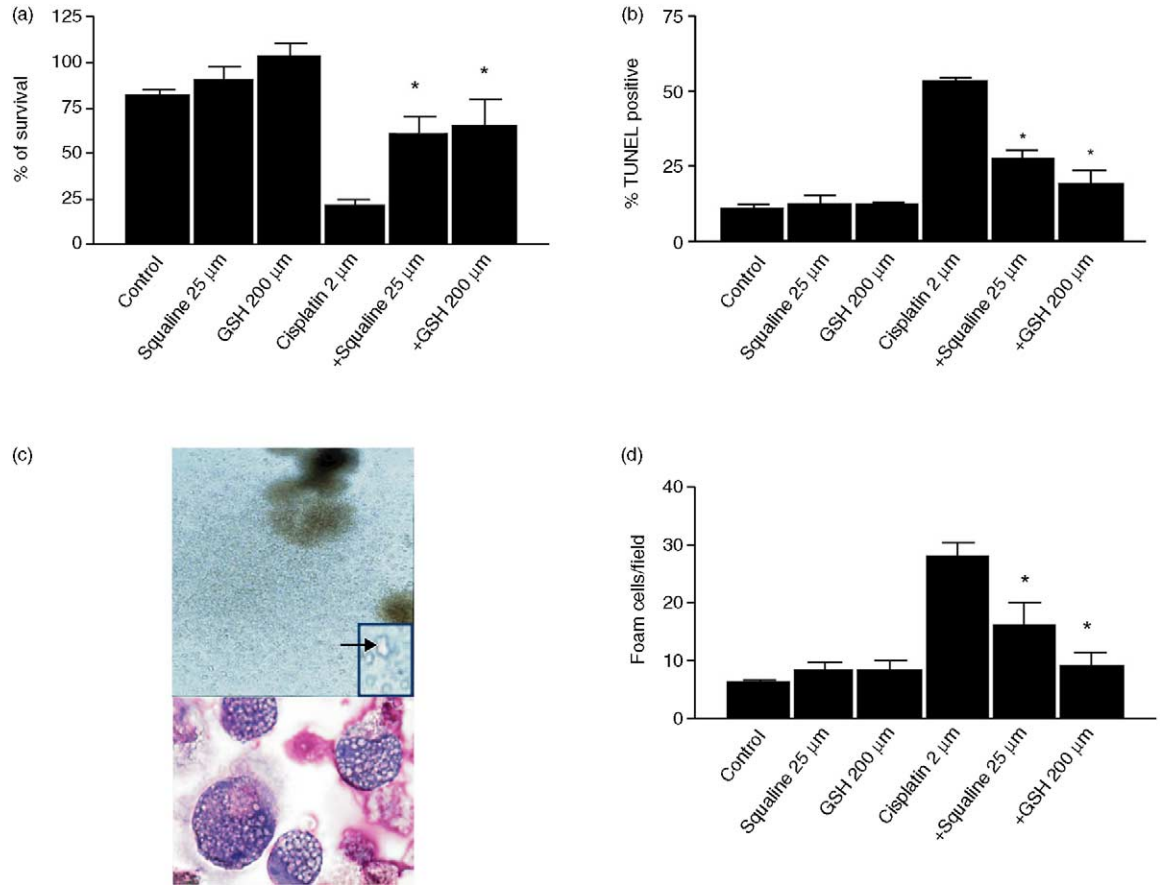

Fig. 5. Colony-forming unit-granulocyte, erythrocyte, monocyte and megakarcyte (CFU-GEMM) colonies were counted after 2 and 4 weeks to obtain the percentage of survival by comparing colony numbers at 2 and 4 weeks. Apoptosis was measured as described in Material and methods (a) cisplatin markedly reduced the colony survival, whereas the addition of squalene and glutathione (GSH) increased the survival (b) addition of 25 μM squalene and 200 μM GSH decreased apoptosis in the CFU-GEMM colonies compared with cisplatin treatment alone (c) the microscopic appearance of a 4-week-old cisplatin (2 μM)-treated CFU-GEMM colony filled with large cells (arrow). Wright-Giemsa staining shows these large cells as lipid-filled foam cells. (d) Both squalene and GSH decreased foam cell accumulation compared with cisplatin. Mean CFU-GEMM colony numbers in control cultures were: 24±5 at the 2nd week and 19±4 at the 4th week. *$P<0.05$. Abbreviations: +squalene 25 μM; squalene 25 μM plus Cisplatin 2 μM; +GSH 200 μM; GSH 200 μM plus Cisplatin 2 μM.

protective effect of squalene has been attributed to its antioxidant nature [11]. We found that squalene increased CFU-C growth in a dose-dependent manner; the growth stimulatory effect was maintained even in the presence of cisplatin (0.1–2 μM dose range, Fig. 4). Thus, the observed protective effect of squalene against cisplatin-induced toxicity may be related to the antioxidant effects of squalene.

However, the antioxidant activity alone cannot explain the protective activity against 4-week-old BM colonies, including a reduction in apoptosis. We have found that the protective activity of squalene was similar to GSH (Fig. 5), which is known to detoxify platinum compounds by enhancing the GSH-GST detoxification system [6]. Squalene may also have a similar detoxification activity against deposited platinum compounds. In addition, we found that squalene significantly decreased cisplatin-induced foam cell accumulation (Fig. 5c and d). Foam cells are lipid-laden macrophages presumably present in an environment of oxidative stress [31]. We believe that the deposited cisplatin created an environment of oxidative stress in the culture leading to an increased accumulation of foam cells. Squalene and GSH reduced foam cell accumulation suggesting that these two agents detoxified platinum compounds resulting in decreased oxidative stress in the culture.

Squalene did not protect NBL cell lines from cisplatin-induced toxicity. In addition, squalene did not protect NBL cell lines from cyclophosphamide, etoposide, carboplatin and doxorubicin-induced toxicity *in vitro*. Interestingly, squalene (25 μM) demonstrated a slight growth inhibition and potentiation of cisplatin toxicity in the SKNSH cell line; however, considering that we did not observe similar activities in other NBL cell lines, it can be assumed that the findings in SKNSH cells are cell-line specific.

We recognise that in our study comparison was made between primary bone marrow stem cells and established

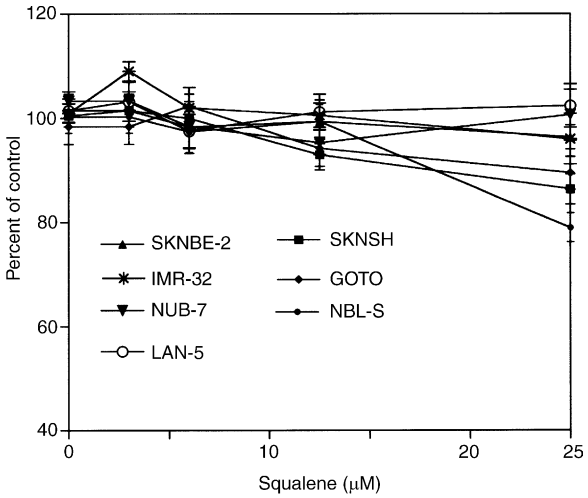

Fig. 6. Effect of squalene on NBL colony growth as measured by a clonogenic assay. Squalene (25 μM) slightly inhibited tumour growth in SKNSH and NBL-S cell lines ($P<0.05$). Data were normalised to the percentage of colony growth compared with the control, and the results represent three independent experiments expressed as the means±SEM. Mean colony numbers in the control groups were: SKNSH, 229±24; SKNBE-2, 399±29; NUB-7, 315±38; IMR-32, 456±27; GOTO, 167±35; NBL-S, 220±21; LAN-5, 188±26.

neuroblastoma cell lines in terms of the squalene-induced protection against cisplatin toxicity which may not be reflective of the clinical situation.

Overall, we found that squalene exerted a selective protective activity against cisplatin-induced toxicity in the BM versus NBL cells. Several antioxidants, including alpha-tocopherol, GSH and GSH-enhancing agents (i.e. drugs that increase the cellular GSH level) such as N-acetyl-cysteine (NAC), have been found to have selective protective activity against cisplatin toxicity in normal versus tumour cells [32,33]. The precise mechanism of such selective protection is not known. Recently, it has been demonstrated that GSH and NAC protect neuronal cells from cisplatin-induced toxicity by modulating the p53-mediated stress response [33,34]; these agents inhibited cisplatin-induced apoptosis in normal neuronal cells, but not in neuroblastoma cells. It can be hypothesised that squalene-induced protection may also be p53-dependent. Normal BM cells having intact p53 would be protected, whereas tumour cells having a deficient p53 function would not be protected. It would be important to examine such p53-mediated selective protection for the future development of more potent cytoprotective agents.

In summary, our investigations suggest a novel biological effect of squalene against cisplatin-induced BM toxicity and the absence of such protection against cisplatin-induced toxicity in neuroblastoma. The cytoprotective activity of squalene resembles GSH, a known protective agent against cisplatin-induced toxicity. In addition, we found that squalene protected CFU-GEMM colonies from persistent cisplatin toxicity; such protection may contribute to a reduction in the cumulative toxicity, which is a major clinical concern relating to platinum toxicity.

Squalene is found in dietary components such as olive, palm and wheat germ oils [35]. Squalene ingestion (900 mg daily for 7 days) in adults has been found to raise the plasma level of squalene to approximately 10±8 μM (the data has been converted from μg of squalene/100 mg cholesterol to μM of squalene) [36]. Squalene can be administered both orally and intravenously, and a recent study indicates that the plasma squalene levels remain above baseline, even 3 h after the injection [37], which suggests that squalene is relatively stable in plasma compared with GSH and amifostine. Although data is lacking about the tissue deposition of squalene after oral/parenteral administration, very high levels of squalene have been found in adipose tissue, kidney and lymphoid tissues. Liu and colleagues [38] found that adipose tissue contains 300 μg/g of squalene in subcutaneous fat and 160 μg/g of squalene in abdominal fat. However, the study did not measure the squalene level in BM. Considering that bone marrow contains adipose tissue, it may be presumed that the squalene level could also be high in the BM. It is therefore likely that a tissue level of 6–25 μM (2.4–10 μg/ml) of squalene used in our study is achievable after oral/parenteral administration of squalene. We are now investigating the tissue level of squalene in bone marrow, kidney and other tissues after oral/parenteral administration of squalene in mice.

Table 1
The effect of squalene against cisplatin-induced neurblastoma (NBL) toxicity as measured by alamar blue assay

NBL cell lines	Cisplatin EC_{50} in μM	+Squalene 12.5 μM	+Squalene 25 μM
NUB-7	8.12±0.23	7.52±0.34	8.24±0.35
SKNBE-2	5.22±0.73	5.12±0.54	4.51±0.65
NBL-S	2.50±0.32	2.21±0.42	2.11±0.32
GOTO	4.51±0.83	4.11±1.2	4.03±1.54
SKNSH	3.52±0.65	3.14±0.74	1.64±0.24*
LAN-5	2.53±0.56	3.04±0.65	2.51±0.34

Cell were grown in 24-well plates and treated with cisplatin, with or without squalene. After 48 h of treatment, cells were incubated with fresh media containing 10% Alamar blue for 3 h and a fluorescence reading was obtained. Data were converted to percent of controls and analysed by non-linear regression (sigmoidal dose response). *$P=0.0025$. Abbreviations: +squalene 12.5 μM; cisplatin EC_{50} in presence of squalene 12.5 μM; +squalene 25 μM; cisplatin EC_{50} in presence of squalene 25 μM.

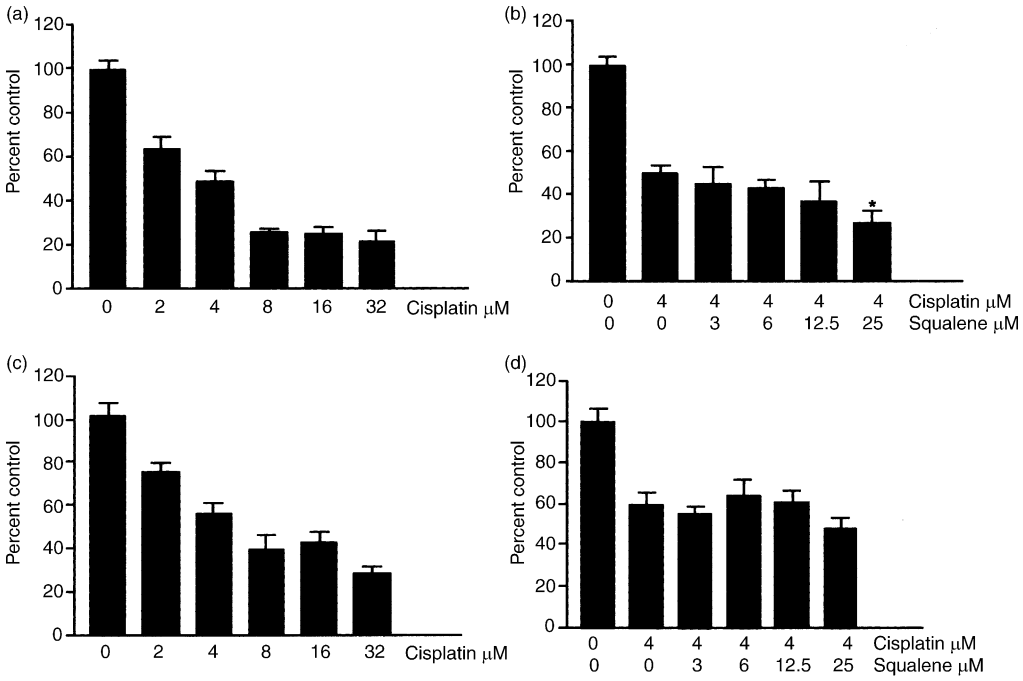

Fig. 7. Effect of squalene on cisplatin-induced neuroblastoma (NBL) colony toxicity as measured by a clonogenic assay. (a) effect of cisplatin against SKNSH colony growth; (b) squalene (25 μM) potentiated the cisplatin (4 μM) toxicity against SKNSH colonies by 22% ($P<0.05$); (c) effect of cisplatin against SKNBE-2 colony growth; (d) 6 μM squalene decreased, whereas 25 μM squalene increased the cisplatin-induced toxicity of SKNBE-2 colonies; however, the differences were not significant. Mean colony numbers in the control groups were: SKNBE-2, 402±22; SKNSH, 206±12.

There is an urgent need for the clinical development of safe and non-toxic cytoprotective agents for the adequate management of cancer chemotherapy [1]. A phase I trial demonstrated that oral squalene is safe and tolerable. In the study, adult males were given 860 mg of squalene daily for 20 weeks to study the cholesterol-lowering effect of squalene. Squalene was found to be safe and tolerable; out of 26 patients studied, only one patient complained of diarrhoea [39].

Even though we have demonstrated selective cytoprotection in an *in vitro* model, it is possible that oxygenation in the culture media may affect the antioxidant activity and therefore, the protective activity of squalene. The *in vivo* cytoprotective activity of squalene may differ significantly from the *in vitro* protective activity. We are developing a NB xenograft mouse model to test the cytoprotective activity of squalene *in vivo*.

In conclusion, the selective cytoprotection of squalene observed in our studies warrants further investigation, including *in vivo* examination of cytoprotection against cisplatin, carboplatin and other alkylating agents such as cyclophosphamide, carboplatin, melphalan and busulphan.

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