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ORIGINAL ARTICLE

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Effect of NQO1 induction on the antitumor activity of RH1 in human tumors in vitro and in vivo

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Abstract NQO1 is a reductive enzyme that is important for the activation of many bioreductive agents and is a target for an enzyme-directed approach to cancer therapy. It can be selectively induced in many tumor types by a number of compounds including dimethyl fumarate and sulforaphane. Mitomycin C is a bioreductive agent that is used clinically for treatment of solid tumors. RH1 (2,5-diaziridinyl-3-(hydroxymethyl)- 6-methyl-1,4-benzoquinone) is a new bioreductive agent currently in clinical trials. We have shown previously that induction of NQO1 can enhance the antitumor activity of mitomycin C in tumor cells in vitro and in vivo. As RH1 is activated selectively by NQO1 while mitomycin C is activated by many reductive enzymes, we investigated whether induction of NQO1 would produce a greater enhancement of the antitumor activity of RH1 compared with mitomycin C. HCT116 human colon cancer cells and T47D human breast cancer cells were incubated with or without dimethyl fumarate or sulforaphane followed by mitomycin C or RH1 treatment, and cytotoxic activity was measured by a clonogenic (HCT116) or MTT assay (T47D). Dimethyl fumarate and sulforaphane treatment increased NQO1 activity by 1.4- to 2.8-fold and resulted in a significant enhancement of the antitumor activity of mitomycin C, but not of RH1. This appeared to be due to the presence of a sufficient constitutive level of NQO1 activity in the tumor cells to fully activate the RH1. Mice were implanted with HL60 human promyelocytic leukemia cells, which have low levels of NQO1 activity. The mice were fed control or dimethyl fumarate-containing diet and were treated with RH1. NQO1 activity in the tumors increased but RH1 produced no antitumor activity in mice fed control or dimethyl fumarate diet. This is consistent with a narrow window of NQO1 activity between no RH1 activation and maximum RH1 activation. This study suggests that selective induction of NQO1 in tumor cells is not likely to be an effective strategy for enhancing the antitumor activity of RH1. In addition, we found that RH1 treatment produced significant leukopenia in mice that may be of concern in the clinic. These results suggest that the ease of reduction of RH1 by NQO1 makes it a poor candidate for an enzyme-directed approach to cancer therapy.

Keywords RH1 · Antitumor activity · NQO1 · Diet · Induction

Abbreviations DCPIP: 2,6-Dichlorophenol-indophenol · DMF: Dimethyl fumarate · EO9: 3-Hydroxymethyl-5-aziridinyl-1-methyl-2-(1H-indole-4,7-dione) prop-β-en-α-ol · FBS: Fetal bovine serum · HBSS: Hank's balanced salt solution · MMC: Mitomycin C · NQO1: NAD(P)H:quinone oxidoreductase 1 · RED: NADPH:cytochrome P450 reductase · RH1: 2,5-Diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone · WBC: White blood cell

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Introduction

Bioreductive agents are a class of anticancer drugs that are inactive in their administered form and must be reduced within cells to the active cytotoxic form. As solid tumors generally have low oxygen tensions, the reduction of these compounds occurs more readily in this environment, leading to preferential activation of the drugs in hypoxic cells. These drugs have been actively studied in terms of their clinical potential [51] and mode of action [31, 34]. The use of the prototype drug, MMC in the clinic has been recently reviewed [1]. The activation of bioreductive agents is generally by one-electron reducing enzymes such as RED (EC 1.6.2.4) [26, 34] or two-electron reducing enzymes such as NAD(P)H:quinone oxidoreductase 1 (NQO1) (DT-diaphorase) (EC 1.6.99.2) [32, 35]. RED and NQO1 are the major activators for current bioreductive agents [2, 6, 7, 16, 28, 32, 35], but the relative contribution of each of these enzymes to the reductive activation is dependent on the drug, enzyme and oxygen levels, [7, 16, 23] and pH [2, 38]. The two-electron reduction of MMC results in the activation of the aziridine and carbamate groups that can then form covalent bonds with DNA [22, 32]. The alkylation of each complementary strand of double-stranded DNA by a single MMC molecule produces the interstrand cross-links that are believed to be the major mechanism of cytotoxicity for this agent [22, 27, 32, 41]. In the presence of oxygen, redox cycling can also occur, resulting in the formation of reactive oxygen species that contribute to the cytotoxic activity [12, 22, 32].

NQO1 catalyses the two-electron reduction of a wide variety of compounds including quinones and nitrogen oxides [13, 32]. Although several human diaphorases have been identified [17, 18, 32], NQO1 is the most important in terms of the activation of bioreductive agents [8, 17, 32]. NQO1 is found predominantly in the cytosol; however 5–10% is membrane-bound [32], and the presence of this enzyme in the nucleus has also been reported [49]. The enzyme is ubiquitous in eukaryotes; however, the levels vary in different tissues of multicellular organisms [32]. The importance of this differential expression for cancer treatment lies in the low levels found in the hematopoietic cells [37, 39], and the overexpression of the enzyme in malignant cells as compared to normal cells [8, 32, 37]. The expression of NQO1 is regulated at the transcriptional level [32] and can be induced by a wide variety of dietary and synthetic inducers [30]. Although the induction pathway has not been fully elucidated it is believed to be initiated by a cytosolic redox signal. This signal alters the expression and/or interaction of transcriptional factors such as Jun, Nrf, Maf, Fos, and Fra with the xenobiotic and antioxidant response elements [8, 14, 20, 25, 44, 45, 53]. Known inducers of NQO1 include: dithiolethiones such as Oltipraz, an antiparasitic agent; isothiocyanates such as sulforaphane, found in cruciferous vegetables; and dietary metabolites such as DMF, a metabolite of fumaric acid which is found in fruits and vegetables [30, 40]. NQO1 is a member of the phase II detoxifying enzymes that help to remove xenobiotics from cells and are important in early defense against carcinogenesis [30].

Previous studies have demonstrated that the selective induction of NQO1 in tumor cells results in enhanced MMC antitumor activity in vitro [11, 47] and in vivo [5]. In a similar study with another bioreductive agent, EO9, enhanced antitumor activity was observed in vitro [11]. The addition of the NQO1 inhibitor, dicoumarol, reversed the effect of the inducer on EO9 activity confirming that the enhancement was due to elevated NQO1 activity [11]. As MMC may be primarily activated by RED [34], this approach to enhancing the antitumor activity of bioreductive agents may be more effective with agents that are selectively activated by NQO1 such as EO9 [11] and the diaziridinylbenzoquinone, RH1 [48]. This has indeed been found to be the case when the activities of MMC and EO9 were compared in mouse lymphoma cells that had been pretreated with the NQO1 inducer 1,2-dithiole-3-thione [3].

RH1 is a particularly potent bioreductive drug that has demonstrated significant antitumor activity against malignant cells in vitro [36, 48] and in vivo [10, 21]. This drug is a very efficient substrate for NQO1 [48] and the reduced form is very stable in comparison to the reduced form of MMC [24, 52]. Reduction of RH1 by NQO1 results in activation of the aziridine groups and subsequent DNA alkylation with interstrand crosslinks formed preferentially at 5′ GCC 3′ sequences [9, 10]. RH1 is currently in clinical trials [10]. In this study we investigated the effect of selective induction of NQO1 by DMF or sulforaphane in human tumors in vitro and in vivo on the antitumor activity of RH1.

Materials and methods

Materials

HCT116, human colon carcinoma cells, were obtained from ATCC (Manassas, Va.), T47D, breast ductal carcinoma cells, were from Dr. S. Mai (Manitoba Institute of Cell Biology, Winnipeg, MB, Canada) and HL60, promyelocytic leukemia cells, were obtained from Dr. A.H. Greenberg (Manitoba Institute of Cell Biology). RPMI 1640, RPMI 1640 with HEPES and DMEM:-Ham's F12 (1:1) media and HBSS were obtained from Invitrogen (Burlington, Ont., Canada). FBS was obtained from Cansera (Etobicoke, Ont.). Sulforaphane was obtained from LKT Laboratories (St. Paul, Minn.). RH1 was a generous gift from Dr. D. Ross (University of Colorado Health Sciences Center, Denver, Colo.). DMF, dicoumarol and reagents for the NQO1 activity assay were from Sigma-Aldrich Canada (Oakville, Ont., Canada).

Female CD-1 nude mice (6–8 weeks of age) were obtained from Charles River Canada (Montreal, QC,

Canada) and were maintained according to institutional regulations. The mice were fed standard irradiated rodent chow except during the experimental diet period. The experimental diet for the mice was a custom powdered autoclavable, semipurified diet with antioxidant-free corn oil and vitamin K instead of menadione from ICN Biochemical Division (Aurora, Ohio).

Microvettes CB 300 with potassium EDTA, used for blood collection were obtained from Sarstedt (St. Leonard, QC). Zap-oglobin II lytic reagent and Isoton II were obtained from Beckman Coulter (Mississauga, Ont.). Ketalean and Rompun were obtained from Central Animal Care, University of Manitoba (Winnipeg, Manit.).

Cell lines and cytotoxicity assays

Human colon carcinoma HCT116 cells were grown in DMEM/Ham's F12 medium supplemented with 10% FBS. Clonogenic assays were performed as previously described [2]. Cloning efficiencies ranged from 51% to 90%. Cells were incubated at 37°C for 48 h in the presence or absence of 5 μM DMF or 1.5 μM sulforaphane and then were treated with various concentrations of RH1 for 1 h. Cells were plated and the resulting colonies (> 50 cells) were counted 6 days later, and the surviving cell fraction determined.

Human breast ductal carcinoma T47D cells were grown in RPMI 1640 with HEPES supplemented with 10% FBS. MTT assays were performed as previously described [19]. Cells were incubated for 48 h at 37°C with or without 5 μM DMF or 1.5 μM sulforaphane and then were treated with various concentrations of RH1 for 1 h. The surviving cell fraction was determined after 11–12 days.

A linear regression analysis of each concentration-survival curve was obtained for the HCT116 and T47D cells and the D_{10} was derived from the negative reciprocal of the regression slope [7]. The cytotoxicities were compared statistically using a *t*-test comparing the significance of the differences for the slopes of the concentration-survival curves.

NQO1 activity measurements

NQO1 activity was measured as described previously [11] using menadione as the electron acceptor and is reported as nanomoles MTT reduced per minute per milligram protein. Protein concentrations were determined using a Bio-Rad DC kit with gamma-globulin as the protein standard. NQO1 activity was measured in 0.25 *M* sucrose sonicates of HCT116 cells, T47D cells and homogenized mouse kidneys or HL60 tumors. Mouse kidneys and tumor xenografts were excised and stored frozen at −80°C in 0.25 *M* sucrose until analysis. NQO1 activities from cell lines or tissues treated with or without an inducer were compared statistically using *t*-tests.

In vivo combination treatment studies

For the in vivo combination treatment studies, female CD-1 nude mice were implanted with 5×10^6 viable HL60 cells (determined by trypan blue exclusion) into both flanks of each mouse 3 days following 150 mg/kg i.p. cyclophosphamide treatment to facilitate tumor take [29]. The mice were fed a standard irradiated mouse diet for the first 10 days following tumor implantation and then randomly assigned to four groups of equal size. Two of the groups received experimental diet containing 0.3% (w/w) DMF and the other two received experimental diet without DMF. Tumor volumes were monitored three times weekly using digital calipers to measure the length, width and depth of the tumor and tumor volume was calculated using the formula, l×w×d×0.5236 [33]. When either of the tumors reached a volume of approximately 200 mm^3 the mice were started on a series of five daily i.p. injections. Two of the groups (one receiving DMF diet and one receiving control diet) were given HBSS and the other two groups received 0.4 mg/kg RH1 in HBSS daily for a total cumulative dose of 2 mg/kg. Tumor volumes and body weights were determined daily during the 5-day treatment period and every second day thereafter. Mean body weights and tumor volumes were compared for each of the time points by ANOVA.

WBC and platelet counts

On days 0, 3, 6, 9, and 12 after the first i.p. injection of HBSS or RH1 approximately 25 µl of blood was collected from the saphenous vein of 5 to 11 mice selected randomly from each treatment group [15]. The blood was collected using a CB 300 microvette coated with potassium EDTA and two 10-µl aliquots were taken, one for WBC counts and the other for platelet counts. The counts were determined using a Coulter Z2 particle counter and cell analyzer (Beckman Coulter, Mississauga, Ont.). The Coulter Z2 counter was optimized for mouse WBCs and platelets according to the manufacturer's protocols and all counts were analyzed using the Coulter AccuComp software program. For WBC counts, the aliquot was diluted in 10 ml Isoton II and counted using a 100 µm aperture tube (set for a lower threshold of 3 µm) following the addition of 50 µl Zap-oglobin II lytic reagent. The aliquot for platelet counts was diluted with the addition of 240 µl Isoton II and spun at 83 *g* for 3 min to remove red blood cells. A 100-µl aliquot of the supernatant was diluted to 10 ml in Isoton II and counted using a 70 µl aperture tube with a lower threshold of 1.4 fl and an upper threshold of 24.4 fl. Differences in the WBC and platelet counts in different treatment groups were analyzed statistically by ANOVA for each time point.

RH1 toxicity in vivo

CD-1 nude mice from each of the four treatment groups that failed to develop tumors were treated as described above for evaluation of a number of toxicological markers. The mice were anesthetized with Ketalean/Rompum 7 days following the final HBSS or RH1 injection, and approximately 1 ml of blood collected by cardiac puncture. The mice were then killed by CO_2 asphyxiation and the kidneys, heart, lungs, liver and stomach were collected and preserved in neutral buffered formalin. The blood was allowed to clot and the serum was collected following centrifugation at 1500 *g* for 15 min and stored at −80°C until analyzed. The serum was analyzed in a Roche Hitachi 917 (Health Sciences Centre, Department of Clinical Chemistry, Winnipeg, Manit.) for the following parameters: Na, K, Cl, blood urea nitrogen, serum creatinine, alkaline phosphatase, alanine transaminase, aspartate transaminase, gamma-glutamyl transpeptidase and lactate dehydrogenase. The organs were sectioned, stained with hematoxylin and eosin and examined histologically.

Results

Effect of induction of NQO1 on cytotoxic activity of RH1 in vitro

HCT116 human colon carcinoma and T47D human ductal breast carcinoma cells were incubated in vitro at 37°C with or without 5.0 μM DMF or 1.5 μM sulforaphane for 48 h and NQO1 activity in the cells was measured. In the HCT116 cells DMF resulted in an increase in NQO1 activity from 94.0 ± 3.0 in the control cells to 194.0 ± 5.3 nmol min^{-1} mg $protein^{-1}$ ($P < 0.001$; Fig.1). Similarly sulforaphane increased the NQO1 activity from 121.0 ± 14.2 to 268.0 ± 17.7 nmol min^{-1} mg $protein^{-1}$ ($P < 0.001$; Fig. 2). In the T47D cells DMF increased the NQO1 activity from 47.6 ± 12.1 to 69.0 ± 12.3 nmol min^{-1} mg $protein^{-1}$ ($P < 0.002$; Fig. 3) while sulforaphane increased the activity from 26.0 ± 4.1 to 72.4 ± 5.7 nmol min^{-1} mg $protein^{-1}$ ($P < 0.0001$; Fig. 4). The increase in NQO1 activity by either DMF or sulforaphane had no effect on the sensitivity of the HCT116 cells to RH1 (Figs. 1 and 2). The D_{10} for HCT116 cells treated with RH1 alone was 149.0 ± 10.1 n *M* while that for cells treated with DMF and RH1 was 147.0 ± 12.5 n *M*. With sulforaphane the D_{10} in the cells treated with RH1 alone was 164.0 ± 7.1 n *M* while that for the cells treated with sulforaphane and RH1 was 196.0 ± 12.6 n *M*. Similarly there was no significant difference in the sensitivity of the T47D cells to RH1 with NQO1 induction (Figs. 3 and 4). The D_{10} for T47D cells treated with RH1 alone was 199.0 ± 14.9 n *M* while that for cells treated with DMF and RH1 was 218.0 ± 9.2 n *M*. With sulforaphane the D_{10} in the cells treated with RH1 alone was 176.0 ± 14.8 n *M* while that for the cells treated with sulforaphane and RH1 was 189.0 ± 11.6 n *M*. None of the changes in the D_{10} values were statistically significant at the $P = 0.05$ level.

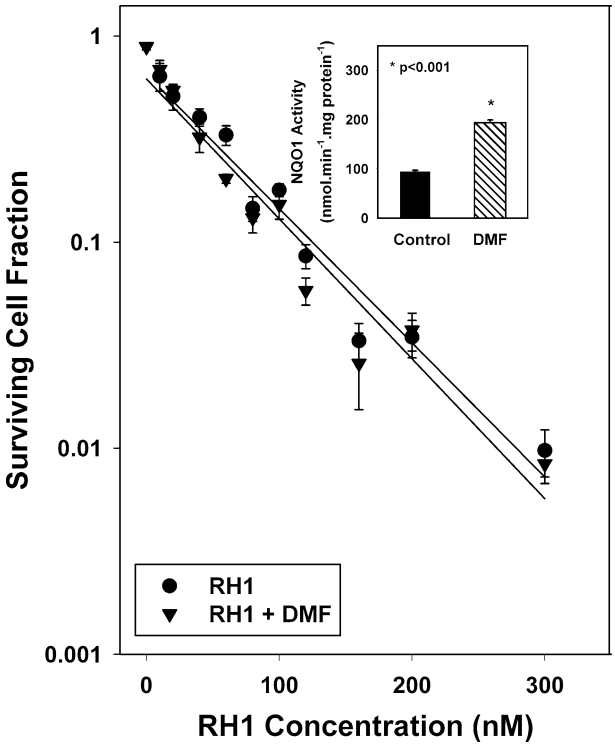

Fig. 1 Effect of DMF on the activity of RH1 in HCT116 human colon cancer cells in vitro. Cells were incubated at 37°C with or without 5 μM DMF for 48 h. Cells were then treated with various concentrations of RH1 for 1 h. The surviving cell fraction was determined using a clonogenic assay [6]. The points represent the mean ± SE surviving cell fractions of 4 to 22 determinations. The lines are linear regression lines. *Inset* level of NQO1 in control cells and cells treated with DMF. NQO1 activity was determined using menadione as the electron acceptor [11]. The *bars* represent the mean ± SE NQO1 activities of 26 to 27 determinations. The means were compared using a *t*-test to determine the significance of the difference between control and DMF-treated cells

Effect of DMF diet on NQO1 activity in vivo

CD-1 nude mice were implanted with 5×10^6 viable HL60, human promyelocytic leukemia cells subcutaneously in both flanks and were fed standard irradiated rodent chow for 10 days. The mice were then randomly split into two groups of three animals per group with one group receiving control experimental diet and the other group receiving experimental diet supplemented with 0.3% DMF. After 7 days on the experimental diets the mice were killed, the kidneys and tumors excised, and the level of NQO1 in the tissues was determined using menadione as the electron acceptor [11]. The DMF diet significantly increased the NQO1 activity in the kidneys from 100 ± 9 ($n = 6$) to 368 ± 36 ($n = 4$) nmol min^{-1} mg $protein^{-1}$ ($P < 0.00005$), and in the tumors from 3.6 ± 0.3 ($n = 6$) to 5.5 ± 0.6 ($n = 4$) nmol min^{-1} mg $protein^{-1}$ ($P < 0.02$).

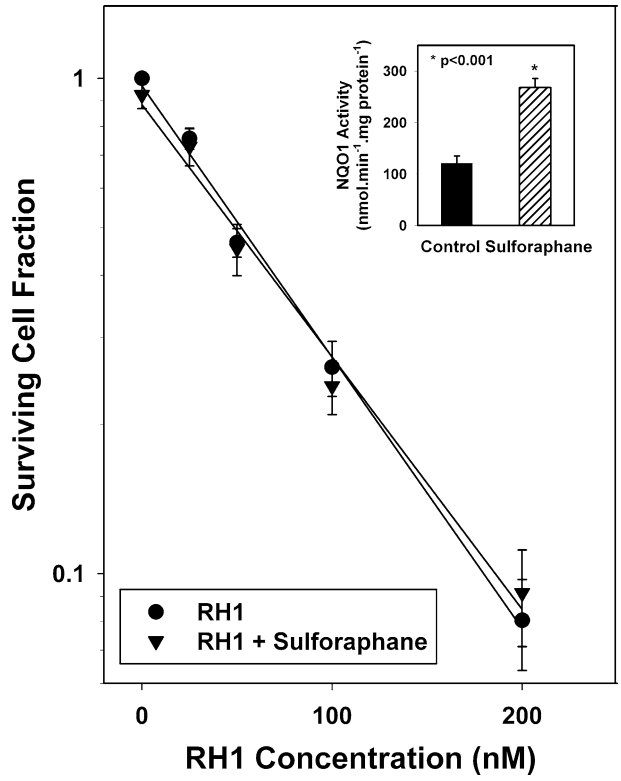

Fig. 2 Effect of sulforaphane on the activity of RH1 in HCT116 human colon cancer cells. Cells were incubated at 37°C with or without 1.5 μM sulforaphane for 48 h. Cells were then treated with various concentrations of RH1 for 1 h. The surviving cell fraction was determined using a clonogenic assay [6]. The points represent the mean ± SE surviving cell fraction of 6 to 12 determinations. The lines are linear regression lines. *Inset* level of NQO1 in control cells and cells treated with sulforaphane. NQO1 activity was determined using menadione as the electron acceptor [11]. The *bars* represent the mean ± SE NQO1 activity of 10 determinations. The means were compared using a *t*-test to determine the significance of the difference between control and sulforaphane-treated cells

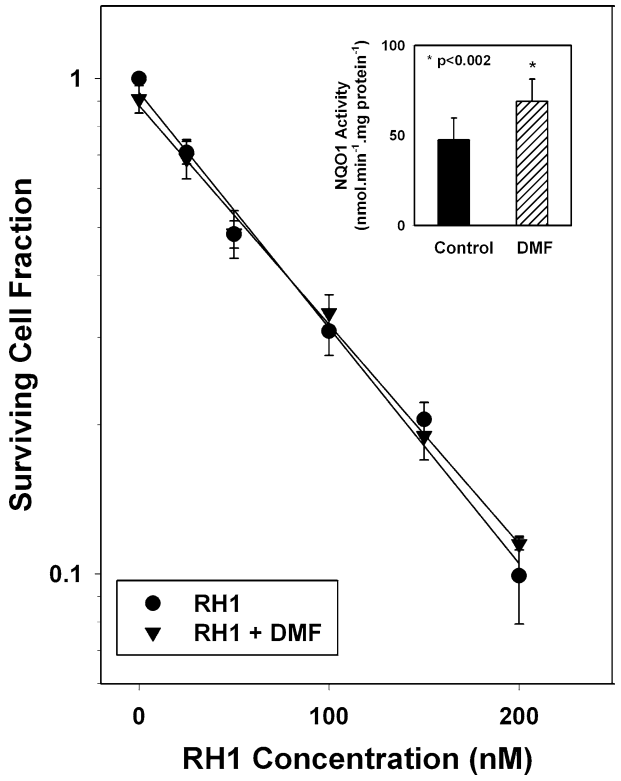

Fig. 3 Effect of DMF on the activity of RH1 in T47D human breast cancer cells. Cells were incubated at 37°C with or without 5 μM DMF for 48 h. Cells were then treated with various concentrations of RH1 for 1 h. Surviving cell fraction was determined using the MTT assay [19]. The points represent the mean ± SE surviving cell fraction of four determinations. The lines are linear regression lines. *Inset* level of NQO1 in control cells and cells treated with DMF. NQO1 activity was determined using menadione as the electron acceptor [11]. The *bars* represent the mean ± SE NQO1 activity of seven determinations. The means were compared using a *t*-test to determine the significance of the difference between control and DMF-treated cells

Effect of induction of NQO1 on RH1 antitumor activity and toxicity in vivo

HL60 human promyelocytic leukemia cells were implanted subcutaneously in both flanks of 48 female CD-1 nude mice and the mice were fed standard irradiated rodent chow for 10 days. The mice were then randomly assigned to four groups of 12 mice each with two of the groups receiving control experimental diet and the other two receiving the experimental diet supplemented with 0.3% DMF (w/w) throughout the treatment period. One control diet and one experimental diet group were randomly selected to receive RH1 treatment while the other two groups received injections of HBSS. The RH1 or HBSS injections were initiated on an individual basis when either of the two tumors reached a volume of approximately 200 mm^3 (8–18 days after diet change). Tumor volumes and mouse weights were measured every day during the treatment period and every second day thereafter. Blood samples were obtained from randomly selected mice (six to ten per group) every third day for WBC and platelet counts.

Tumor volumes on the first day of treatment ranged from 149 to 262 mm^3, the mean tumor volumes continued to increase during and after treatment for each of the four groups (Fig. 5). When tumor volumes were compared using ANOVA to determine if there was a statistically significant difference in the groups for each of the time points, only day 9 showed a significant difference ($P < 0.05$). Further analysis using Tukey's test determined that the DMF-alone group had tumors that were significantly larger than the other three groups. On day 9, in relation to their size on day 0, the tumors were 356 ± 53% in the control group, 512 ± 66% in the DMF-alone group, 320 ± 39% in the RH1-alone group, and 319 ± 35% in the DMF + RH1 combination group.

The presence or absence of DMF in the diet did not have a significant effect on WBC counts in either the RH1-treated or HBSS-treated groups at any of the time points. There was a significant reduction ($P < 0.01$ for days 3, 6 and 9) in WBC counts in the two groups

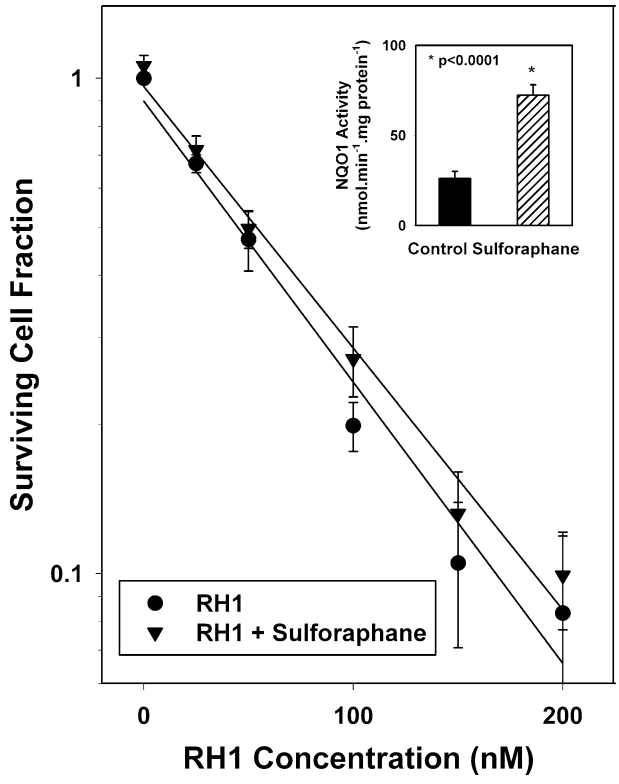

Fig. 4 Effect of sulforaphane on the activity of RH1 in T47D human breast cancer cells. Cells were incubated at 37°C with or without 1.5 μM sulforaphane for 48 h. Cells were then treated with various concentrations of RH1 for 1 h. The surviving cell fraction was determined using the MTT assay [19]. The points represent the mean ± SE surviving cell fraction of four or five determinations. The lines are linear regression lines. *Inset* level of NQO1 in control cells and cells treated with sulforaphane. NQO1 activity was determined using menadione as the electron acceptor [11]. The *bars* represent the mean ± SE NQO1 activity of six determinations. The means were compared using a *t*-test to determine the significance of the difference between control and sulforaphane-treated cells

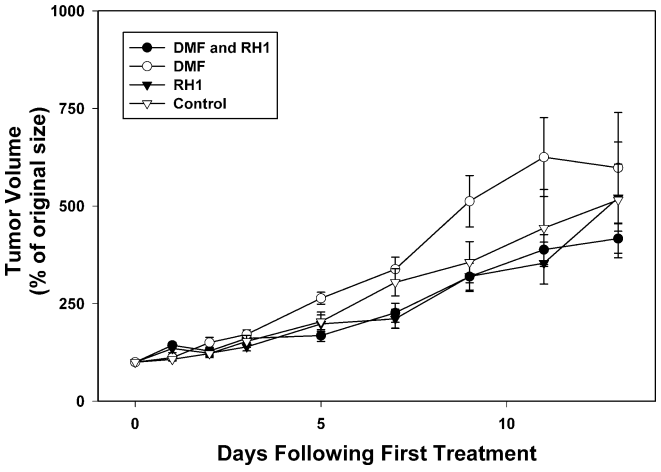

Fig. 5 Effect of DMF diet on RH1 antitumor activity against HL60, human promyelocytic leukemia xenografts in female CD-1 nude mice. Each mouse was implanted with 5×10^6 viable HL60 cells subcutaneously in both flanks. After 10 days the mice were fed a custom experimental diet containing 0 or 0.3% DMF (w/w) for 8–18 days. When either of the tumors reached a volume of approximately 200 mm^3 the mice were weighed and received five daily i.p. injections of HBSS or 0.4 mg/kg RH1. On days 0, 1, 2, 3, 5, 7, 9, 11, and 13 tumor diameters in three dimensions were measured using digital calipers, and tumor volumes calculated. The points represent the mean ± SE tumor volume as a percentage of the day-0 volume for 4 to 12 mice

that received RH1 injections beginning on day 3, with the nadir occurring on day 6 (1 day after the final injection; Fig. 6). The WBC counts in the circulating blood of the RH1- and RH1 + DMF-treated mice were 2.5 and 3.3×10^6 cells/ml or 27% and 36%, respectively, of the control value of 9.3×10^6 cells/ml. The observed leukopenia was reversible with WBC counts returning to control levels by day 12 (7 days following the final RH1 injection). Similarly, platelet concentrations in the blood were determined (Fig. 7). There were no significance differences between any of the four groups for this measurement, for any of the days measured.

Evaluation of mouse body weight showed a reduction in body weight for the mice given RH1 throughout the 5-day treatment period (Fig. 8). On day 5 mice in the RH1 group had lost 3% of their body weight and mice in the RH1 + DMF group had lost 5% of their initial body weight. Immediately following cessation of the RH1 injections the weights began to increase again. Comparison of the four groups for each time point using

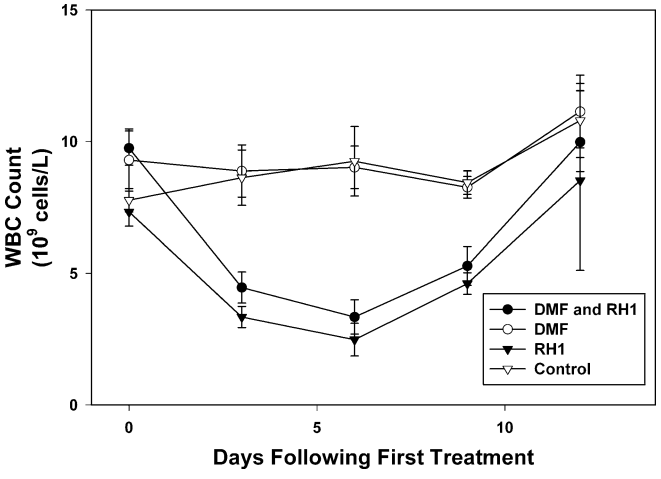

Fig. 6 Effect of DMF diet on RH1 toxicity to WBC in female CD-1 nude mice. Each mouse was implanted with 5×10^6 viable HL60 cells subcutaneously in both flanks. After 10 days the mice were fed an experimental diet containing 0 or 0.3% DMF (w/w) for 8–18 days. When either of the tumors reached a volume of approximately 200 mm^3 the mice were weighed and received five daily i.p. injections of HBSS or 0.4 mg/kg RH1. On days 0, 3, 6, 9, and 12, approximately 25 µl of blood was collected from the saphenous vein of some of the mice in each treatment group [15]. The blood was collected into a microvette CB300 with potassium EDTA and 10-µl aliquots were used for the determination of WBC counts. The counts were determined with a Coulter Z2 particle counter and cell analyzer. The points represent mean ± SE WBC counts for 5 to 11 mice. Differences in the WBC counts for the different treatment groups were analyzed statistically by ANOVA for each time point

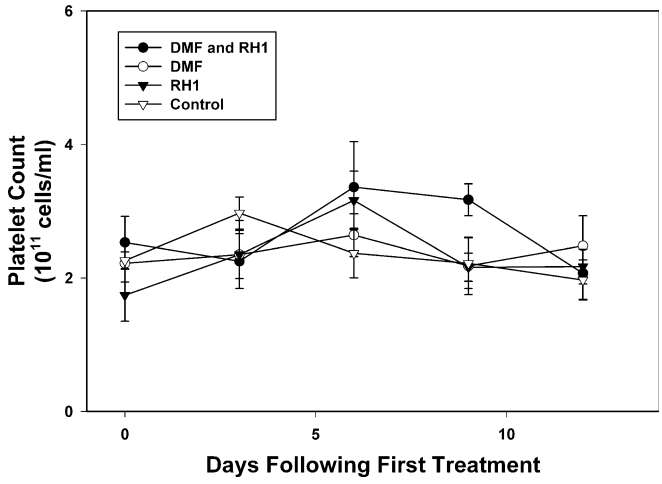

Fig. 7 Effect of DMF diet on RH1 toxicity to platelets in female CD-1 nude mice. Each mouse was implanted with 5×10^6 viable HL60 cells subcutaneously into both flanks. After 10 days the mice were fed a custom experimental diet containing 0 or 0.3% DMF (w/w) for 8–18 days. When either of the tumors reached a volume of approximately 200 mm^3 the mice were weighed and received five daily i.p. injections of HBSS or 0.4 mg/kg RH1. On days 0, 3, 6, 9, and 12, approximately 25 µl of blood was collected from the saphenous vein of some of the mice in each treatment group [15]. The blood was collected into a microvette CB300 with potassium EDTA and 10-µl aliquots were used for the determination of platelet counts. The counts were determined with a Coulter Z2 particle counter and cell analyzer. The points represent the mean ± SE platelet counts for 6 to 10 mice. Differences in the platelet counts for the four treatment groups were analyzed statistically by ANOVA for each time point

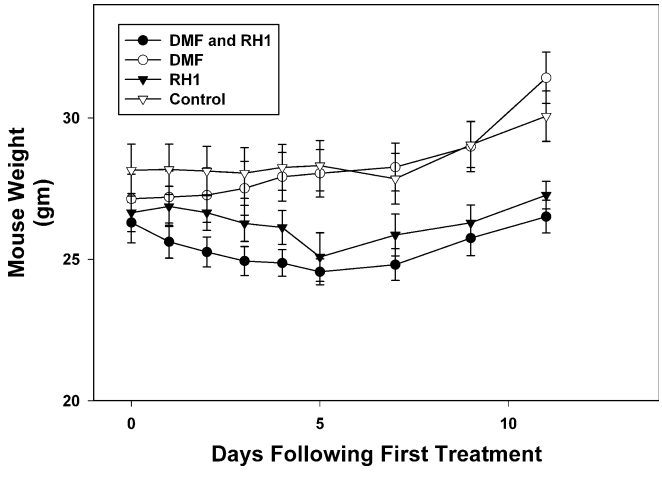

Fig. 8 Effect of DMF diet and RH1 on body weights of female CD-1 nude mice. Each mouse was implanted with 5×10^6 viable HL60 cells subcutaneously into both flanks. After 10 days the mice were fed a custom experimental diet containing 0 or 0.3% DMF (w/w) for 8–18 days. When either of the tumors reached a volume of approximately 200 mm^3 the mice were weighed and received five daily i.p. injections of HBSS or 0.4 mg/kg RH1. On days 0, 1, 2, 3, 4, 5, 7, 9, 11, and 13 mice were weighed. The points represent the mean ± SE body weights of 5 to 12 mice. Differences in the body weights for the four treatment groups were analyzed statistically by ANOVA for each time point

ANOVA showed that from day 3 to day 11 the group receiving the combination therapy was significantly lighter than both the control group and the group receiving DMF in their diet. Although not statistical significant at the $P = 0.05$ level, a trend existed suggesting that RH1 alone may also result in treatment-associated weight loss.

Mice from each of the four groups that failed to develop tumors ($n = 5$–11) were treated in a similar fashion to those with tumors. The mice were anesthetized with Ketalean and Rompun 7 days following the final HBSS or RH1 injection, and blood samples obtained by cardiac puncture. The mice were killed by CO_2 asphyxiation, and the kidneys, heart, lungs, liver and forestomach were collected for histological examination. The clinical chemistry analysis of the serum obtained from each of the four experimental groups showed no significant changes for any of the toxicological markers examined. The levels of gamma-glutamyl transpeptidase and serum creatinine were too low to be measured. Histological examination of each of the tissues collected from the four groups showed no evidence of toxicity (Fig. 9).

Discussion

MMC, the prototype bioreductive agent, was first isolated in 1958 from the bacteria *Streptomyces caespitosus* [46] and has been used in the clinic since the early 1960s [1]. NQO1 is one of the enzymes involved in the activation of MMC and a number of other bioreductive agents [32]. NQO1 has a number of characteristics that make it a very promising target for enzyme-directed therapy. It is often overexpressed in tumor tissue [8, 32, 37], is found at low levels in hematopoietic cells [37, 39], and can be selectively induced in tumor tissues [4]. Studies have demonstrated that the antitumor activity of MMC can be enhanced by increasing the level of expression of NQO1 in human tumor cells in vivo and in vitro [5, 11]. As MMC may be primarily activated by RED [34], it is reasonable to expect that this approach may be more effective with agents that are selectively activated by NQO1 such as EO9 [11] and RH1 [48]. This hypothesis was confirmed when the activities of MMC and EO9 were compared in mouse lymphoma cells that had been pretreated with the NQO1 inducer 1,2-dithioloe-3-thione [3].

RH1 is a new bioreductive agent that is a potent antitumor agent and is an excellent substrate for NQO1 [48]. RH1 is also metabolized by RED [24]; however the efficiency of NQO1 reduction of RH1 is so great that nearly all of the activation of the drug is due to NQO1 activity [48, 50]. RH1 has demonstrated significant antitumor activity against malignant cells expressing high levels of NQO1 in vitro [36, 48] and in vivo [10, 21]. RH1 is currently involved in phase I clinical trials in the United Kingdom [10].

In this study we investigated the effect of induction of NQO1 on the antitumor activity of RH1. We used DMF

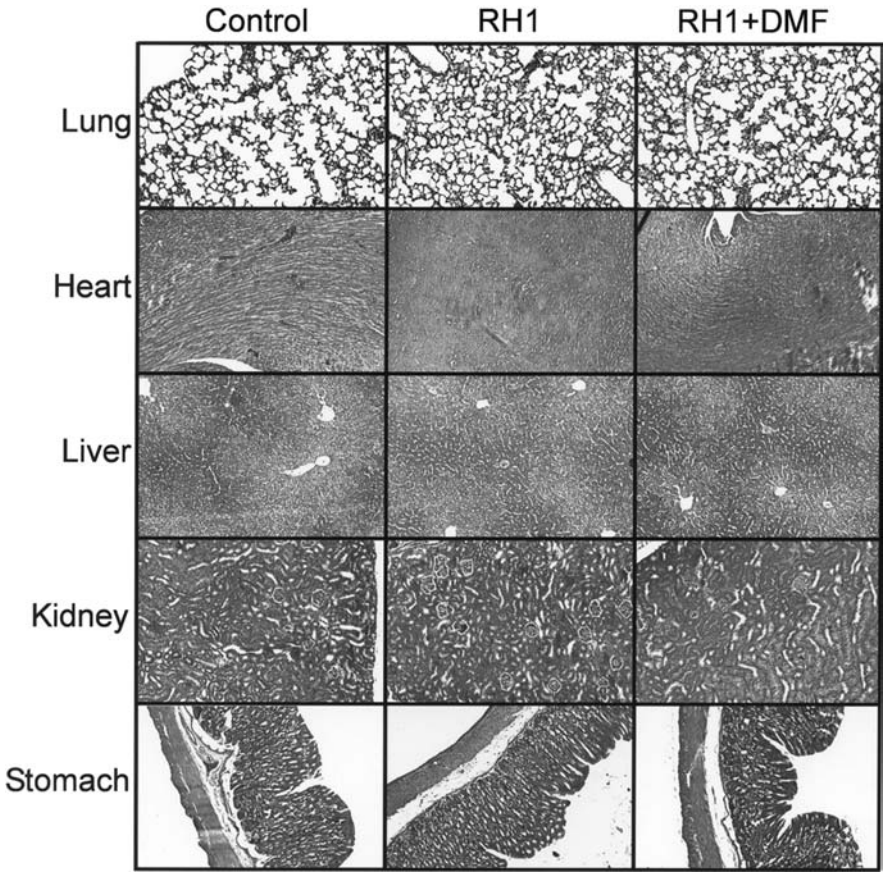

Fig. 9 Effect of DMF diet and RH1 treatment on tissues from female CD-1 nude mice. The mice were fed custom experimental diet containing 0 or 0.3% DMF (w/w) for 10 days. The mice were weighed and received five daily i.p. injections of HBSS or 0.4 mg/kg RH1. The mice were killed by CO_2 asphyxiation 7 days after the final injection, and the tissues were removed and fixed in neutral buffered formalin. The organs were sectioned, stained with hematoxylin and eosin and examined histologically. Shown are light micrographs of various tissues from mice fed control diet and treated with HBSS (*Control*), mice fed control diet and treated with RH1 (*RH1*) and mice fed 0.3% DMF diet and treated with RH1 (*RH1 + DMF*)

and sulforaphane as the NQO1 inducers in vitro on HCT116, human colon carcinoma cells, and T47D, human ductal breast carcinoma cells. We studied the effects of dietary DMF on the in vivo antitumor activity of RH1 against HL60, human promyelocytic leukemia xenografts in female CD-1 nude mice.

Treatment of the HCT116 cells with 5 μM DMF or 1.5 μM sulforaphane resulted in 2.1- and 2.2-fold increases in NQO1 activity, respectively. Similarly, when T47D cells were exposed to the DMF and sulforaphane the increase in NQO1 activity was 1.4- and 2.8-fold, respectively. Although NQO1 activity was increased by the inducers in these cell lines, there was no effect on the antitumor activity of RH1. A likely explanation for the lack of RH1 enhancement is that the base levels of NQO1 found in the cells were sufficient for full activation of the drug. This hypothesis is supported by studies conducted by Winski et al. [50] involving a series of cell lines derived from BE human colon adenocarcinoma cells, which have no NQO1 activity due to the presence of a polymorphism in the NQO1 gene [42, 43]. Winski et al. [50] developed a series of BE cell lines stably transfected with the NQO1 gene that expressed various levels of NQO1. They used these cells to demonstrate that NQO1 activity of 77 nmol DCPIP reduced min^{-1} mg $protein^{-1}$ resulted in maximum activation of RH1, while NQO1 activity of 23 nmol DCPIP reduced min^{-1} mg $protein^{-1}$ resulted in no activation of RH1. Reduction of DCPIP by NQO1 is generally two or three times higher than the reduction of menadione (unpublished observations) suggesting maximum activation of RH1 at NQO1 levels of 25–40 nmol menadione reduced min^{-1} mg $protein^{-1}$ or lower. The base levels of NQO1 activity observed in the HCT116 cells were 94–121 nmol menadione reduced min^{-1} mg $protein^{-1}$ and 26–48 nmol menadione reduced min^{-1} mg $protein^{-1}$ in the T47D, and therefore it is possible that maximum RH1 activation was obtained in both HCT116 and T47D cells without NQO1 induction.

The addition of 0.3% (w/w) DMF to the diet of female CD-1 nude mice implanted with HL60 human promyelocytic leukemia cells resulted in a 1.5-fold increase in NQO1 activity in the HL60 tumors ($P < 0.02$). However, the NQO1 activity in the tumors was low in both the control and DMF-fed mice. Treatment of these mice with RH1 produced no antitumor activity either when given alone or when combined with the DMF diet. The lack of RH1 antitumor activity was a surprising result as other studies have demonstrated significant antitumor activity against non-small-cell lung carcinoma and colon carcinoma tumors in vivo [10]. The most likely explanation for this lack of activity is the low levels of NQO1 in the HL60 cells in vivo. The observed levels of 3.6 ± 0.3 and 5.5 ± 0.6 nmol menadione reduced min^{-1} mg $protein^{-1}$ for the control diet and 0.3% (w/w) DMF diet, respectively, are below the lower

threshold of 23 nmol DCPIP reduced min^{-1} mg $protein^{-1}$ determined by Winski et al. [50].

This is consistent with the narrow window of NQO1 activity between no RH1 activation and maximum RH1 activation described by Winski et al. [50]. This study suggests that the selective induction of NQO1 in tumor cells is not likely to be an effective strategy for enhancing antitumor activity of RH1. However, this does not rule out the use of this strategy as a way of enhancing the antitumor activity of a bioreductive agent selectively activated by NQO1 that have a more favorable window of NQO1 activation.

Our findings also raise some serious concerns regarding the use of RH1 in the clinic. The low levels of NQO1 required for maximum activation is likely to be clinically significant. This would indicate a failure of RH1 to specifically target tumors with high NQO1 activity as maximum activation would be obtained in many healthy tissues of the body. In this study RH1 treatment resulted in a significant decrease in WBC counts that was reversible with full recovery seen 7 days after the final dose. The addition of DMF to the diet did not increase this toxicity. RH1 treatment alone or in combination with DMF did not produce a decrease in platelet counts. In addition, RH1 with or without DMF did not produce any significant changes in blood chemistry or obvious histological damage to kidney, heart, lung, liver and forestomach tissues. The combination of RH1 with DMF-supplemented diet resulted in a significant reduction in body weight ($P < 0.05$).

The observation of leukopenia and possible RH1 treatment-associated weight loss are similar to the toxic effects observed previously with MMC [5] and suggest that similar toxicities may be observed with RH1 in the clinic.

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