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Original Contribution

Protection of human cultured cells against oxidative stress by *Rhodiola rosea* without activation of antioxidant defenses

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ABSTRACT

Rhodiola rosea root has been long used in traditional medical systems in Europe and Asia as an adaptogen to increase an organism's resistance to physical stress. Recent research has demonstrated its ability to improve mental and physical stamina, to improve mood, and to help alleviate high-altitude sickness. We have also recently found that *R. rosea* is able to extend the life span of *Drosophila melanogaster*. The mode of action of *R. rosea* is currently unknown; it has been suggested by some to act as an antioxidant, whereas others have argued that it may actually be a pro-oxidant and act through a hormetic mechanism. We found that *R. rosea* supplementation could protect cultured cells against ultraviolet light, paraquat, and H_2O_2. However, it did not alter the levels of the major antioxidant defenses nor did it markedly activate the antioxidant response element or modulate heme-oxygenase-1 expression levels at relevant concentrations. In addition, *R. rosea* extract was not able to significantly degrade H_2O_2 in vitro. These results suggest that in human cultured cells *R. rosea* does not act as an antioxidant and that its mode of action cannot be sufficiently explained through a pro-oxidant hormetic mechanism.

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Rhodiola rosea, also known as golden root, is a member of the Crassulacea family of plants and grows in mountainous regions throughout the world. Its root is used in traditional medicine in Eastern Europe and Asia and has numerous physical and mental health benefits attributed to its consumption. *R. rosea* has been most intensively studied in Russia and Scandinavia, where it has been purported to improve stamina, memory, and mood and protect against high altitude sickness, as well as acting as a cardioprotective agent [1]. It has also been found to attenuate tumor progression in a rat model [2], reduce blood glucose levels in diabetic mice [3], and improve endurance and muscle ATP levels in rats [4]. *R. rosea* is considered to contain components belonging to a group of compounds known as adaptogens; compounds that afford a generalized resistance to a variety of stresses. The adaptogenic properties of *R. rosea* were demonstrated by protection of snail larvae against three different types of insults: heat shock, oxidative stress, and heavy metals [5].

Despite the documentation of a variety of health benefits, there is a paucity of knowledge regarding its molecular mode of action, although it has been suggested to act as an antioxidant. Significant antioxidant activities have been documented for the extracts of various *Rhodiola* species, which have been attributed to a variety of antioxidant compounds including gallic acid, tyrosol, and flavonoids. One study reported that 19 compounds isolated from a *Rhodiola* species, *R. sacra,* had scavenging activities against superoxide anion and hydroxyl radicals [6]. And as mentioned above, *R. rosea* itself can protect snail larvae against a superoxide-generating agent. Twenty-eight compounds have been identified in *R. rosea,* with its activity attributed to *p*-tyrosol, salidroside, and 5 salidroside-like glycosides (rhodiolin, rosiridin, rosarin, rosavin, and rosin). Three of these glycosides, rosarin, rosavin, and rosin, seem to be specific to *R. rosea* [1].

It has also been proposed that *R. rosea* may actually be a pro-oxidant and mediate its benefits through a hormetic mechanism [7]. This is when a toxic, but sublethal, agent or dose induces a defense response that confers a protective effect against further challenge [8]. In a study in which *R. rosea* was found to extend the life span of the worm *Caenorhabditis elegans,* it catalyzed the nuclear localization of the transcription factor DAF-16 and elevated expression of a heat shock promoter, prompting the authors to conclude that *R. rosea* is mildly toxic and its beneficial effects are secondary [9]. In a related cell culture study, *R. rosea* was found to increase expression of heme-oxygenase-1 (HO-1), a protein that can be activated by the antioxidant-response element (ARE) in response to oxidative challenge [7]. The ARE is a *cis*-acting motif located in the promoter of a number of genes that confer enhanced protection against oxidative and/or chemical challenge when expressed [10] and is an important component for the protection of cells against oxidative insult [11].

Whether *R. rosea* acts directly as an antioxidant or initially as a pro-oxidant, its protection against oxidative stress is a possible mechanism explaining its beneficial effects. The main aim of this study was to determine if *R. rosea* is able to protect human cells against oxidative stress and to determine if it does so through an antioxidant or a pro-

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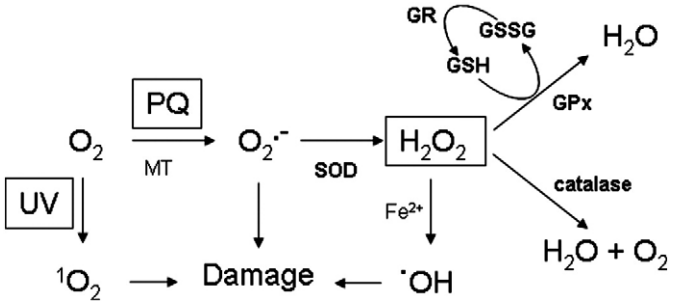

Fig. 1. Oxidants, antioxidants, and their relationships relevant to this work. The agents used to induce oxidative stress are boxed. The antioxidants examined were SOD, catalase, GPx, and GR. Abbreviations used: MT, mitochondria; PQ, paraquat; UV, ultraviolet light, 254 nm; SOD, superoxide dismutase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione.

oxidant mechanism. The antioxidants and oxidants relevant to this work are diagrammed in Fig. 1. Although we initially used immortalized human cells to understand the mechanism, we found that *R. rosea* can exert its beneficial effects on nontransformed human cells as well.

Materials and methods

Reagents

All reagents except those listed below were obtained from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), penicillin/streptomycin, trypsin, and cumene hydroperoxide were obtained from Thermo Fisher Scientific (Waltham, MA, USA) for the 143B cell culture study. Versene and DMEM were obtained from Invitrogen, and FBS from HyClone, for the IMR-32 cell culture study.

Cells and cell culture

Human osteosarcoma-derived 143B, human diploid fibroblast IMR-90, and human neuroblastoma IMR-32 cells were maintained in humidified incubators at 37°C and 5% CO_2 and fed DMEM supplemented with 10% FBS and 100 units of penicillin and 100 μg of streptomycin per milliliter. IMR-90 and 143B cells were subcultured by treatment with trypsin every week at a dilution of 1/4 or every 2–3 days at a dilution of 1/10, respectively. IMR-32 cells were subcultured by detachment with versene (dilution 1:3 or 1:4).

IMR-90 cells were obtained from the American Type Culture Collection at a population doubling level (PDL) of 25. All experiments in this work were completed with cells derived from two to four additional passages, each at a dilution of 1/4, corresponding to a PDL of approximately 29–33. As these cells reach replicative senescence at a PDL of 58–71 [12], the studies described here were conducted with cells in the midrange of their replicative life span.

Measurement of toxicity

Cells were counted using a hemocytometer and plated at a density of approximately 1000 cells per well in a 96-well microtiter plate in medium supplemented with 0, 0.1, 1, 10, or 100 μg/ml *R. rosea* extract or ascorbate at a final volume of 200 μl. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to measure cell viability [13]. After 24 h, the medium was removed and the cells were supplied medium containing 0.45 mg/ml MTT. The cells were incubated at 37°C for an additional 3 h, after which the medium was removed, the cells were washed once with 1× PBS, and a solution of 50% dimethylformamide, 10% sodium dodecyl sulfate (DMF/SDS) was added to disrupt the cells and to solubilize the MTT precipitate. The concentration of MTT was measured at 570 nm using a SpectraMax plate reader (Molecular Devices, Sunnyvale, CA, USA). Percentage survival was calculated as the MTT absorbance of a particular condition (compound or concentration) divided by the absorbance of the appropriate control (cells supplied with medium only) multiplied by 100. All values obtained from *R. rosea*-supplemented cells were normalized by subtracting the absorbance at 570 nm of control cells in DMF/SDS without the addition of MTT.

Growth curves

Cells were exposed to *R. rosea* at the indicated concentrations for 48 h. Approximately 1000 cells were plated per well in a 96-well microtiter plate. MTT was added each day up to day 6. Data were reported as the absorbance at 570 nm ($n = 4$ wells per data point).

Oxidative challenges

Cells were plated at a 1/10 (143B) or 1/4 (IMR-90) dilution of an approximately confluent monolayer into a 25-cm^2 tissue culture flask containing 5 ml of medium, FBS, and antibiotics supplemented with or without (vehicle control) the desired concentration of *R. rosea* extract. Cells were fed fresh medium (*R. rosea* supplemented and control) after 24 h. Twenty-four h later, 48 h after exposure to *R. rosea*, approximately 1000 cells were plated per well in a 96-well microtiter plate in 100 μl DMEM with serum and antibiotics. For UV challenge, cells were exposed to UV light generated from a 254-nm 30-W germicidal lamp in the cell culture hood with the plastic top of the microtiter plate removed for 45, 60, or 75 s. Aluminum foil was used to block UV light from the control cells and regulate the time of UV exposure. To challenge the cells to paraquat (PQ) and H_2O_2, 100 μl of the appropriate concentrations of PQ or H_2O_2 was added to the original 100-μl volume of cells plated. The cells were exposed to all three oxidative challenges, UV light, PQ, and H_2O_2, within 30 min of being plated. Forty-eight h after initial exposure, cell survival was measured via the MTT assay as described above and was reported as percentage survival relative to the untreated control of each dose of *R. rosea*. For the reversal assay, cells were plated in medium without *R. rosea* for 48 h and fed once after 24 h, before being subjected to oxidative challenge.

Measurement of antioxidant defenses

Superoxide dismutase (SOD) activity was determined by an indirect method using xanthine/xanthine oxidase as a superoxide generator and nitroblue tetrazolium (NBT) as the target [14]. One unit of SOD is defined as the amount of protein required to inhibit NBT reduction (at 560 nm) by 50%. Catalase activity was measured by the direct decomposition of H_2O_2 at 240 nm [15]. Glutathione (GSH) peroxidase activity was measured by an enzyme-linked system in which cumene hydroperoxide oxidizes GSH, which is then reduced by glutathione reductase with a concomitant oxidation of NADPH at 340 nm [16]. Glutathione reductase (GR) activity was determined by the oxidation of NADPH in the presence of oxidized glutathione (GSSG) with concomitant reduction of DTNB (5,5′-dithiobis(2-nitrobenzoic acid)) detected at 412 nm [17]. Total glutathione levels were determined at 412 nm by a recycling reaction between GSH and DTNB in which the product GSSG is reduced by GR and NADPH [18]. To determine GSSG levels, GSH initially present was removed by preincubation with 2-vinylpyridine [19,20].

H_2O_2 degradation

Direct decomposition of H_2O_2 was measured at 240 nm for 5 min at room temperature with a molar extinction coefficient of 43.6 M^{-1} cm^{-1}. The reaction contained 45 mM H_2O_2 in 50 mM sodium phosphate buffer with the desired concentration of *R. rosea* extract or catalase.

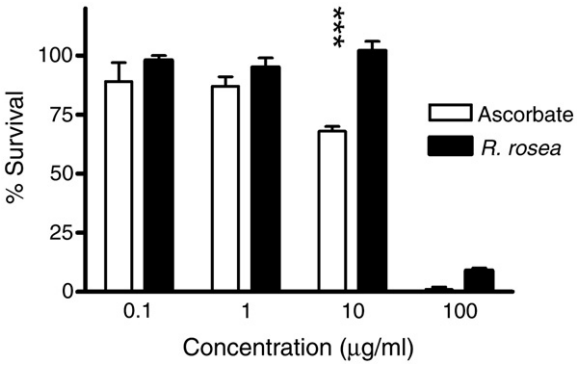

Fig. 2. Relative toxicity of *R. rosea* extract and ascorbate in human osteosarcoma-derived 143B cells. Approximately 1000 cells were exposed to the indicated doses of *R. rosea* or ascorbate for 24 h. Percentage survival was calculated from the MTT absorbance at each treatment divided by the untreated control. Each bar represents the mean ± SEM, $n = 8$. ***$P<0.001$, two-way ANOVA, Bonferroni's posttest for 10 μg/ml *R. rosea* vs 10 μg/ml ascorbate.

Reporter gene assay

IMR-32 human neuroblastoma cells were plated at a density of 3×10^6 per 10-cm dish. Twenty-four h later, transfections were performed using FuGENE HD (Roche). Briefly, for each well of the 96-well plates, 50 μl transfection mix consisting of OptiMEM, FuGENE HD, 50 ng ARE–luciferase reporter construct, 50 ng actin–lacZ (for normalization), and 10 ng CMV–GFP (to monitor transfection efficiency) was added. After incubation for 30 min at room temperature, 50 μl of culture medium containing 30,000 IMR-32 cells was added to each well. Twenty-four h after transfection, cells were treated with various concentrations of *R. rosea* (from 100 ng/ml to 100 μg/ml) or solvent control. Luciferase and β-galactosidase activities were measured after 24 h treatment using Britelite (Perkin–Elmer) and the Gal-Screen System (Applied Biosystems), respectively, according to the suppliers' instructions. Normalized ARE activities were expressed as ratios of both activities. Transfection efficiency was estimated to be approximately 60–70%.

Immunoblot analysis

IMR-32 cells (5×10^5 cells/well) were seeded in six-well plates and 24 h later treated with various concentrations of *R. rosea* (from 100 ng/ml to 100 μg/ml) or solvent control. After incubation for 24 h, whole-cell protein lysates were prepared using PhosphoSafe lysis buffer (Novagen) and protein concentration was measured using the BCA protein assay kit (Pierce). Cell lysates were separated by SDS–PAGE, transferred to polyvinylidene difluoride membranes, probed with antibodies, and detected with the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). Anti-NAD(P)H:quinone oxidoreductase (NQO1) antibody was obtained from Abcam, anti-HO-1 antibody was purchased from Stressgen, anti-β-actin and anti-rabbit antibodies were from Cell Signaling, and anti-goat antibody was from Santa Cruz Biotechnology.

Statistical analyses

Results were analyzed for statistical significance using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Data for toxicity and survival after oxidative challenge (except 10 μM H_2O_2) were analyzed by two-way ANOVA, with the two factors being the agent (ascorbate or *R. rosea*) and the concentration, for the toxicity assay, or the concentration of the oxidative agent (UV, PQ, or H_2O_2) and the concentration of *R. rosea* for the survival assays. Growth curves were assayed by repeated-measures ANOVA. The unpaired *t* test was used to analyze the antioxidant defenses data, except for catalase, for which the variances were unequal (*F* test, $P<0.05$). In that case the data were analyzed using the nonparametric Mann–Whitney test. Activation of the ARE, degradation of H_2O_2, and 10 μM H_2O_2 challenge were analyzed by one-way ANOVA. Bonferroni's posttest was used when appropriate to determine significance between groups. For all cases, a *P* value of less than 0.05 was considered to be statistically significant.

Results

Relative toxicity of R. rosea versus ascorbate

Our initial aim was to estimate the relative toxicity of the *R. rosea* extract compared to a commonly used antioxidant, ascorbate (vitamin C), and also to identify an appropriate dose that offers beneficial effects with no toxicity. Our findings in 143B cells (Fig. 2) demonstrated that the *R. rosea* extract is comparable in toxicity to ascorbate, or may be less toxic ($P<0.001$) at 10 μg/ml, in our experimental system. It should also be noted that the putative active compounds (rosin, rosavin, and rosarin) constitute less than 5% of the *R. rosea* extract and may have a toxicity level much lower than what was suggested by these results. Thus, we can conclude that, at least in our cell culture model, *R. rosea* extract is no more toxic and possibly less toxic than the commonly used antioxidant ascorbate, though some of its individual constituents may be relatively more toxic than ascorbate.

Growth rates of human cells after treatment with R. rosea

We permitted cells to grow for 48 h after oxidative challenge to allow for toxic effects to be adequately exerted. However, an observed increase in survival could be due to enhanced growth rates in the

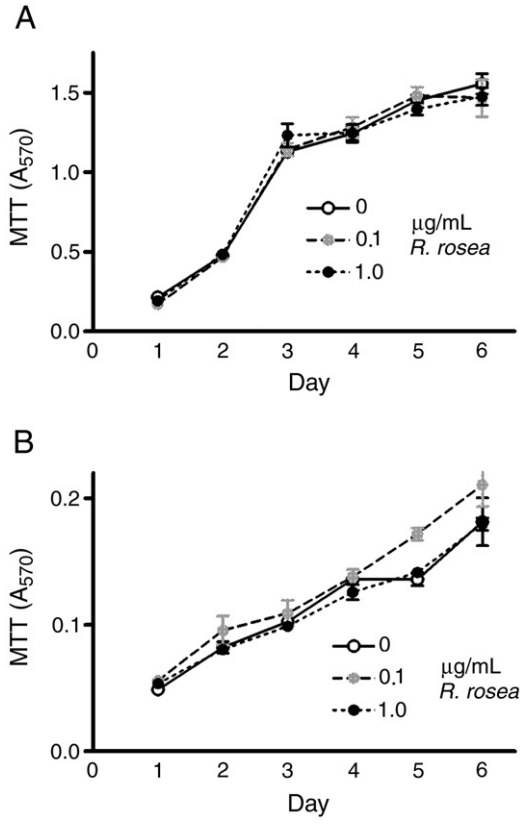

Fig. 3. Growth rates of (A) 143B and (B) IMR-90 cells after 48 h exposure to *R. rosea.* Cells were supplemented with the indicated dosages of *R. rosea* before approximately 1000 cells per well were plated in 96-well plates. MTT was added to each column (four wells) daily in a consecutive manner. $P=0.90$ for 143B cells and $P<0.01$ for IMR-90 cells, repeated-measures ANOVA. For IMR-90 cells, only 0.1 μg/ml affected growth rates ($P<0.05$ vs 0 μg/ml and $P<0.01$ vs 1 μg/ml, Tukey's multiple comparison test).

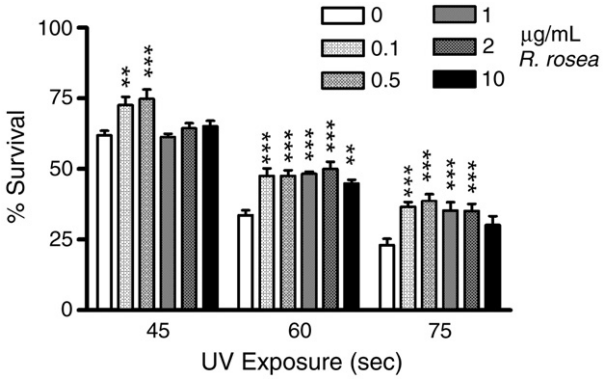

Fig. 4. Protection against UV exposure by *R. rosea* in human osteosarcoma-derived 143B cells. Approximately 1000 cells were exposed to the indicated doses of ultraviolet light. Percentage survival was calculated by the MTT absorbance at each treatment divided by the untreated control after 48 h. Each bar represents the mean ± SEM, $n = 8$. $^{**}P<0.01$, $^{***}P<0.001$, two-way ANOVA, Bonferroni's posttest vs 0 µg/ml.

surviving cells resulting from the prior treatment with *R. rosea.* We found that prior treatment of *R. rosea* had no effect on the growth rates of 143B cells up to 6 days (Fig. 3A). Growth rates did seem to be mildly affected in IMR-90 cells at the dose of 0.1 µg/ml, particularly after 4 days of growth (Fig. 3B). However, no effect was detected at the higher dose of 1 µg/ml (Fig. 3B).

R. rosea protects immortalized and nontransformed human cells against oxidative stress

The defining characteristic of an antioxidant is that it should protect cells, or an organism, against oxidative stress. To determine the antioxidant capability of *R. rosea,* we fed cells various concentrations of *R. rosea* and subjected them to oxidative challenge via ultraviolet light, paraquat, and H_2O_2 (Fig. 1). In all three cases, we found that *R. rosea* feeding was able to afford a protective effect at some dose (Figs. 4, 5, and 6) in immortalized cells. However, we also observed that the beneficial effects of *R. rosea* began to diminish as the dose was increased (Figs. 4 and 5) and it even became sensitizing in the case of challenge with H_2O_2 (Fig. 6). These results are consistent with the ability of *R. rosea* to function as an antioxidant in a dose-dependent manner. Depending on the oxidative challenge, the protective dose range may be very narrow, as in the case of paraquat (Fig. 5), or broader, as with UV and H_2O_2 (Figs. 4 and 6), which have a protective dose range over 3 orders of magnitude. We also found that *R. rosea* was able to exert a protective effect on the nontransformed

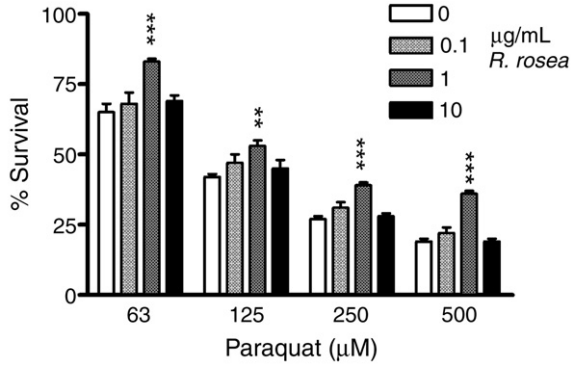

Fig. 5. Protection against paraquat exposure by *R. rosea* in human osteosarcoma-derived 143B cells. Approximately 1000 cells were exposed to the described doses of paraquat. Percentage survival was calculated by the MTT absorbance at each treatment divided by the untreated control 48 h later. Each bar represents the mean ± SEM, $n = 8$. $^{**}P<0.01$, $^{***}P<0.001$, two-way ANOVA, Bonferroni's posttest vs 0 µg/ml.

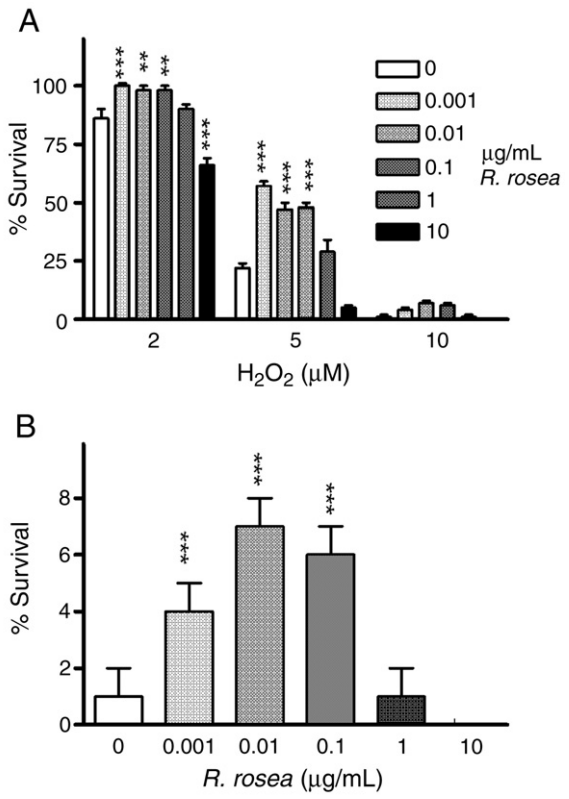

Fig. 6. Protection against and sensitization to H_2O_2 by *R. rosea* in human osteosarcoma-derived 143B cells. Approximately 1000 cells were exposed to the indicated doses of H_2O_2. Percentage survival was calculated by the MTT absorbance at each treatment divided by the untreated control 48 h later. Each bar represents the mean ± SEM, $n = 8$. (A) Challenge of 2, 5, and 10 µM H_2O_2. $^{**}P<0.01$, $^{***}P<0.001$, two-way ANOVA, Bonferroni's posttest vs 0 µg/ml. (B) Expanded view of 10 µM H_2O_2 challenge. This was analyzed separately as it is likely that the marked differences in absolute values contributed to the lack of statistical significance in the overall two-way ANOVA. $^{***}P<0.001$, one-way ANOVA, Bonferroni's posttest vs 0 µg/ml *R. rosea.*

human fibroblast cell line IMR-90, which was derived from healthy lung tissue and has a normal diploid karyotype [12], against all three agents tested (Fig. 7).

Reversibility of R. rosea treatment

R. rosea could potentially act by imparting heritable changes in gene expression, e.g., changes in methylation status. In this case, cells subsequently cultured in the absence of *R. rosea* would retain its protective effects. However, if *R. rosea* did not impart heritable changes, its beneficial effects would be lost upon its removal from the medium. To distinguish between these two possibilities, we supplemented 143B cells with *R. rosea* for 48 h. They were then passaged at a 1/10 dilution and cultured for an additional 48 h before oxidative challenge. In this experiment, no protective benefits were seen against paraquat or H_2O_2 (Fig. 8). However, some protective benefit was still present against UV challenge at 1 µg/ml (Fig. 8).

R. rosea does not alter the major cellular antioxidant defenses

One of the predictions we might make about an antioxidant compound is that it should not result in an increase in antioxidant defenses. Such an increase would suggest that the compound is working through a hormetic mechanism, in that it is actually stressful and its benefits are secondary to its initial insult. To evaluate this possibility, we assayed the levels of the major antioxidant defense systems in response to 48 h feeding of 1 µg/ml *R. rosea.* These included the superoxide dismutases, catalase, and glutathione peroxidase and

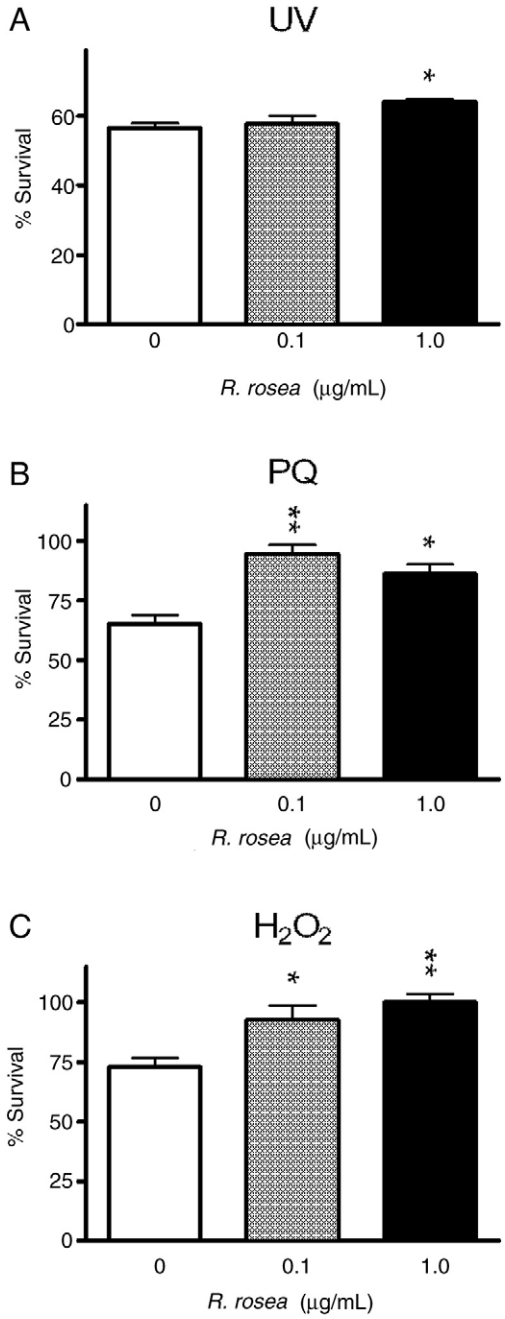

Fig. 7. Protection against UV light, paraquat, and H_2O_2 afforded to human fibroblast cell line IMR-90 by *R. rosea*. Approximately 1000 cells were exposed to the indicated doses of *R. rosea*. Percentage survival was calculated by the MTT absorbance at each treatment divided by the untreated control 48 h later. Each bar represents the mean ± SEM, $n = 8$. *$P<0.05$, **$P<0.01$, Dunn's multiple comparison test vs 0 µg/ml.

reductase and the levels of GSH and the GSH/GSSG ratio (Fig. 1). In all cases, no significant difference was found between the *R. rosea*-fed and unfed cells (Table 1).

Activation of the antioxidant response element is not required for the protective action of R. rosea

To test whether *R. rosea* could protect cells through the activation of the ARE, we carried out a reporter gene assay using a human NQO1–ARE reporter construct in which the luciferase gene is under control of the ARE-containing promoter which is recognized by transcription factor Nrf2 [21]. We chose IMR-32 human neuroblastoma cells for this assay, as they are a validated model for oxidative stress that is highly responsive to ARE activation [21–23]. These cells were transfected with the reporter and treated with various doses of *R. rosea* extract and with *tert*-butylhydroquinone (tBHQ; 10 µM), a model activator of the ARE, as the positive control. *R. rosea* did exhibit a dose-dependent activation of the ARE (up to 3-fold at 100 µg/ml), but this was substantially lower in magnitude than the 60-fold activation caused by 10 µM tBHQ under these conditions (data not shown). However, no detectable activation of the ARE was seen at doses below 1 µg/ml (Fig. 9A), which do confer protection against H_2O_2 and UV. To further characterize ARE activation, we carried out immunoblot analyses for NQO1 and HO-1, two gene products regulated by the ARE, with lysates derived from IMR-32 cells treated with *R. rosea* or tBHQ for 24 h.

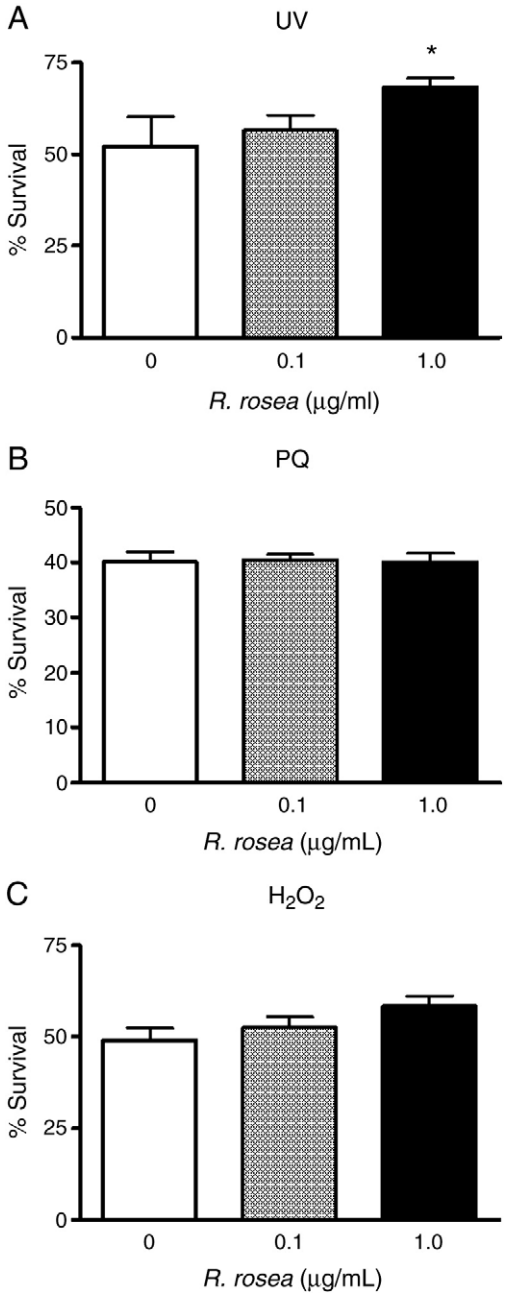

Fig. 8. Protection against UV light, paraquat, and H_2O_2 afforded to human osteosarcoma-derived 143B cells 48 h post-*R. rosea* supplementation. Approximately 1000 cells were exposed to the indicated doses of *R. rosea*. Percentage survival was calculated by the MTT absorbance at each treatment divided by the untreated control 48 h later. Each bar represents the mean ± SEM, $n = 8$. *$P<0.05$, Dunn's multiple comparison test vs 0 µg/ml.

Table 1
Antioxidant defenses in *R. rosea* fed (1 μg/mL) and control 143B cells

Antioxidant	Control	*R. rosea*	Units	*P*-value*
SOD	87.8 ± 18.7	80.3 ± 12.3	U/mg	0.74
Catalase	3.3 ± 0.5	3.4 ± 0.2	U/mg	0.39**
GPX	1.7 ± 0.2	1.6 ± 0.1	U/mg	0.78
GR	2.3 ± 0.1	2.3 ± 0.1	U/mg	0.70
GSH	1.7 ± 0.1	1.4 ± 0.3	nmol/mg	0.28
GSH:GSSG	77 ± 18	63 ± 9	None	0.50

*Unpaired *t* test,
**Mann Whitney test; unpaired *t* test, $P = 0.53$. *F* test, $P = 0.03$ $n = 5$ for SOD control, $n = 6$ for all other data.
Results are shown as means ± SEM.

Again, in contrast to tBHQ, *R. rosea* had no measurable effect on NQO1 or HO-1 levels (Fig. 9B).

R. rosea does not directly degrade H_2O_2

Our finding that *R. rosea* extract could protect cells against H_2O_2 insult (Fig. 5) could have resulted from the direct decomposition of H_2O_2 or from an increase in the activities of H_2O_2-degrading enzymes. *R. rosea* exhibited no appreciable degradation of H_2O_2 (Fig. 10) when the two were incubated together in phosphate buffer ($P = 0.25$, one-way ANOVA, catalase was excluded).

Discussion

R. rosea belongs to a growing list of herbal remedies that are attracting attention in the modern medical research community [24,25]. It is a member of a class of extracts containing compounds known as adaptogens, which are natural herbal extracts that are capable of protecting an individual from stress, anxiety, and fatigue [1,26–28]. But, despite its demonstrative benefits in both human trials and animal studies, its molecular mode of action is not known. Twenty-eight potentially active compounds have been identified in *R. rosea* extract [1], including several polyphenols, which have been proposed to function as antioxidants. Furthermore, *R. rosea* extract was also able to protect snail larvae against menadione-induced superoxide [5]. These observations support the plausibility that *R. rosea* acts through an antioxidant mechanism. However, it has been recently suggested that *R. rosea* may actually be a pro-oxidant and function by inducing defense systems that confer its protective benefits [7,9]. The aim of this study was to determine whether *R. rosea* functions either as an antioxidant or as a pro-oxidant using human cultured cells as a model system.

As *R. rosea* has been previously shown to confer improved resistance against oxidative insults in other systems, we wished to verify that it could also function as an antioxidant in human cells. Osteosarcoma-derived cells (143B) and nontransformed human fibroblasts (IMR-90) were supplemented with *R. rosea* extract and subjected to three types of oxidative insult: ultraviolet light, which generates singlet oxygen ($^1O_2$); paraquat, a potent superoxide generator ($O_2^{\cdot-}$); and hydrogen peroxide. *R. rosea* extract afforded enhanced protection in both of these cell lines against all three insults, though its benefits were restricted to certain dose ranges (Figs. 4, 5, 6, and 7). Although these findings are consistent with its action as an antioxidant, it is possible that *R. rosea* could mediate its effects through the up-regulation of antioxidant defense systems. We found that this was not the case with respect to the major antioxidant defense systems, as these were unchanged by *R. rosea* supplementation in 143B cells (Table 1). This is in contrast to the effect of green tea extract in *Drosophila,* which does seem to up-regulate SOD and catalase, at both the RNA and the enzyme activity levels [29]. These results demonstrate that up-regulation of the major antioxidant defenses is not required for the protective action of *R. rosea.* We also found that *R. rosea* extract had no appreciable ability to directly degrade H_2O_2 (Fig. 10), suggesting that it itself is probably not acting as an antioxidant with respect to H_2O_2, and its benefits must come from some other mechanism.

R. rosea is also not likely to be imparting a heritable change in cells. When cells were supplemented with *R. rosea* for 48 h, and then cultured for an additional 48 h without *R. rosea,* no protective benefit was seen against paraquat or H_2O_2 (Fig. 8). Protection was seen against UV when cells were previously supplemented with 1 μg/ml *R. rosea*, but not at 0.1 μg/ml. We interpret this observation as a dilution effect and not a heritable change, meaning that some constituents of the *R. rosea* extract may still be present within cells, but at a lower level. This is because 0.1 μg/ml *R. rosea,* which was previously protective (Fig. 4), was no longer beneficial 48 h after treatment.

Much of our work was undertaken in cell lines that were originally derived from cancerous growths. Our original rationale for this was to

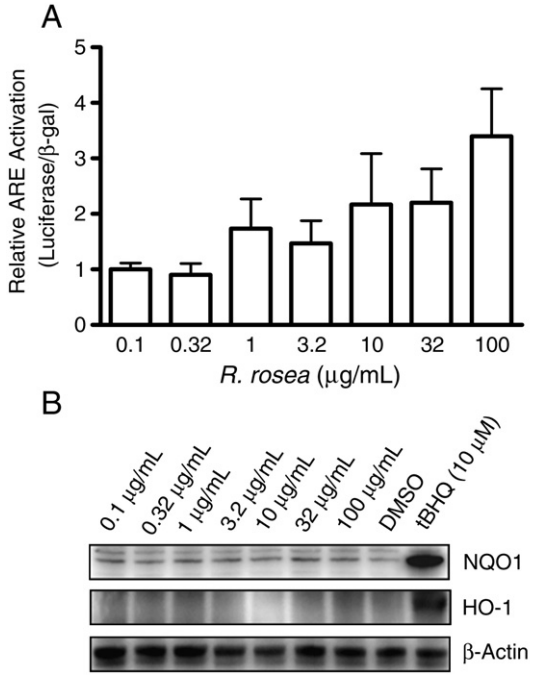

Fig. 9. (A) ARE reporter gene assay in IMR-32 human neuroblastoma cells. There was a dose-dependent activation of the ARE in response to *R. rosea* supplementation ($P = 0.0028$, linear regression analysis after doses were transformed to a log scale). However, no activation of the ARE was detected at 0.1 or 0.32 μg/ml ($P = 0.82$, one-way ANOVA including 0.5% DMSO control). IMR-32 cells were transfected with ARE–luciferase and actin–lacZ reporter constructs and treated with various concentrations of *R. rosea* and luminescence was detected 24 h later. ARE activity is shown as normalized to respective controls, 1% DMSO for 100 μg/ml, 0.5% DMSO for all other doses. (B) Immunoblot analysis for Nrf2–ARE target gene products. IMR-32 cells were treated with various doses of *R. rosea* for 24 h, and proteins were isolated, resolved by SDS–PAGE, and subjected to Western blot analyses for NQO1 and HO-1 (β-actin was the control).

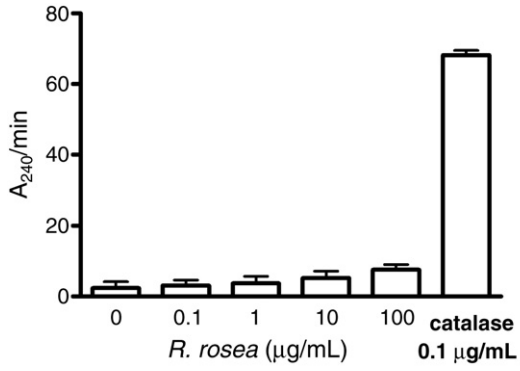

Fig. 10. *R. rosea* extract did not appreciably degrade H_2O_2 in vitro. Catalase was used as a positive control ($P = 0.25$, one-way ANOVA; catalase was excluded from the statistical analysis).

assay the effects of *R. rosea* in rapidly replicating cells, in contrast to those of a postmitotic nature previously studied in worms [9] and flies [30]. However, our results should not be interpreted as implying that *R. rosea* has a beneficial effect to tumor cells *in vivo*. In fact, *R. rosea* has been shown to retard tumor growth in an animal model [2], and the relationship between cancer, reactive oxygen species, and antioxidants may be very complex. *R. rosea* was also found to impart protective effects in nontransformed human fibroblasts (Fig. 7), demonstrating that its beneficial effects are not limited to immortalized cells and can be extended to cell lines representing a more normal physiological state.

The molecular mechanism of action of *R. rosea,* and adaptogens in general, has been compared to that of hormesis [9]. This is a phenomenon in which low doses of a mildly toxic agent induce a stress response, such as up-regulation of antioxidant defenses, heat shock proteins, DNA repair activities, etc., which result in an enhanced protection against additional stress later on [28]. Recently, *R. rosea,* along with another adaptogen, *Eleutherococcus senticosus,* was reported to extend the life span of the worm *C. elegans* [9]. The authors argued that these herbal extracts may mediate their effects through changes in gene expression patterns and, in particular, in those related to DAF-16. DAF-16 and the insulin-like signaling pathway is well known for its ability to modulate life span and stress resistance in experimental organisms [31], and the authors convincingly show nuclear translocation of the DAF-16 gene product after *R. rosea* supplementation. They also found that *R. rosea* could modulate heat shock protein (HSP) expression, though not to the same degree as heat shock at 35°C. However, this is at odds with findings in snail larvae, in which *R. rosea* did not activate HSP expression, but still afforded protection against heat stress [5]. This discrepancy suggests that, although *R. rosea* may activate HSP expression in some systems, this activation may not fully explain its beneficial effects.

To evaluate the possibility of *R. rosea* operating through a hormetic mechanism, we assayed its ability to activate the ARE, a *cis*-acting enhancer element in the 5′ flanking region of these cytoprotective enzymes. This element regulates many antioxidant enzymes including the glutathione *S*-transferases, HO-1, and NQO1, and its activation by transcription factor Nrf2 confers a resistance to oxidative damage. Using a luciferase reporter tagged downstream of the ARE-containing *NQO1* promoter, we found that *R. rosea* did not activate the ARE at doses (<1 μg/ml) that provided protection against H_2O_2 and UV (Fig. 9A). In particular, protection against H_2O_2 was observed at an *R. rosea* dose level 1000-fold lower (Fig. 6) than the minimum dose required to detectably activate the ARE (Fig. 9A). However, a dose-dependent activation of the ARE, up to threefold, was observed at doses of 1 μg/ml and greater (Fig. 9A). This suggests the potential involvement of a hormetic effect at higher doses and does not preclude the possibility that a mild, but undetectable, hormetic effect may even occur at the lower doses examined. In addition, we found no evidence for the activation of the ARE via Western analysis of two ARE-responsive proteins, HO-1 and NQO1, at all doses examined (Fig. 9B), including 100 μg/ml, at which *R. rosea* exhibits significant toxicity and activation of the ARE. These results do suggest that activation of the ARE element, specifically HO-1 and NQO1, is not required for the protective effects of *R. rosea* in cultured cells. This is in contrast to the results of Wiegant et al. [7], in which HO-1 expression was induced after *R. rosea* administration, albeit at doses approaching toxic levels. This difference may reflect differences between the cell lines used or differences between the organisms or tissues from which the cells were derived. For the assay of HO-1 expression, Wiegant et al. [7] used rat hepatoma cells, whereas we used human neuroblastoma cells.

Whereas this work has focused on the beneficial effects of *R. rosea* supplementation, based on our data, it is clear that in cell culture, the extract becomes toxic as dose levels are increased (Fig. 2), though no more than the commonly used antioxidant ascorbate. In addition, *R. rosea* at higher doses begins to sensitize cells to oxidative stress. For example, 0.1 μg/ml *R. rosea* protects cells against H_2O_2, whereas 1 μg/ml had no effect, and 10 μg/ml had sensitizing effects (Fig. 6A). Though this finding is consistent with the action of a hormetic agent, with low doses being beneficial and higher doses being toxic, the lack of activation of the ARE, or increased expression of HO-1 or NQO1, at protective doses argues against a pro-oxidative hormetic mechanism. However, the potential impact of a modest, or even undetectable, hormetic effect cannot be discounted. This has been argued by Rattan [32], who proposed that a modest stress may result in the "biological amplification of adaptive responses," leading to a benefit much greater than would be expected from the initial insult.

R. rosea extract is composed of at least 28 identifiable compounds. Therefore, it is not unreasonable to suppose that some of these are beneficial, whereas others are detrimental, and as dose levels increase, the toxicity of the detrimental components begin to outweigh the advantages of the beneficial agents. As a result, it would be attractive and imperative to identify the beneficial components of the *R. rosea* extract. One might guess that a good starting point would be with the three rosavins, as these three compounds are unique to the most-used species, *R. rosea,* of about 200 members of the genus *Rhodiola* [1]. Counter to this argument is the idea that herbal extracts act as a concert of many different compounds that collectively result in a synergistic effect [33,34] and that the individual components alone may afford little or no beneficial effects. Nevertheless, a worthwhile question that should be addressed in the future is what proportion of the benefits, i.e., protective activity, of *R. rosea* is due to the rosavins.

In conclusion, our findings are not consistent with *R. rosea* acting either as an antioxidant or as a pro-oxidant, despite its protective effects against oxidative insults. This conclusion is supported by the ability of *R. rosea* to enhance survival against oxidative stress at dose levels that do not elevate the major antioxidant defenses, activate the ARE, or degrade H_2O_2. Nevertheless, we have not ruled out the possibility that *R. rosea* may act through another hormetic mechanism or through a mild, but undetectable, hormetic effect, or that it may act on other pathways such as DNA repair. The finding that *R. rosea* is beneficial at a particular dose, but becomes toxic at higher levels, is consistent with what would be predicted from a hormetic agent. Thus, future studies should be pursued to determine the effects of *R. rosea* on other defense systems not directly related to oxidative stress and to determine which individual components of its extract are primarily responsible for its benefits. We therefore advocate a rigorous bioassay-guided isolation of beneficial components of *R. rosea.*

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