Friedreich Ataxia (FRDA), the most prevalent of the inherited ataxias, is a multi-systemic disease with loss of sensory neurons and life-threatening hypertrophic cardiomyopathy as its most severe manifestations. Reduced levels of the mitochondrial protein frataxin lead to cell-damaging oxidative stress and consequently FRDA is considered as a model for more common neurodegenerative disorders in which reactive radicals and oxidative stress are involved. We have developed a cellular assay system that discriminates between fibroblasts from FRDA patients and unaffected donors on the basis of their sensitivity to pharmacological inhibition of de novo synthesis of glutathione. With this assay we observed that supplementation with selenium effectively improved the viability of FRDA fibroblasts, indicating that basal selenium concentrations are not sufficient to allow an adequate increase in the activity of certain detoxification enzymes (such as GPX). Furthermore, we characterized potential drug candidates and found that idebenone, a mitochondrially localized antioxidant that ameliorates cardiomyopathy in FRDA patients, as well as other lipophilic antioxidants protected FRDA cells from cell death. Our results also demonstrate for the first time that small-molecule GPX mimetics have potential as a novel treatment strategy for Friedreich Ataxia and presumably also for other neurodegenerative diseases with mitochondrial impairment.

INTRODUCTION

Friedreich Ataxia (FRDA), an autosomal recessive neurodegenerative disease, is the most prevalent of the inherited ataxias. Progressive loss of sensory neurons starts at early childhood and culminates in gait and limb ataxia, absent tendon reflexes and dysarthria (1,2). Life-threatening hypertrophic cardiomyopathy is found in two-thirds of the patients at the time of diagnosis (2–4). Most FRDA patients are homozygous for large expansions of GAA triplet repeats in the first intron of the gene encoding for the nuclear encoded mitochondrial protein frataxin. Early studies indicated a link between reduced frataxin levels and an impaired iron homeostasis based on iron deposits seen in cardiac tissue of FRDA patients (5,6) and in the yeast Δyyh1 model (7,8). This has led to the hypothesis that elevated levels of mitochondrial iron as a consequence of frataxin deficiency generate cell-damaging superoxide and hydroxyl radicals. In support of this, several studies have directly demonstrated increased levels of oxidative stress in patients as well as in Δyyh1-yeast (9–13). More recent experiments in mouse models for FRDA indicate, however, that iron accumulation appears to be a late-onset and secondary effect (14,15). Nevertheless, the existing experimental data support the observation that impaired function of iron/sulfur (Fe/S) cluster proteins such as aconitase and respiratory chain complexes I–III and concomitant lack of stress-induced increase of defense enzymes (such as superoxide dismutase) causes mitochondrial damage in frataxin deficient cells (9,16). This link between pathology and oxidative stress qualifies FRDA as an excellent model for more common neurodegenerative diseases in which reactive radicals and oxidative stress contribute to the progression of the disease.

Idebenone, a benzoquinone originally developed for the treatment of cerebral ischemia-induced lesions (17–19) has been shown to inhibit lipid peroxidation (17,20–22), to stimulate mitochondrial functions (23) and to improve the myocardial energy state in cardiac hypertrophy (24). Therefore, idebenone is considered a good candidate for antioxidant-based treatment of FRDA. Indeed, clinical reports have demonstrated the improvement of cardiomyopathy (25–27) and markers for DNA damage (11) in FRDA patients. Unfortunately, however, the clinical data currently available do not support idebenone for the treatment of neurological signs (e.g. ataxia) in FRDA (27). Consequently, besides idebenone additional drug candidates will have to be considered for the treatment of the neurological pathology in FRDA patients.
Here we report a cell culture model using FRDA-patient-derived fibroblasts that is suitable for screening and validation of novel drug candidates. We observed that FRDA fibroblasts are discriminated from normal cells when cultured in medium with restricted selenium content and upon pharmacological depletion of cellular glutathione (GSH) pools. Motivated by our observation that idebenone was able to prevent cell toxicity in this assay system, we have screened additional chemical compounds for their FRDA cell protecting effect. We found that not only antioxidants (such as idebenone and coenzyme Q analogs) but also small molecule, non-protein mimetics of glutathione peroxidase were able to rescue FRDA cells from endogenous oxidative stress. This cellular assay using readily available patient skin fibroblast cultures is an important tool for the discovery of novel drug candidates that also have potential as therapy for other neurodegenerative diseases that are caused and/or aggravated by oxidative stress.

RESULTS

FRDA fibroblasts are extremely sensitive to BSO treatment

Motivated by preliminary observations that report abnormalities of glutathione metabolism in FRDA patients (28–30), we quantified survival of skin fibroblasts taken from FRDA patients and unaffected normal donors under conditions of partial GSH depletion. For this, we cultured cells under conditions where γ-glutamylcysteine synthase (EC 6.3.2.2), the rate-limiting enzyme in the de novo synthesis of glutathione, had been blocked pharmacologically with L-buthionine (S,R)-sulfoximine (BSO). Under this condition, the cells are able to recycle GSH through NADPH-dependent glutathione reductase, however, de novo synthesis of GSH is blocked (31), and cells become depleted for GSH and susceptible to endogenously generated oxidative stress. Exposure of normal fibroblasts to BSO at concentrations below 100 μM had no detrimental effect on cell viability as determined in a fluorogenic assay that allows quantification of live cells by their cellular esterase activity (Fig. 1A). This was clearly different for FRDA fibroblast cultures where cell viability was already reduced to less than 10% at BSO concentrations as low as 50 μM. Closer microscopic examination using double-fluorogenic labeling revealed that normal cells treated with 1 mM BSO remained essentially viable with the majority of cells stained for esterase activity. In contrast, the large majority of the BSO-treated FRDA cells displayed nuclear ethidium homodimer staining, an indication of severe plasma membrane damage leading to cell death (Fig. 1B). This elevated susceptibility of FRDA fibroblasts towards BSO was observed in cultures derived from three different, molecularly diagnosed FRDA patients. Specifically, upon exposure to 1 mM BSO, FRDA fibroblasts showed less than 10% cell viability in all cases studied, whereas under these conditions normal fibroblasts displayed only moderate sensitivity to GSH-depletion retaining an average of 60% cell viability (Fig. 1C). Taken together, inhibition of de novo GSH synthesis provided for a discriminating assay that can be used in 96-well microtiter format for drug discovery and validation.

Idebenone and coenzyme Q10 analogs differently protect FRDA fibroblasts from cell death upon GSH depletion

We next tested whether idebenone [2,3-dimethoxy-5-methyl-6-(10-hydroxydecyl)-1,4-benzoquinone], a membrane-permeable antioxidant (32,33) could protect FRDA cells from BSO-mediated cell death. Indeed, preincubation of FRDA fibroblasts with increasing concentrations of idebenone ~24 h prior to the BSO treatment effectively prevented cell death for at least 10 days (the longest period observed) with an EC50 of ~0.5 μM (Fig. 2A and B). We have also tested the influence of 5 μM idebenone on control cells which were treated with various concentrations of BSO. However, one has to bear in mind that in this case the consequences of BSO-mediated toxicity are not as apparent as in FRDA fibroblasts and manifest themselves as uniform growth retardation and not in selective and acute cell death. Only at concentrations above 1 mM BSO did we observe that idebenone significantly improved overall cell viability, e.g. at 3.3 mM BSO from 24.3 ± 0.7% to 62.9 ± 2.8% compared with non-BSO-treated control cells (n = 4).

To exclude the possibility that idebenone simply prevented BSO-mediated GSH depletion, we directly measured the cellular GSH content under these experimental conditions. For this we treated FRDA fibroblasts during 24 h with 5 μM idebenone and then added BSO up to a final concentration of 1 mM. After 8 h of incubation the cellular GSH content was measured with the fluorescent GSH-specific dye monochloro-bimane. This experiment revealed that in FRDA cells BSO alone mediated a drop in GSH levels to 32.2 ± 2.2%, (n = 3) of the untreated control, whereas in the presence of idebenone, cellular GSH levels dropped to similar levels (33.4 ± 2.7%, n = 3). This indicates that idebenone did not inhibit BSO-mediated GSH depletion but instead protected the frataxin deficient cells from its consequences, most likely elevated levels of endogenous oxidative stress.

Interestingly, vitamin E was almost as potent as idebenone in preventing cell death (EC50 = 0.7 μM), whereas Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble derivative of vitamin E lacking the 16-carbon-atom long side chain, was both less potent and less efficient in preventing cell death (Fig. 2A). Specifically, Trolox preserved only 60% cell viability at the optimal concentration of 25 μM. Another well-known antioxidant, astaxanthin (3,3′-dihydroxy-4,4′-diketo-β-carotene) (34) was even less potent, resulting in only 50% cell viability at 50 μM, the highest concentration tested (data not shown).

Not only idebenone but also several idebenone analogs protected FRDA cells from oxidative stress, provided that a certain lipophilicity was retained. Decylubiquinone, for example, lacks the hydroxy group at the end of the alkyl side chain and has an increased overall lipophilicity compared with idebenone, as indicated in the respective calculated ClogP values (Fig. 3). This elevation of lipophilicity by two orders of magnitude in ClogP value correlated with a ~10-fold increase in the potency of decylubiquinone to restore FRDA cell viability (EC50 = 0.04 μM) compared with idebenone. Likewise, analogs of the electron transport chain factor coenzyme Q10 (CoQ10) showed a similar correlation between lipophilic GSH and FRDA cell protection upon GSH-depletion. For instance, CoQ0
Figure 1. Differential sensitivity of control and FRDA fibroblasts to BSO treatment. Cellular viability after BSO treatment was measured by calceinAM fluorescence of live, unfixed cells. (A) Dose–response curve of BSO treatment of control (solid circles) and FRDA fibroblasts (open circles). Data are expressed as percentages of the corresponding untreated cells ($n = 3$ for each data point). (B) Fluorescence microscopy analysis of control and FRDA fibroblasts co-stained with calceinAM and ethidium homodimer. Upon treatment with BSO, most FRDA fibroblasts were dead (indicated by the red nuclear staining with ethidium homodimer) while most control fibroblasts were alive, indicated by calceinAM staining (green). (C) Disease-specific BSO sensitivity of different control (C1–C3) and FRDA (F1–F3) cell lines as expressed as percentage cell viability ($n = 4$ for each bar). A two-way ANOVA revealed a significant effect of cell type ($F = 1391.1$, d.f. = 5, $P < 0.001$), BSO treatment ($F = 1538.8$, d.f. = 1, $P < 0.001$) and the interaction between the two ($F = 100.2$, d.f. = 5, $P < 0.001$).
lacking the carbon chain tail of CoQ10 had the lowest logP value (−0.15) and was not able to protect FRDA fibroblasts from the detrimental effects of BSO treatment at concentrations as high as 50 μM. In contrast, CoQ1 with a five-carbon-atom tail (logP = 1.87) and CoQ2 with a 10-carbon-atom tail (logP = 3.90) protected FRDA fibroblasts from cell death with decreasing EC₅₀ values (Fig. 3). Taken together, these results demonstrate that this disease-relevant cellular assay system not only discriminates between normal and FRDA fibroblasts but allows analysis of structure–activity relations and validation of drug candidates such as idebenone and active idebenone analogs.

Importance of selenium for FRDA cell survival

To investigate the mechanism underlying the cytotoxic effect of GSH depletion in FRDA fibroblasts, we next analyzed the composition of various cell culture media and their contribution to the outcome of the assay. We found that the susceptibility of FRDA cells towards GSH depletion is correlated with the concentration of selenium in the medium. FRDA fibroblasts which were preincubated for 24 h with sodium selenite did survive in a dose-dependent manner while a selenium supplementation simultaneous to the BSO treatment was ineffective in the protection of BSO-challenged FRDA cells. Under non-stressed conditions and at low concentrations selenium supplementation did not change cell viability, while at higher concentrations (≥10 μM) a slightly negative influence could be observed (Fig. 4). Since preincubation for several hours was required for the cell-protecting effect of selenium supplementation we tested the hypothesis that in our assay selenium could have become a strong rate-limiting factor for the de novo synthesis of selenoproteins. Of particular interest in the context of this assay was the influence of selenium supplementation on the activity of glutathione peroxidase (GPX), an enzyme known to protect from cell-damaging oxidative stress (35). To investigate this, we compared the effect of selenium starvation with selenium supplementation in combination with BSO treatment on the level of GSH and enzyme activity of GPX in FRDA cells. Incubation with 1 mM BSO reduced the level of cellular GSH content to ~34% in both control and FRDA fibroblasts. Preincubation with 500 nM selenium for 24 h did not prevent BSO-mediated GSH depletion in either cell type (Fig. 5A). This result shows that selenium, like idebenone, exerts a cell-protecting activity without preventing the BSO-mediated depletion of the cellular GSH pool.

We next measured the effect of selenium supplementation on the activity of GPX. Under conditions where selenium was limited in the culture medium, both control and FRDA fibroblasts had comparable GPX activities which dropped to ~65% in both cell types upon BSO-mediated depletion of GSH (Fig. 5B, black bars). Selenium complementation, however, increased GPX activity only in FRDA fibroblasts by ~60% while GPX activity in selenium-complemented control cells was not different from selenium-starved cells. This increase in GPX activity upon selenium supplementation was even more pronounced under conditions of GSH depletion. Selenium supplementation of BSO-treated control cells increased GPX activity ~70% over the activity seen in selenium-starved, BSO-treated cells. In contrast, selenium supplementation increased GPX activity ~2.9-fold in the same assay with FRDA cells. In conclusion, these data show that selenium supplementation leads to an upregulation of the GPX enzyme activity which is more pronounced in FRDA cells than in normal fibroblasts and which is particularly evident under conditions of GSH depletion (Fig. 5B). This supports our hypothesis that FRDA cells suffer from a selenium deficiency that limits GPX activity and becomes lethal under conditions where GSH levels are limited.

Small-molecule GPX mimetics protect FRDA fibroblasts from cell death

Recent data demonstrated a link between frataxin expression levels, glutathione peroxidase activity and oxidative stress. Frataxin overexpression in 3T3L1 murine fibroblasts, for example, increased glutathione peroxidase activity by 50% (36), while yeast cells deficient for frataxin and GPX (Δyhf1/Δgpx double knockout) had elevated peroxide levels and increased DNA damage (13). Therefore, we tested the possibility that small-molecule GPX mimetics could effectively rescue FRDA
cells from BSO-mediated GSH depletion and oxidative stress. For this, FRDA cells were first incubated for 24 h in the presence of ebselen [2-phenyl-1, 2-benzisoselenazol-3 (2H)-one], a well-known GPX mimetic (37). The cells were then subjected to 1 mM BSO treatment. We found that ebselen was able to prevent cell death of BSO-challenged FRDA fibroblasts with an EC$_{50}$ of 10$^{-10}$ M (Fig. 6). In an attempt to discover active GPX mimetics with lower EC$_{50}$ values we screened a selection of monoselenide and diselenide compounds (38–44), some of which had been shown previously to act as GPX mimetics in vitro (42). The cell protecting effect of these compounds was determined in BSO-challenged FRDA fibroblasts and dose–response curves and EC$_{50}$ values for cell protection were determined in each case. All GPX mimetics tested were effectively rescuing cell viability from the BSO effect, however with clear differences in potency and efficacy. In contrast to antioxidants such as idebenone and decylubiquinone which showed at least 50% cell rescue over a wide concentration range, typically 3 orders of magnitude, all small-molecule GPX mimetics had a more narrow working range, typically 1.5 orders of magnitude. Diselenides (compound nos 1–3, 6, 7, 9–16) were in general more potent than monoselenides (compound nos 4, 5, 8) and, interestingly, among the most effective compound was a diselenide GPX mimic with a centrally coordinated iron atom (compound no. 13). Taken together our results show that this cellular model can be used for drug screening purposes for Friedreich Ataxia and it allowed the identification of a new class of molecules with therapeutic potential for this disease.

**DISCUSSION**

Systematic screening for small molecule compounds to be used for the treatment of FRDA is hampered by the lack of disease-relevant and validated cellular assay systems. The use of frataxin-deficient yeast as well as immortalized cells from frataxin-deficient mouse models turn out to be less appropriate for drug screening since in FRDA patients a residual expression level of frataxin is retained (45). FRDA-patient derived cells, in particular skin fibroblasts, offer an alternative source of cell material to be used in disease-specific drug screening assays. However, such a cell-based assay not only has to be applicable for screening of large compound collections, but, even more important, it must discriminate between patient cells and cells derived from healthy donors and, ideally, it has to be validated with idebenone. In previous attempts to establish such an assay, patient-derived fibroblasts or lymphoblasts were used in culture medium supplemented with pro-oxidant agents such as H$_2$O$_2$, tert-butyl hydroperoxid, butyl sulfonide, trifluoroperazone and menadione or transition metal ions, in particular FeCl$_3$ (16,46,47). A potential drawback of

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this assay type, however, is the possible interference of the pro-oxidant culture conditions with the chemical stability of test compounds to be used. In addition, none of the aforementioned cellular tests have been validated with idebenone or standardized to allow study of structure–activity relationships (SARs), a prerequisite for successful drug screening. Here we report a cellular assay system that qualifies as a disease-relevant drug screening assay, since it discriminates between fibroblasts from FRDA patients and normal donors on the basis of differential susceptibility to endogenously generated oxidative stress induced pharmacologically by inhibition of de novo synthesis of GSH in selenium-restricted medium. This assay is robust, does not interfere with the chemical stability of test compounds, allows semi-automated assay readout and, therefore, is compatible with large-scale compound screening and validation. Upon validation with idebenone, we have shown that this assay is suitable for analysis of SARs, shown here for benzoquinones.

One of the most surprising results of this study was the observation that small-molecule GPX mimetics can rescue FRDA fibroblasts. This is of particular interest since clinical trials documented that hypertrophic cardiomyopathy in FRDA patients can be effectively treated with the antioxidant idebenone (25–27,48) while there is currently no effective treatment strategy for the neurodegenerative manifestation (i.e. ataxia) in FRDA. Interestingly, the GPX mimetic ebselen mediated protection in an experimental ischemia model in rodents (49), indicating that GPX mimetics could also be considered as a novel treatment strategy for FRDA. Further experiments need to address in more detail the mechanism of action of GPX mimetics in this FRDA cell model. However, the interpretation that the assay itself substantially inhibits GPX activity which is then rescued by GPX mimetics is an oversimplification since GPX activity is moderately inhibited by BSO in both normal and FRDA fibroblasts (Fig. 5) while the survival of FRDA cells is particularly affected under these stress conditions (Fig. 1).

Instead, we conclude that the disease-correlated effect of GPX mimetics demonstrates that FRDA patient cells are apparently exposed to increased levels of peroxide radicals while at the same time induction of superoxide dismutase activity is prevented in frataxin-deficient cells (16). This limit in detoxification activities can be overcome by an increase in GPX activity through selenium supplementation. Consequently we postulate that at least in vitro cells have a higher demand for this micronutrient.

In conclusion, the cellular model system described here not only reveals a novel class of small-molecule drug candidates that hold the potential for FRDA treatment, but it also points towards the involvement of selenium and selenoproteins in this neurodegenerative disease.

Figure 4. Preincubation with selenium increases resistance of FRDA cells to 1 mM BSO challenge. FRDA cells were incubated with increasing concentration of sodium selenite and cell viability was measured (n = 4 for each data point). Sodium selenite preincubation for 24 h prior to BSO challenge (open squares); sodium selenite preincubation for 24 h without BSO challenge (open circles); and sodium selenite applied simultaneously with BSO (solid squares).

Figure 5. Effect of BSO and selenium on intracellular GSH levels and GPX activity. Black bars, no selenium supplementation; open bars, supplementation with 500 nM sodium selenite; C, control fibroblasts; F, FRDA fibroblasts. (A) BSO treatment reduces cellular GSH content in both control and FRDA cells. Selenium does not influence GSH concentrations (expressed as nmol GSH/mg protein, n = 3 for each bar). (B) Selenium supplementation increases GPX activity upon BSO challenge in FRDA fibroblasts compared with control fibroblasts (expressed as mU/mg protein, n = 3 for each bar; P-values for significance are indicated).
Figure 6. Small-molecule GPX mimetics improve resistance of FRDA fibroblasts to BSO challenge. (A) FRDA fibroblasts were preincubated with the listed compounds for 24 h prior to BSO challenge. Dose–response and EC50 values of idebenone, decylubiquinone, ebselen and other small-molecule GPX mimetics are provided. The concentration ranges for which a given compound displayed at least 50% activity were taken from dose–response curves and plotted as a bar histogram (the left-hand side of each bar represents the EC50-value). (B) Chemical structures of ebselen and the small-molecule GPX mimetics used in this study.
**METHODS**

**Cell culture and reagents**

Primary fibroblasts were derived from donors with a molecular diagnosis of FRDA (F1–F3) and control donors with no mitochondrial disease (C1–C3). Cell lines C1 and F1 were provided by the Swiss Network on Friedreich Ataxia Research, line F3 was provided by Hopital Necker, Paris (France), and lines F2, C2 and C3 were obtained from Coriell Cell Repositories (Camden, NJ, USA; catalog nos GM04078, GM08402 and GM08399, respectively). All cell lines were diagnosed at the molecular level for intronic GAA triplet repeat length using a PCR-based method (50). FRDA-cell types had shown 400–450 repeats (F2 line) or more (F1 and F3), whereas control cell lines displayed PCR products of normal length (not shown). Unless indicated differently, all data shown were obtained with cell lines F2 and C1 and confirmed with all other cell lines. For experiments, the cells were seeded in microtiter plates at a density of 4000 cells per 100 μl in growth medium consisting of 25% (v/v) M199 EBS and 64% (v/v) MEM EBS without phenol red (Bioconcept, Allschwil, Switzerland) supplemented with 10% (v/v) fetal calf serum (PAA Laboratories, Linz, Austria), 100 U/ml penicillin, 100 μg/ml streptomycin (PAA Laboratories, Linz, Austria), 10 μg/ml insulin (Sigma, Buchs, Switzerland), 10 ng/ml EGF (Sigma, Buchs, Switzerland), 10 ng/ml bFGF (PreproTech, Rocky Hill, NJ, USA) and 2 μM glutamine (Sigma, Buchs, Switzerland).

The cells were incubated in the presence of various test compounds for 24 h before addition of BSO.

**Cell viability measurements**

Cell viability was measured after the first signs of toxicity appeared in the BSO-treated controls (typically after 16–48 h). The cells were stained for 60 min at room temperature in PBS with 1.2 μM calceinAM and 4 μM ethidium homodimer (Live/Dead assay, Molecular Probes, Eugene, OR, USA). Fluorescence intensity was measured with a Gemini Spectramax XS spectrophuorometer (Molecular Devices, Sunnyvale, CA, USA) using excitation and emission wavelengths of 485 and 525 nm, respectively. Live cell imaging was performed with a Zeiss Axiovert 135 M fluorescence microscope equipped with a cooled CCD camera (Sensicam, PCO Computer Optics, Kelheim, Germany). Image acquisition was performed with the ImagePro Plus software (Media Cybernetics, Silver Spring, MD, USA).

**Glutathione content**

Cells were removed from 100 mm culture dishes by trypsination, washed twice with PBS, snap-frozen in 100 μl PBS and lysed in PBS supplemented with a protease inhibitor cocktail (Complete, Roche Diagnostics, Rotkreuz, Switzerland) by four freeze–thaw cycles. Total protein content was measured with the BioRad protein assay (BioRad, Hercules, CA, USA). Reduced glutathione content was determined essentially as described previously (51) with a final monochlorobimane (mCIB, Molecular Probes, Eugene, OR, USA) concentration of 25 μM. The GSH-mCIB adduct fluorescence was measured with a Gemini spectrofluorimeter using excitation and emission wavelengths of 380 and 470 nm, respectively.

**Glutathione peroxidase assay**

Cell extracts obtained for total GSH measurements were adjusted to a final protein concentration of 1.85 mg/ml. Enzymatic activity was measured with the Glutathione peroxidase cellular activity assay kit (Sigma, St Louis, MO, USA) according to manufacturer’s instructions with minor modifications. Enzymatic activity was measured with 40 μg total protein extract in a final reaction volume of 100 μl monitoring NADPH consumption as a decrease in NADPH fluorescence. GPX enzymatic activities were determined by measuring the maximum velocity of the NADPH consumption using tert-butyl hydroperoxide as substrate and purified bovine erythrocyte GPX (Sigma, St Louis, MO, USA) as standard. All measurements were done in triplicate in 96-well plates with a Gemini spectrofluorimeter using excitation and emission wavelengths of 340 and 445 nm, respectively.

**Data and statistics**

All data are expressed as mean ± SD. Unpaired t-test and two-way ANOVA were used for statistical evaluation.

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