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Ruthenium red-mediated suppression of Bcl-2 loss and Ca^{2+} release initiated by photodamage to the endoplasmic reticulum: scavenging of reactive oxygen species

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Abstract

The photosensitizer 9-capronyloxytetrakis (methoxyethyl) porphycene localizes predominantly in the endoplasmic reticulum (ER) and, to a lesser extent, in mitochondria of murine leukemia L1210 cells. Subsequent irradiation results in the loss of ER > mitochondrial Bcl-2 and an apoptotic response. Although an increase in cytosolic Ca^{2+} was observed after irradiation, apoptosis was not inhibited by either the presence of the calcium chelator BAPTA or by the mitochondrial uniporter inhibitor ruthenium amino binuclear complex (Ru360). Moreover, neither reagent prevented the loss of Bcl-2. Ruthenium red (RR) devoid of Ru360 prevented Bcl-2 loss, release of Ca^{2+} from the ER and the initiation of apoptosis. Since RR was significantly more sensitive than Ru360 to oxidation by singlet oxygen, we attribute the protective effect of RR to the quenching of reactive oxygen species. Although cytosolic and (to a lesser extent) mitochondrial Ca^{2+} levels were elevated after photodynamic therapy, these changes were apparently insufficient to contribute to the development of apoptosis.
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Abbreviations: AM, acetoxymethyl ester; BAPTA-AM, (acetyoxymethyl)-1,2-bis(*o*-amino phenoxy)ethane *N,N,N′,N′*-tetra (acetoxymethyl)ester; CPO, 9-capronyloxytetrakis (methoxyethyl) porphycene; DCF, 2′,7′-dichlorofluorescein; DEVD-R110, asp-glu-val-asp-rhodamine 110; ER, endoplasmic reticulum; ERTr, endoplasmic reticulum tracker; FHS, Fischer's medium with 20 mM HEPES buffer pH 7.2 replacing $NaHCO_3$; H_2DCF, dichlorohydrofluorescein; H_2DCFDA, dichlorodihydrofluorescein diacetate; LTB, lysotracker blue; NAO, nonyl acridine orange; NPe6, *N*-aspartyl chlorin e6; PDT, photodynamic therapy; ROS, reactive oxygen species; RR, ruthenium red; Ru360, ruthenium amino binuclear complex; SnET2, tin etiopurpurin; THP, thapsigargin; TMRM, tetramethylrhodamine methyl ester; Trolox, 6-Hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid (water-soluble derivative of Vitamin E); $\Delta\Psi_m$, mitochondrial membrane potential

Introduction

Photodynamic therapy (PDT) is a procedure whereby cells and tissues are sensitized to light by photosensitizing agents. Subsequent irradiation catalyzes the localized production of predominantly singlet molecular oxygen.[1,2] This reactive product causes photodamage to nearby organelles and macromolecular molecules along with the initiation of an apoptotic and/or necrotic response.[1–10] PDT has been successfully employed for tumor eradication, and for the treatment of macular degeneration and atherosclerotic plaque.[1,2] In the case of cancer therapy, the efficacy of PDT is related to both direct killing of tumor cells and shutdown of the tumor vasculature.[1,2]

While many agents function as photosensitizing agents, their intracellular targets can vary. The photosensitizer termed *N*-aspartyl chlorin e6 (NPe6) localizes predominantly in acidic organelles. Subsequent irradiation causes disruption of lysosomes and endosomes, and the release of proteases.[9,10] Apoptosis initiated by this sensitizer is characterized by the cleavage of Bid, release of cytochrome *c* and activation of the apoptosome.[9] These events occur in the absence of measurable loss of Bcl-2 or of mitochondrial membrane potential ($\Delta\Psi_m$).[9,11] In contrast, lysosomes are unaffected by PDT using either the porphycene CPO (9-capronyloxytetrakis (methoxyethyl) porphycene) or the phthalocyanine Pc 4. For both of these latter agents, activation of the intrinsic apoptotic pathway is accompanied by the loss of Bcl-2 and $\Delta\Psi_m$.[3,11–13]

The chlorin derivative meta-hydroxy phenyl chlorin preferentially localizes in the Golgi and endoplasmic reticulum (ER).[4] Subsequent irradiation results in photodamage at these sites, with mitochondria apparently spared from perturbation. Nevertheless, irradiated cells die by the intrinsic apoptotic pathway.[5] Similarly, the photosensitizer verteporfin activates the intrinsic apoptotic pathway in PDT protocols. This activity is preceded/accompanied by the release of ER Ca^{2+} stores.[14]

The antiapoptotic protein Bcl-2 resides in both the mitochondria and the ER.[15] Although the antiapoptotic properties of mitochondrial-associated Bcl-2 are widely appreciated, recognition of the role of ER-associated Bcl-2

in the regulation of ER Ca^{2+} stores, and the role of Ca^{2+} in the initiation/potentiation of the intrinsic apoptotic pathway, are relatively recent developments.[16–24] We previously demonstrated that the porphycene photosensitizer CPO catalyzes the loss of Bcl-2 in murine leukemia L1210 cultures, an effect correlated with the intensity of the proapoptotic response.[11] As the induction of apoptosis was associated with the loss of $\Delta\Psi_m$, we initially concluded that mitochondria were the target in CPO PDT protocols. However, with the recent finding that CPO may localize to the ER, it seemed possible that the ER may be a target.[11] Since the ER is a major storage site for calcium ion, we have investigated the possible role of Ca^{2+} release, after CPO-catalyzed ER photodamage, in initiating or potentiating the apoptosis occurring in this model. Our data indicate that both mitochondria and the ER are targets in this PDT protocol, and that associated release of Ca^{2+} into the cytosol is insufficient for the development of apoptosis. In addition, our studies identified a novel anti-apoptotic property of ruthenium red (RR), an agent widely used as a putative inhibitor of the mitochondrial Ca^{2+} uniporter,[25,26] which is unrelated to the latter activity.

Results

CPO localization

Colocalization studies were performed using lysotracker blue (LTB), nonyl acridine orange (NAO) and endoplasmic reticulum tracker (ERTr) as probes for lysosomes, mitochondria, and the endoplasmic reticulum (ER), respectively (Figure 1). Fluorescence overlap with CPO was optimal with the ERTr probe, while LTB and NAO showed substantially lesser degrees of colocalization. These studies all involved 30 min incubations with CPO at 37°C, corresponding to the time used in PDT protocols.

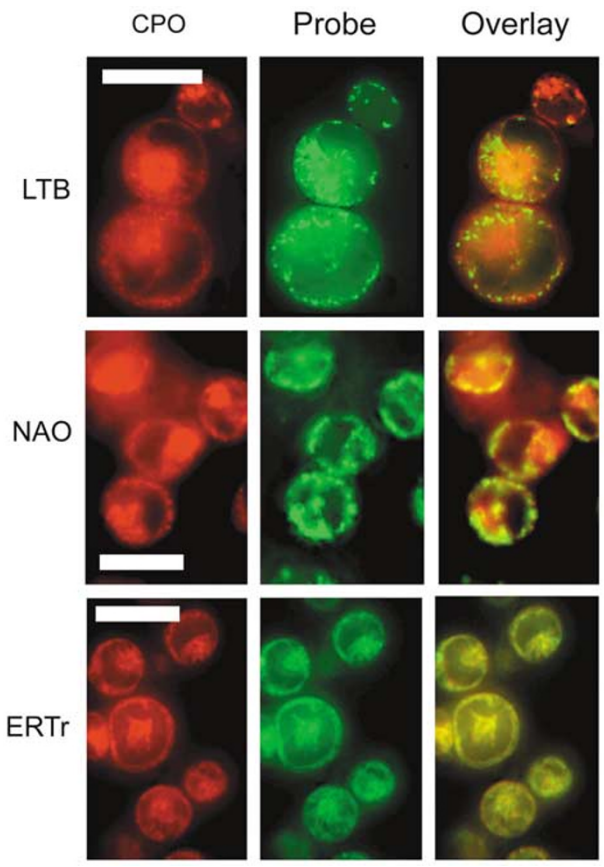

Figure 1 Colocalization of CPO with selected fluorescent probes in L1210 cells. Cultures were coincubated with CPO (2 μM) and LTB (2 μM), NAO (2 μM) or ERTr (3 μM) for 30 min prior to being washed and imaged. MetaMorph software was used to overlay the images. Column 1 = fluorescence of CPO; column 2 = fluorescence of probes; column 3 = overlay of images. White bars in panels = 10μ

PDT effects on viability and asp-glu-val-asp-rhodamine 110 (DEVD)ase activation

Irradiation of L1210 cultures loaded with 2 μM CPO elevated DEVDase activities ~27-fold (Table 1). This increase in DEVDase was paralleled by a marked loss of viability, as scored in clonogenic assays. RR alone, at a concentration of 5 μM, neither activated DEVDase nor was cytotoxic to L1210 cells (Table 1). However, a six-fold higher concentration of RR (30 μM) dramatically elevated DEVDase activity and was cytotoxic (Table 1). The morphology of cells treated with 30 μM RR suggested that they were dying by an apoptotic mechanism (data not shown).

Cotreatment of irradiated, CPO-sensitized L1210 cultures with RR suppressed both cell killing and DEVDase activation (Table 1). The ID_{50} for suppression of DEVDase activity by RR was ~0.5 μM. A 10-fold higher concentration of RR totally suppressed DEVDase activation. In contrast, 5 μM ruthenium amino binuclear complex (Ru360) neither inhibited DEVDase activation nor offered protection against the phototoxicity of CPO (Table 1). In the presence of the reactive oxygen species (ROS) scavenger Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (water-soluble derivative of Vitamin E)), both caspase activation and loss of viability were prevented. Cotreatment of cultures with the Ca^{2+} chelator BAPTA-AM did not protect cells from the proapoptotic effects of PDT (Table 1).

Table 1 Effects of RR on DEVDase activity and viability after PDT

Conditions	DEVDase activity	Viability (%)
Control	0.41±0.16	100
CPO PDT	10.8±1.0	12±4
CPO PDT+RR (0.5 μM)	4.3±0.3	65±3
CPO PDT+RR (5 μM)	0.33±0.08	96±5
CPO PDT+Ru360 (5 μM)	10.3±0.9	5±3
CPO PDT+BAPTA (10 μM)	11.1±0.9	9±2
CPO PDT+10 mM Trolox	0.51±0.20	94±2
NPe6 PDT	10.1±1.1	15±4
NPe6 PDT+RR (5 μM)	11.2±1.0	17±3
NPe6 PDT+Ru360 (5 μM)	11.3±0.8	18±3
RR (5 μM)	0.26±0.06	96±4
RR (30 μM)	11.8±1.1	11±3
Ru360 (5 μM)	0.23±0.04	98±3

Effects of an LD_{90} PDT dose with CPO or NPe6 on DEVDase activity and viability of L1210 cells. RR, Ru360 or Trolox were present, as specified, at the time of loading with sensitizer. Data represent the mean±S.D. for three determinations. DEVDase activity = nmol product/min/mg protein. Viability was measured by clonogenic assays and is expressed as % control (untreated cells)

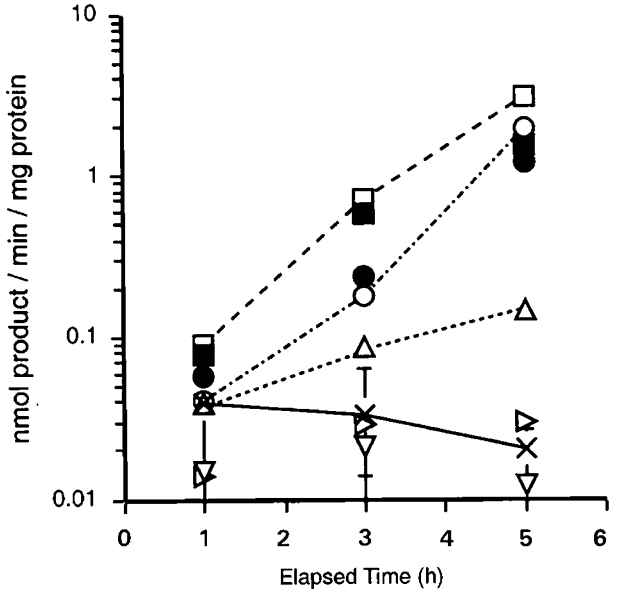

Figure 2 Effect of RR on DEVDase activity after NPe6-induced photodamage. Subconfluent cultures of 1c1c7 cells were sensitized with 66 μM NPe6 for 45 min prior to being washed and irradiated. In some groups, cultures were coincubated with 5 μM RR at the time of sensitization. Symbols are: $\times$ = control, Δ = NPe6, ∇ = light alone (80 mJ/cm^2), $\triangle$ = RR alone, $\bullet$ = NPe6 + light (40 mJ/cm^2, an LD_{50} light dose), $\circ$ = NPe6 + light (40 mJ/cm^2) + RR, $\blacksquare$ = NPe6 + ight (80 mJ/cm^2, an LD_{95} light dose), $\square$ = NPe6 + light (80 mJ/cm^2) + RR

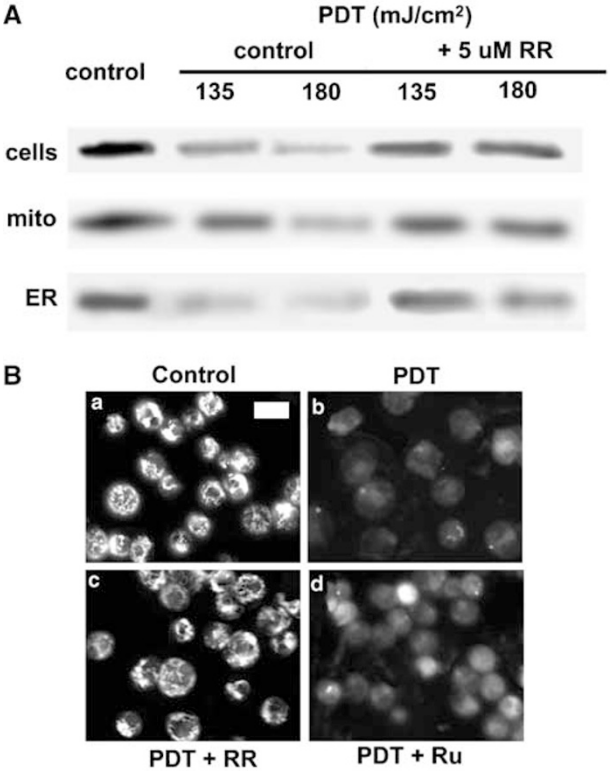

Figure 3 Effects of RR and Ru360 on PDT-induced losses of Bcl-2 and $\Delta\Psi_m$. (**A**) Cultures of L1210 cells were sensitized with 2 μM CPO, in the absence or presence of 5 μM RR, for 30 min prior to irradiation with two different light doses, followed by isolation mitochondrial and ER fractions. Western blot analyses of Bcl-2 utilized of 40 μg of whole-cell protein. (**B**) Cultures of L1210 cells were cotreated with 2 μM CPO and 5 μM RR or Ru360 for 30 min prior to being washed and irradiated. After irradiation, cells were loaded with TMRM and imaged to detect $\Delta\Psi_m$. Panels are: (a) control, (b) CPO + light and (c) CPO + light + RR, (d) CPO + light + Ru360. White bar in panel a = 10μ

We previously reported that the photosensitizer NPe6 localizes in the lysosomes of L1210 and murine hepatoma 1c1c7 cells, and causes lysosomal destruction following irradiation.[9,10] Cotreatment of NPe6-sensitized L1210 cultures with 5 μM RR or Ru360 affected neither DEVDase activation nor cell killing after irradiation (Table 1). Similar effects were observed in 1c1c7 cells: a 5 μM concentration of RR did not alter the activation of DEVDase in NPe6-sensitized cultures irradiated with either LD_{50} or LD_{95} doses of light (Figure 2). Hence, we found no protective effect of RR with regard to lysosomal photodamage.

Effects of RR and Ru360 on Bcl-2 and $\Delta\Psi_m$

Cell fractionation and immunofluorescence colocalization analyses have shown that Bcl-2 is found in both mitochondria and the ER in a variety of cell types.[15,18,19] Analyses of enriched organelle fractions show a similar distribution in L1210 cells (Figure 3A). Irradiation of CPO-sensitized L1210 cultures resulted in the loss of both mitochondrial and ER-associated Bcl-2, as determined by Western blot analyses, with the latter more sensitive to PDT. Cotreatment of CPO-sensitized cultures with 5 μM RR markedly suppressed the loss of Bcl-2 that occurred following irradiation (Figure 3A). RR also protected against the loss of $\Delta\Psi_m$, while cotreatment with 5 μM Ru360 did not afford similar protection (Figure 3B).

Release of Ca^{2+} into the cytosol

As the level of ER-associated Bcl-2 was decreased following irradiation of CPO-sensitized L1210 cultures, and recent studies have indicated that ER-associated Bcl-2 modulates Ca^{2+} release from the ER, we reasoned that our PDT protocol might cause release of ER Ca^{2+} stores into the cytosol.[18,19,24] This possibility was assessed using the fluorescent probe Calcium Green-1 (Figure 4). Virtually no Calcium Green-1 signal was detected in untreated L1210 cultures (Figure 4a). However, a strong fluorescence signal was detected throughout the cells following exposure to thapsigargin (THP), a cell-permeable agent that facilitates accumulation of cytosolic Ca^{2+} as a consequence of its inhibition of endoplasmic Ca^{2+}-ATPase activity (Figure 4b). RR alone, at a concentration of 5 μM, had little effect on cytosolic Ca^{2+} levels (Figure 4c). At a higher concentration (30 μM), RR clearly elevated cytosolic Ca^{2+} levels (Figure 4d). Ru360 alone (5 μM) slightly elevated the cytosolic Ca^{2+} concentration (Figure 4e). Cytosolic calcium levels were also elevated after irradiation of CPO-sensitized cultures with an LD_{90} PDT dose (Figure 4f). This effect was markedly inhibited by 5 μM RR (Figure 4g), but not by 5 μM Ru360 (Figure 4h). The ROS scavenger Trolox also antagonized the ability of PDT to promote the level of cytosolic Ca^{2+} (Figure 4i). The ER was presumably the source of the cytosolic Ca^{2+} in PDT protocols since a comparable Calcium

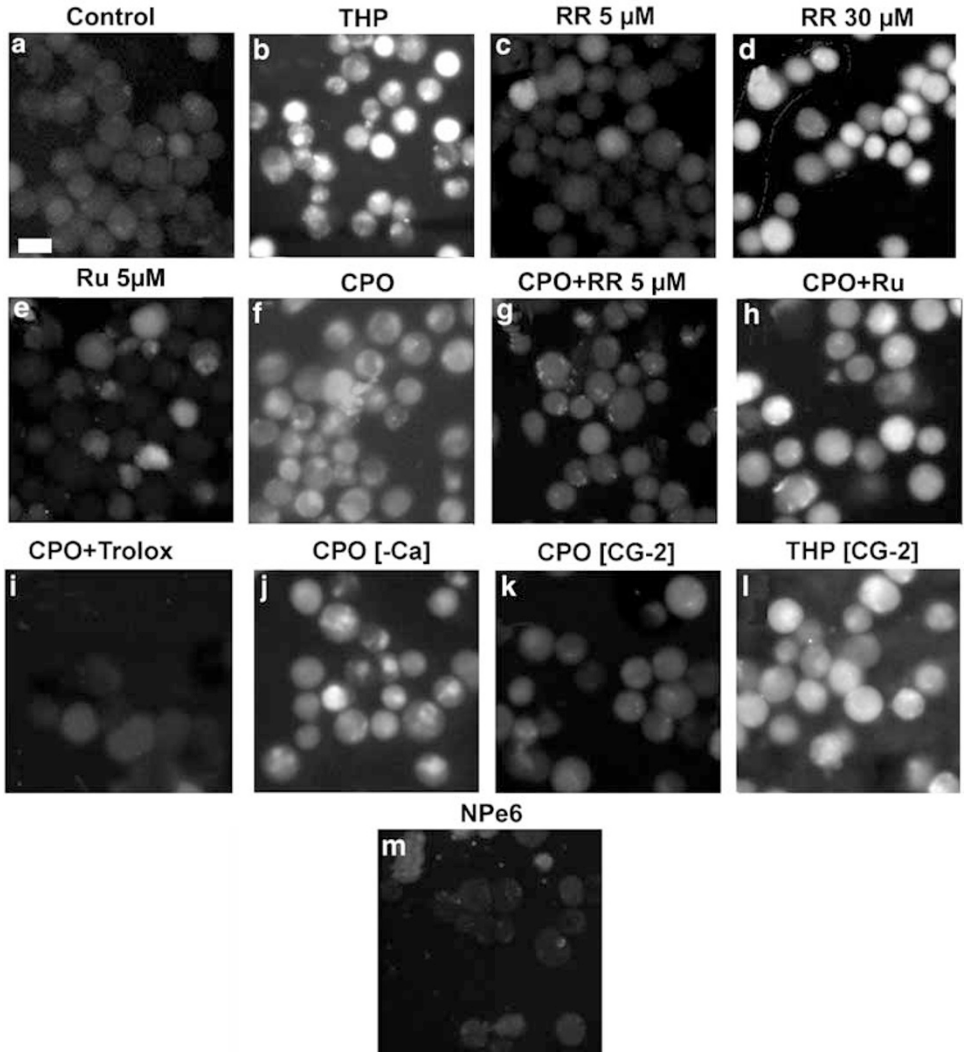

Figure 4 Detection of cytosolic Ca^{2+} with Calcium Green-1 (a–j, m) or Calcium-Green-2 (k, l). Cultures of L1210 cells were treated with nothing, or singularly with THP, RR or Ru360. Other cultures were sensitized with CPO or NPe6 and irradiated either in the absence or presence of RR or Ru360. Treatments were: (**a**) control cells, (**b**) 5 μM THP, (**c**) 5 μM RR, (**d**) 30 μM RR, (**e**) 5 μM Ru360, (**f**) CPO + light, (**g**) CPO + 5 μM RR + light, (**h**) CPO + 5 μM Ru360 + light, (**i**) CPO + 10 mM Trolox + light and (**j**) CPO + light in calcium-free medium. Images shown in panels (**k**) and (**l**) utililized Ca-Green 2 to detect Ca^{2+}: (**k**) CPO + light, (**l**) 3 μM THP and (**m**) NPe6 + light. In all studies involving PDT, the light dose was sufficient to decrease viability by 90%. White bar in panel a = 10μ

Green-1 fluorescence was observed following the irradiation of cells suspended in Ca^{2+}-free medium (compare Figures 4f and j).

The fluorescence intensity of Calcium Green-1 was demonstrably greater after treatment of cells with THP than after the irradiation of CPO-loaded cells (compare Figures 4b and f). Molecular probes has reported that this reagent shows a response to Ca^{2+} levels in the 0.04–1.0 μM range. The less-sensitive probe Calcium-Green 2 can delineate Ca^{2+} between 1 and 9 μM. Analyses with the latter probe indicated that THP stimulated much higher levels of cytosolic Ca^{2+} accumulation than did CPO photodamage (Figures 4k and l).

The sensitizer NPe6 localizes to acidic organelles such as endosomes and lysosomes. Rupture of these organelles using an LD_{90} PDT dose did not perturb cytosolic Ca^{2+} levels (compare Figures 4a and m).

Translocation of Ca^{2+} into mitochondria

Influx of Ca^{2+} into mitochondria was assessed with the fluorescent probe X-Rhod-1-AM (Figure 5). Upon uptake and cleavage of the acetoxy moiety to generate X-Rhod-1, this probe is attracted by the potential difference across the mitochondrial membrane and its fluorescence reflects the level of Ca^{2+} in these organelles.

A very weak X-Rhod-1 fluorescence signal was detected in control L1210 cultures (Figure 5a). The intensity of this signal was not affected by 5 μM RR or 5 μM Ru360 (Figures 5b and d, respectively). Exposure to either a high concentration of RR (30 μM, Figure 5c) or to THP (Figure 5e) dramatically increased X-Rhod-1 fluorescence, which appeared to be quite punctate. As anticipated, cotreatment of THP-exposed cultures with 5 μM RR had no effect upon X-Rhod-1

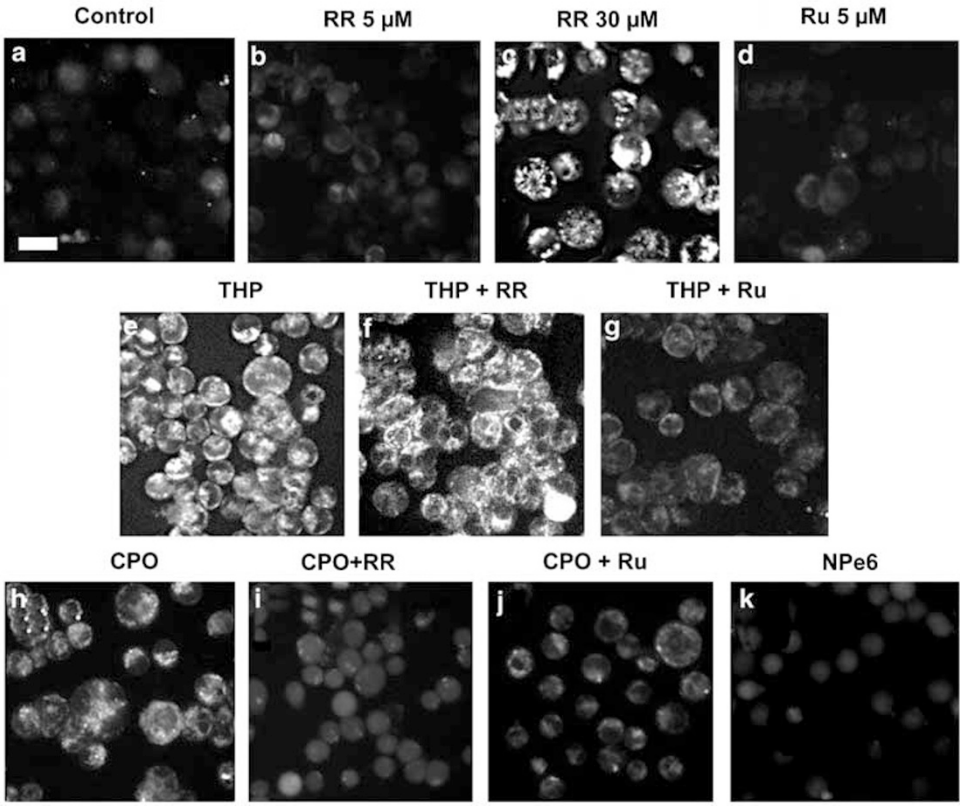

Figure 5 Detection of mitochondrial Ca^{2+} with X-Rhod-1. Cultures of L1210 cells were treated with nothing, or singularly with THP, RR or Ru360. Other cultures were sensitized with CPO or NPe6 and irradiated either in the absence or presence of RR or Ru360. Specific treatments were: (**a**) control cells, (**b**) 5 μM RR, (**c**) 30 μM RR, (**d**) 5 μM Ru360, (**e**) 5 μM THP, (**f**) THP + 5 μM RR, (**g**) THP + 5 μM Ru360, (**h**) CPO + light, (**i**) CPO + light + 5 μM RR, (**j**) CPO + light + 5 μM Ru360 and (**k**) NPe6 + light. In all cases, the light dose was sufficient to decrease viability by 90%. White bar in panel a = 10μ

fluorescence (Figure 5f), while cotreatment with Ru360 suppressed the translocation of Ca^{2+} to the mitochondria (Figure 5g).

Exposure of CPO-sensitized L1210 cultures to an LD_{90} light dose resulted in a slight promotion of mitochondrial Ca^{2+} uptake (Figure 5h). However, the magnitude of Ca^{2+} uptake was considerably less than after exposure to THP. The fluorescence observed after CPO-catalyzed PDT could be suppressed by cotreatment with either 5 μM RR or 5 μM Ru360 (Figures 5i and j, respectively). Irradiation of NPe6-sensitized cultures and the resulting disruption of lysosomes and endosomes did not enhance X-Rhod-1 fluorescence above control levels (Figure 5k). In studies involving PDT or THP, patterns of X-Rhod-1 fluorescence were not altered when cells were suspended in calcium-free medium (not shown).

Photostability of RR and Ru360

Both RR and Ru360 were found to be unstable to irradiation at 535 and 360 nm, respectively (not shown). These results are not significant in the present context since all PDT irradiation protocols involved light at wavelengths >600 nm. However, these results suggested a potential for photosensitivity of RR and/or Ru360. To study the relative sensitivity of Ru360 and RR to oxidation by the singlet oxygen generated during PDT, we used the tin etiopurpurin (SnET2). This photosensitizer absorbs light minimally at the peak absorbance wavelengths of Ru360 (Figure 6a) and RR (Figure 6c). Irradiation of ethanolic solutions containing SnET2 and the ruthenium compounds resulted in the loss of both Ru360 (Figure 6b) and RR (Figure 6d) absorbance. Progressively greater light doses resulted in the progressive loss of absorbance for both compounds. A dose–response curve revealed that RR was substantially more sensitive than Ru360 to oxidation by singlet oxygen (Figure 7).

Suppression of dichlorohydrofluorescein (H_2DCF) oxidation

Excessive amounts of ROS commonly cause oxidative stress. Dichlorodihydrofluorescein diacetate (H_2DCFDA) is a quenched fluorescence probe commonly used to monitor the development of oxidative stress. Although it does not directly interact with ROS, this probe is cooxidized by peroxidases involved in the detoxification of species generated during oxidative stress.[27] Very little fluorescence from 2′,7′-dichlorofluorescein (DCF), the oxidized product of

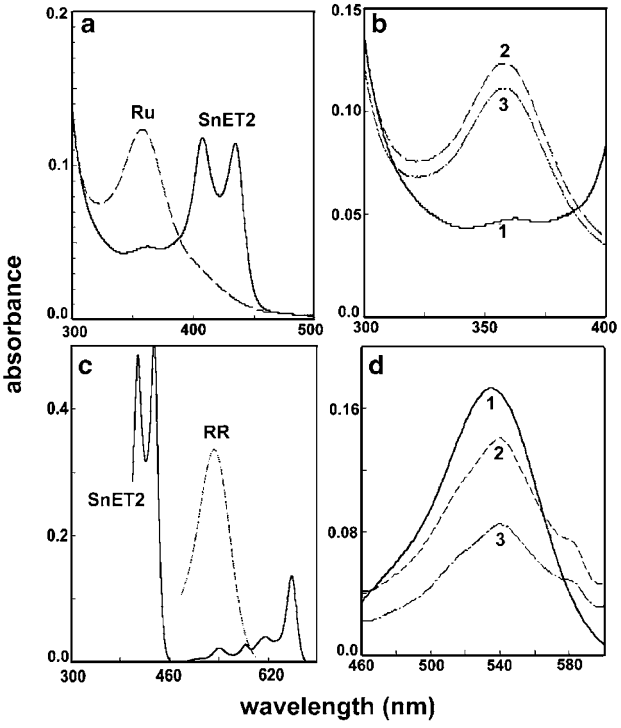

Figure 6 Absorbance spectra showing photooxidation of Ru360 (top) and RR (bottom). Absorbance spectra were collected on solutions of Ru360, RR or SnET2 prior to, and after irradiation. (**a**) SnET2 (solid line) and Ru360 (dashed line). (**b**) 1 = SnET2 alone, 2 = SnET2 + Ru360 and 3 = SnET2 + Ru360 after a light dose of 600 mJ/cm^2; (**c**) SnET2 (solid line), RR (dashed line); and (**d**) 1 = SnET2 + RR, 2 = SnET2 + RR after a light dose of 120 mJ/cm^2 and 3 = SnET2 + RR after a light dose of 360 mJ/cm^2.

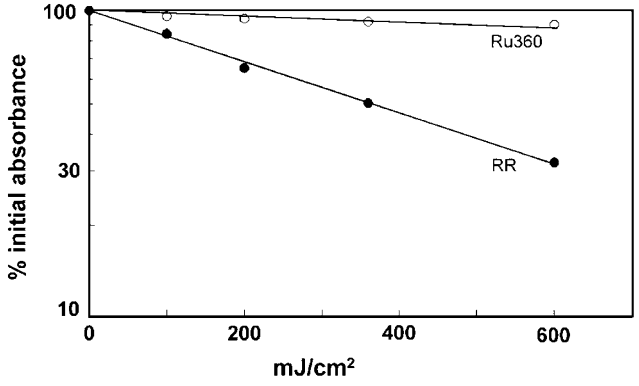

Figure 7 Dose–response curve showing the effects of irradiation, in the presence of SnET2, on the absorbance optima of RR and Ru360.

H_2DCF, was detected in control cells (Figure 8a). However, within minutes of irradiation, a distinct fluorescence could be detected in CPO-sensitized L1210 cultures (Figure 8b).

The appearance of DCF fluorescence was not altered in the presence of 5 μM Ru360 (Figure 8c). In contrast, addition of 5 μM RR strongly suppressed H_2DCF oxidation in irradiated, CPO-sensitized cultures (Figure 8d), as did 10 mM Trolox (Figure 8e). Irradiation of NPe6-sensitized cultures also caused H_2DCF oxidation (Figure 8f). This was not prevented by coincubating NPe6-sensitized cultures with either 5 μM RR or Ru360 prior to irradiation (Figures 8g and h, respectively).

Discussion

Previous investigations of CPO-sensitized L1210 cultures have shown that irradiation causes the loss of Bcl-2 and $\Delta\Psi_m$, and induces cytochrome *c* release and procaspase activation.[3,11,28] We initially attributed the apoptosis occurring in this PDT protocol to a direct effect upon the mitochondria. However, the current study demonstrates that the sensitizer CPO preferentially associates with the ER and, to a lesser extent, with the mitochondria. Subsequent irradiation resulted in the loss of both mitochondrial- and ER-associated Bcl-2, along with slightly increased levels of cytosolic and mitochondrial Ca^{2+}. This Ca^{2+} is presumably derived from the ER since use of calcium-free media did not influence CPO/PDT-induced changes in cytosolic/mitochondrial Ca^{2+} levels.

At least three mechanisms could account for the release of Ca^{2+} after ER photodamage. First, studies with THP demonstrated that suppression of the machinery responsible for Ca^{2+} transport into the ER was paralleled by rapid increases in cytosolic Ca^{2+}. There is precedent for hypothesizing that a similar effect may occur after CPO-induced photodamage: the PDT sensitizer protoporphyrin localizes in the ER and inhibits the ER–Ca^{2+} influx transporting machinery following irradiation.[8] Second, it has been reported that activated Bax stimulates the release of ER Ca^{2+} stores via a mechanism inhibited by Bcl-2.[23,29] Studies with an antibody that only recognizes activated Bax have demonstrated that Bax is activated in L1210 cells following CPO PDT (Kessel, unpublished data). Reduced levels of functional Bcl-2 after PDT may result in an impaired prevention of ER Ca^{2+} release stimulated by activated Bax. Finally, there is the possibility that the cytosolic Ca^{2+} levels seen after irradiation of CPO-sensitized cultures simply reflect ER photodamage and subsequent Ca^{2+} leakage.

Ru360 is a specific and potent inhibitor of the mitochondrial Ca^{2+} uniporter.[30] This reagent is extensively used as a tool to assess the effects of Ca^{2+} influx on mitochondrial function and activation/potentiation of the intrinsic apoptotic pathway.[23,29] In the current study, cotreatment of CPO-sensitized L1210 cultures with Ru360 effectively inhibited the accumulation of mitochondrial Ca^{2+} following irradiation. However, Ru360 cotreatment did not prevent the loss of $\Delta\Psi_m$, activation of DEVDase, or the cytotoxicity of CPO/PDT, as assessed by clonogenic assays. Hence, the influx of ER-derived Ca^{2+} into the mitochondria is not involved in the initiation and development of apoptosis in the CPO PDT model. Indeed, apoptosis occurred in irradiated, CPO-sensitized cultures that were cotreated with the chelator BAPTA. Given our demonstration of losses of both ER and mitochondrial Bcl-2 in the CPO PDT protocol, and the reports that Bcl-2 sequesters/inactivates proapoptotic BH3-only proteins that activate Bak and Bax,[18,19,31] it appears likely that the intrinsic apoptotic pathway is activated in our model system by a mechanism not requiring calcium.

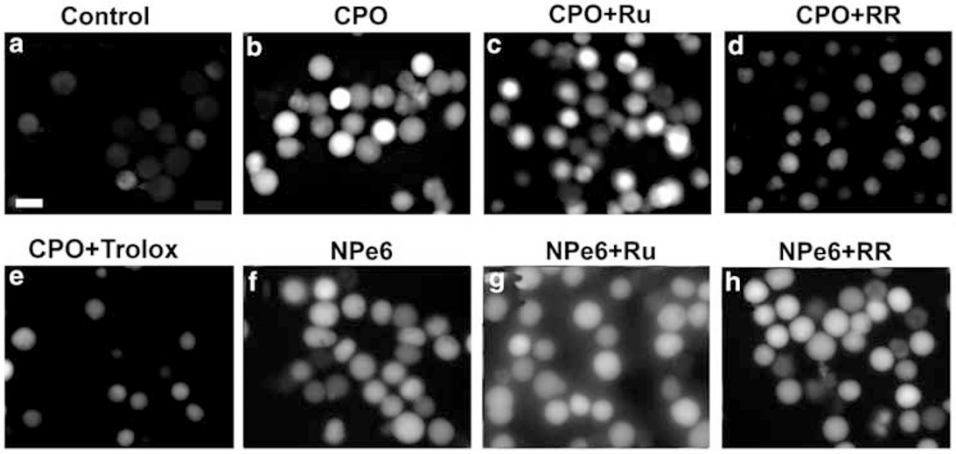

Figure 8 Suppression of PDT-induced H_2DCF oxidation by RR and Ru360. Cultures of L1210 cells were sensitized with 2 μM CPO for 30 min, or 66 μM NPe6 for 1 h, in either the presence or absence of 5 μM RR or Ru360 prior to being washed and irradiated (135 mJ/cm^2 for CPO and 450 mJ/cm^2 for NPe6) at 10°C. Cultures were subsequently loaded with H_2DCFDA and incubated at 37°C for 10 min prior to being imaged. Panels are: (**a**) no treatment, (**b**) CPO + light, (**c**) CPO + Ru360 + light, (**d**) CPO + RR + light, (**e**) CPO + Trolox + light, (**f**) NPe6 + light, (**g**) NPe6 + Ru360 + light and (**h**) NPe6 + RR + light. White bar in panel a = 10μ

The ruthenium–complex RR exhibits several activities that affect Ca^{2+} uptake and release.[32–34] One such activity is its ability to inhibit the mitochondrial calcium uniporter. However, it is not widely appreciated that the 'active' component of RR responsible for this effect is actually Ru360. This product commonly contaminates commercial RR preparations.[35] We screened several commercial preparations of RR before we identified a preparation that was devoid of Ru360. As expected, this product did not prevent mitochondrial Ca^{2+} influx following THP treatment or irradiation of CPO-sensitized cultures. However, cotreatment of CPO-sensitized cultures with 5 μM RR completely inhibited procaspase activation and the cell killing that occurred following irradiation. In particular, PDT-induced loss of Bcl-2, increased cytosolic/mitochondrial Ca^{2+} levels, loss of $\Delta\Psi_m$ and oxidative stress were all suppressed by the presence of RR.

Singlet oxygen is generated following photoactivation of PDT sensitizers, and is responsible for initiating the damage that occurs in PDT protocols. The ROS scavenger Trolox was previously shown to suppress the apoptotic program initiated by PDT.[36,37] Studies presented in Figures 6 and 7 clearly indicate that RR is highly susceptible to oxidation by singlet oxygen, while Ru360 is substantially more resistant. RR can therefore function as a quencher of this ROS species. This property provides a possible mechanism for the global protective effects of RR in the CPO PDT protocol. To our knowledge, this is the first study to identify this property of RR. Other investigators have demonstrated that RR suppresses oxidative injury via a mechanism independent of mitochondrial Ca^{2+} accumulation,[38,39] and that RR can redox cycle and promote the decomposition of H_2O_2 to H_2O and O_2 in an *in vitro* model.[40] This latter study, coupled with our current findings, clearly demonstrate that RR has antioxidant properties.

In the current studies, the antiapoptotic activity of RR was concentration dependent; whereas 5 μM RR was antiapoptotic, a 30 μM level of RR was proapoptotic, even in the dark. While the basis for this concentration-dependent effect is not known, Meinicke *et al*. have reported that RR, in the presence of an electron donor, can substitute for iron in Fenton-type reactions, and contribute to the generation of toxic ROS.[41]

The photosensitizer NPe6 localizes to lysosomes and causes lysosomal damage and leakage following irradiation.[9] Several laboratories have demonstrated that extracts derived from isolated lysosomes are capable of activating Bid by proteolytic cleavage,[9,42,43] and we have demonstrated that Bid cleavage precedes/accompanies cytochrome *c* release/procaspase-9 activation in NPe6-sensitized, irradiated 1c1c7 cultures.[9] Furthermore, unlike the photodynamic effects of CPO, NPe6 does not catalyze loss of Bcl-2, Ca^{2+} release from the ER or loss of $\Delta\Psi$m.[9,11] Hence, the mechanisms by which NPe6 and CPO PDT protocols activate the intrinsic apoptotic pathway are markedly different. Nevertheless, both sensitizers generate singlet oxygen, and cause oxidative stress (as assessed by measurements of H_2DCF oxidation) following irradiation. However, pretreatment of cultures with RR suppressed only the induction of apoptosis in CPO PDT protocols. As singlet oxygen is highly reactive, it interacts with only molecules in close proximity to sites of formation. Presumably, the differential protective properties of RR in NPe6 and CPO PDT protocols reflect either an inability of RR to cross lysosomal/endosomal membranes, or a modification of the chemistry of RR due to the acid environment of these organelles.

In summary, the current study demonstrates that irradiation of CPO-sensitized cells results in loss of both ER- and mitochondrial-associated Bcl-2, and initiates the intrinsic apoptotic pathway. Although cytosolic Ca^{2+} levels were elevated after photodamage, the magnitude of this effect does not appear to be sufficient to evoke apoptosis since neither cotreatment with the Ca^{2+} chelator BAPTA nor inhibition of the mitochondrial uniporter by Ru360 affected the outcome. The apoptotic response to PDT in our model could be prevented by cotreatment with RR. We attribute the protective properties of RR to its ability to quench ROS at their site of

generation, and not its functioning as an inhibitor of the mitochondrial Ca^{2+} uniporter.

Materials and Methods

Chemicals

The porphycene CPO was obtained from Dr Alex Cross, CytoPharm, San Francisco, CA, USA. CPO was dissolved in dimethylformamide to yield a 1 mM solution. SnET2 was provided by Dr Allan Morgan (University of Louisville, KY, USA) and dissolved in dimethylformamide. NPe6 was provided by Dr Kevin Smith (Louisiana State University, Baton Rouge, LA, USA) and dissolved in water. DEVD-R110, a fluorogenic substrate for caspase-3, was provided by Molecular Probes (Eugene, OR, USA), as were the fluorescent probes H_2DCFDA, NAO, Calcium Green-1, LTB, ERTr and X-Rhod-1, and the chelator BAPTA-AM. RR and Trolox were obtained from Sigma-Aldridge, St. Louis, MD, USA, and Ru360 from Calbiochem, San Diego, CA, USA. Several commercial preparations of RR were analyzed for contamination with Ru360 before a sample from Sigma-Aldridge was identified that showed essentially no absorbance at 360 nm, indicating minimal contamination with Ru360. This preparation of RR was used in all studies described here. THP was purchased from Calbiochem.

Cells

Murine L1210 cells were maintained in suspension culture using a medium closely approximating the composition of Fisher's growth medium, a product no longer commercially available. To achieve this formulation, we supplemented α-MEM (GIBCO BRL, Grand Island, NY, USA) with $MgCl_2$ (45 mg/l), methionine (75 mg/l), phenylalanine (30 mg/l), valine (30 mg/l) and folic acid (9 mg/l). Additional components were 10% horse serum, 1 mM glutathione, 1 mM mercaptoethanol and gentamicin. Murine hepatoma Hepa 1c1c7 cells were obtained from Dr JP Whitlock Jr., Stanford University, CA, USA. They were grown in α-MEM supplemented with 5% fetal calf serum and antibiotics. All cell lines were grown in a 5% CO_2 atmosphere, at 37°C. L1210 viability after treatment was assessed by clonogenic assays. This involved serial dilution of cell suspensions, followed by plating on soft agar. After 7–9 days growth in a humidified chamber under 5% CO_2, colonies were counted and compared with untreated control cultures. All such experiments were carried out in triplicate.

Fluorescence microscopy

Fluorescence images were acquired using a SenSys CCD camera (Photometrics, provided by Roper Scientific, Tucson AZ, USA), MetaMorph software (Universal Imaging, Downingtown, PA, USA) and a Nikon microscope fitted with a Uniblitz shutter (Vincent Associates, Rochester, NY, USA). All irradiation studies were carried out on a stage thermoelectrically cooled to 10°C.

Excitation and emission spectra used in fluorescence microscopy were chosen to correspond with the photophysical properties of the probes. In all cases, interference filters were inserted in the emission beam to further limit extraneous fluorescence as described previously.[3,28] LTB, NAO and ERTr were used as fluorescent markers for lysosomes, mitochondria and the ER, respectively. In colocalization protocols, L1210 cultures were incubated with CPO (2 μM) and LTB (2 μM), or NAO (2 μM), or ERTr (3 μM) for 30 min at 37°C, and then washed once with fresh medium before being imaged. The excitation/emission optimum wavelengths used for CPO, LTB, NAO and ERTr were 400 nm/620 nm, 380 nm/420 nm, 500 nm/520 nm, and 380 nm/575 nm, respectively. For the CPO colocalization studies, MetaMorph software was used to overlay the fluorescence images. In order to minimize photo bleaching, the Uniblitz shutter was configured to open and close with the camera shutter, thereby limiting exposure of the samples to exciting light for <1 s. Tetramethylrhodamine methyl ester (TMRM) was used to assess $\Delta\Psi_m$. Cultures were loaded with 5 μM TMRM $\sim$10 min prior to imaging using 510–560 nm excitation and 590–650 nm emission. Calcium Green-1 was used to assess relative Ca^{2+} levels in the cytosol. This probe is sensitive to Ca^{2+} concentrations in the range of 0.4–1.5 μM ($K_d = 0.19$ μM). The probe Calcium Green-2 is less sensitive, and can probe for Ca^{2+} concentrations from 0.6 to 9 μM ($K_d = 0.55$ μM). The cationic probe X-Rhod-1-AM was used to detect mitochondrial Ca^{2+}. This acetoxymethyl ester penetrates cells where it is de-esterified to generate the fluorophore X-Rhod-1, which has a Ca^{2+} dissociation constant of 700 nM (Molecular Probes bulletin MP 01244).

L1210 cultures were incubated for 30 min at 37°C with either 5 μM Calcium Green-1/2 or 2.5 μM X-Rhod-1-AM and, as specified, a photosensitizer and/or ruthenium salt. In some experiments, a 10 μM concentration of the cell-permeant calcium chelator BAPTA-AM was added after 15 min. The cells were subsequently washed, resuspended in fresh medium and irradiated at 10°C if so indicated, and then incubated at room temperature for an additional 30 min prior to imaging. The excitation/emission wavelengths for Calcium Green-1/2 and X-Rhod-1 imaging were 490 nm/530 nm and 580 nm/605 nm, respectively.

PDT protocols

Unless specified, all PDT studies employed CPO as the sensitizer. In these studies, L1210 cells were incubated in FHS (Fischer's medium lacking amino acids and vitamins, supplemented with 10% horse serum, with 20 mM HEPES replacing $NaHCO_3$ for increased buffering capacity) at a density of 7 mg/ml (wet weight). For PDT studies, cells were treated with 2 μM CPO for 30 min at 37°C, and then washed and resuspended in fresh FHS at 10°C prior to irradiation. In order to examine the contribution of extracellular Ca^{2+} to CPO-induced apoptosis, a limited number of studies employed FHS prepared without $CaCl_2$ that had been passed through a column of Chelex (Biorad, 5 g/100 ml).

Cells were irradiated, usually at a light flux of 1.5 mW/cm^2, at 10°C for 2 min (180 mJ/cm^2). This corresponds to an LD_{90} PDT dose, as determined by clonogenic assays. The light source was a 700-W quartz-halogen lamp filtered with 10 cm of water to remove excess infrared radiation, with the transmission wavelength confined to 630 ± 10 nm with an interference filter (Oriel, Stratford, CT, USA). The cells were then collected and viability assessed as described above. For studies involving lysosomal photodamage, we used both murine hepatoma 1c1c7 and L1210 cells sensitized with 66 μM NPe6 for 45 min prior to 660 ± 10 nm irradiation. Both LD_{50} and LD_{95} PDT doses were used. Unless otherwise stated, RR, Ru360 or Trolox were added with sensitizer in PDT protocols, and used at a concentration of 5 μM (RR, Ru360) or 10 mM (Trolox).

Photostability of RR and Ru360

To assess the stability of these reagents to singlet oxygen, 5 μM solutions in 75% ethanol in the presence of 2 μM SnET2 were irradiated with varying light doses at 660 ± 10 nm. Absorption spectra were acquired for the photosensitizer and the ruthenium compounds, and for mixtures as a function of the light dose.

DEVDase assays

Activation of DEVDase after irradiation was assessed using the fluorogenic substrate DEVD-R110 as described previously.[44] Cells were incubated at 37°C for either 10 min (L1210) or 2, 4 or 6 h (1c1c7) after irradiation, prior to being harvested for subsequent analyses.

Bcl-2 Western blots

The procedure used for the Western blot detection of Bcl-2 in L1210 extracts, and mitochondrial and ER preparations has been described in detail.[44] Comparable amounts of protein (40 μg) were loaded onto each lane of the gels.

Isolation of ER and mitochondria

Procedures described by Gottlieb and Adachi[45] and Annis *et al*.[46] were modified and used for the rupture of cells by nitrogen cavitation and isolation of ER and mitochondria. L1210 cells (400 mg) were washed in a salt + glucose solution (100 ml contains 762 mg NaCl, 600 mg HEPES, 40 mg KCl, 9.7 mg $MgSO_4$, 20 mg $CaCl_2$, 100 mg glucose), and then resuspended in lysis buffer (250 mM sucrose, 2 mM $MgCl_2$, 20 mM HEPES, 1 mM EDTA, 1 mM PMSF, 1 mM DTT and 5 $\times$ protease inhibitor cocktail). They were then placed in a Parr nitrogen bomb. Nitrogen was introduced to a pressure of 175 psi, and the cells were stirred for 15 min at 4°C prior to releasing the pressure. Lysates were centrifuged at 100 $\times$ *g* for 10 min to remove unbroken cells and nuclei. The supernatant fraction was centrifuged at 1000 $\times$ *g* for 30 min to pellet the mitochondria. The resulting supernatant fluid was centrifuged at 100 000 $\times$ *g* for 1 h at 4°C to pellet the ER fraction. Mitochondrial and ER preparations were analyzed for cross contamination by Western blotting for the ER marker calreticulin and the mitochondrial marker cytochrome *c*. By such analyses, organelle fractions were judged to be $>$95% free of cross contamination.

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