4-Hydroxymethyl-1,6,8-trimethylfuro[2,3-*h*]quinolin-2(1*H*)-one Induces Mitochondrial Dysfunction and Apoptosis upon Its Intracellular Oxidation

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We investigated the mechanism of cell death induced by a furoquinolinone derivative, namely, 4-hydroxymethyl-1,6,8-trimethylfuro[2,3-*h*]quinolin-2(1*H*)-one (HOFQ), in the dark. Mitochondrial depolarization was found to be a causative event in HOFQ-induced apoptosis that was blunted either by replacing the 4-hydroxymethyl group with a methyl one, or by 4-methylpyrazole, an inhibitor of alcohol dehydrogenase (ADH). In vitro enzymatic assay demonstrated that HOFQ is a substrate of ADH. In isolated mitochondria HOFQ was without effect, whereas in the presence of ADH and NAD^+ it caused the opening of the permeability transition pore, indicating that HOFQ-oxidized products affect mitochondrial function directly. Finally, an analogue bearing the formyl group at the C-4 position mimicked all the effects exerted by HOFQ. In conclusion, these results suggest that the direct action on mitochondria of HOFQ-oxidized products are responsible for their cytotoxicity, which might be exacerbated, but hardly determined, by photodynamic action and/or binding to DNA.

Introduction

In recent years great efforts have been made to search for new antitumor compounds. While searching for new photochemotherapeutic agents with increased antiproliferative activity and decreased undesired toxic effects, as skin phototoxicity and mutagenesis, a new family of molecules was synthesized, namely, the furoquinolinones.[1] Among these molecules 1,4,6,8-tetramethylfuro[2,3-*h*]quinolin-2(1*H*)-one (FQ) is the most investigated compound (Figure 1). Interestingly, some furoquinolinones and other psoralen analogues were found to exert a biological activity not only after UVA irradiation but also in the dark, thus suggesting additional or different mechanisms of action for these compounds.[2,3]

The effects obtained in the absence of irradiation could result from the interaction of a given molecule in its native form with intracellular structures or from its transformation operated by intracellular metabolic processes. The toxicity of the photosensitizers is generally not referred to the formation of intracellular metabolites excluding, obviously, those produced by photodynamic effect. On the other hand, it is well established that the toxicity of several compounds is exerted upon their intracellular activation. In most cases, these reactions are catalyzed by members of the cytochrome P450 family.[4] Another enzyme involved in this transformation is alcohol dehydrogenase (ADH).[5] Examples are given by the toxicity exerted by a wide array of alcohol compounds which is blunted by ADH inhibition. This concept supports the therapeutic use of 4-methylpyrazole (4-MP), an ADH inhibitor, in ethylene glycol and methanol poisoning.[6,7]

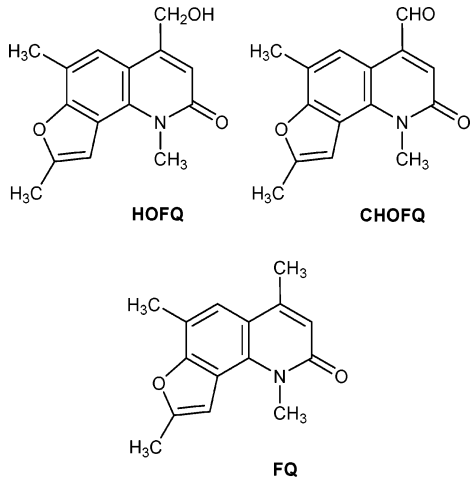

Figure 1. Structures of 4-hydroxymethyl-1,6,8-trimethylfuro-[2,3-*h*]quinolin-2(1*H*)-one (HOFQ), 4-formyl-1,6,8-trimethylfuro[2,3-*h*]quinolin-2(1*H*)-one (CHOFQ), and 1,4,6,8-tetramethylfuro[2,3-*h*]quinolin-2(1*H*)-one (FQ).

To test the possibility that the toxicity of a photoactive compound is exerted upon intracellular activation, we examined a furoquinolinone derivative, namely, 4-hydroxymethyl-1,6,8-trimethylfuro[2,3-*h*]quinolin-2(1*H*)-one (HOFQ) (Figure 1), bearing a hydroxymethyl group at the 4 position. This compound has been reported to exert antiproliferative activity both in the presence and in the absence of light.[3] We hypothesized that its activity might depend on its oxidation catalyzed by intracellular enzymes. Here, we demonstrated that HOFQ induces apoptosis by causing mitochondrial dysfunction. The inhibition of ADH prevented the occurrence of apoptosis and the dysfunction of mitochondria both in intact cells and isolated organelles. Finally, an oxidation product of HOFQ, namely, 4-formyl-1,6,8-trimethylfuro[2,3-*h*]-quinolin-2(1*H*)-one (CHOFQ) (Figure 1), obtained by

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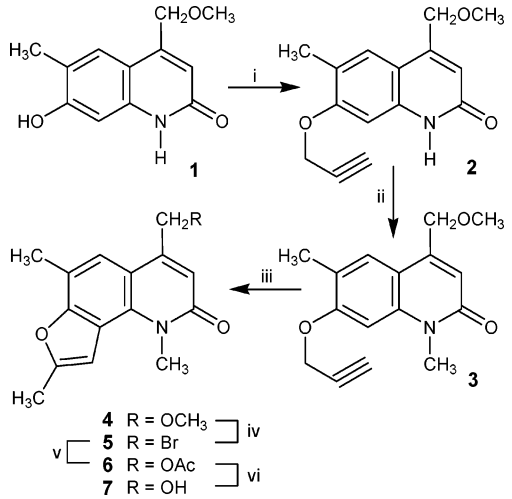
Scheme 1[a]



[a] Reagents and conditions: (i) propargyl bromide, acetone, K_2CO_3, reflux, 47%; (ii) Me_2SO_4, acetone, K_2CO_3, reflux, 42%; (iii) *N*,*N*-diethylaniline, CsF, 210 °C, 86%; (iv) HBr, AcOH, reflux, 55%; (v) Ac_2O, AcONa, reflux, 86%; (vi) KOH, MeOH, reflux, 76%.

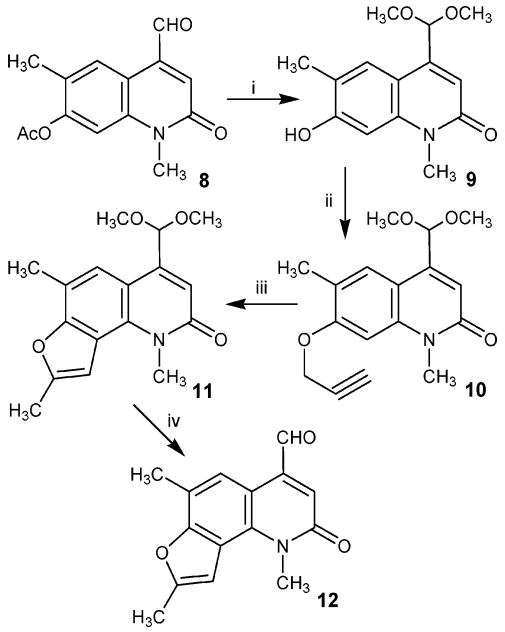
replacing the hydroxymethyl group with the formyl moiety, mimics entirely the apoptogenic potency of HOFQ.

Chemistry

The title compound was synthesized by a new and more convenient synthetic route which allows obtaining the key intermediate 4-methoxymethyl-1,6,8-trimethylfuro[2,3-*h*]quinolin-2(1*H*)-one (**4**) in good yield, as outlined in the Scheme 1. The starting product was the 7-hydroxy-4-methoxymethyl-6-methylquinolin-2(1*H*)-one[8] (**1**), which was condensed with propargyl bromide, giving 4-methoxymethyl-6-methyl-7-propargyloxyquinolin-2(1*H*)-one (**2**). The 7-*O*-propargyl ether **2** was methylated with dimethyl sulfate to give 4-methoxymethyl-1,6-dimethyl-7-propargyloxyquinolin-2(1*H*)-one (**3**) together with a byproduct (probably the 2-*O*-methyl derivative, in analogy with previous synthetic experiments on similar compounds[1,3]), not isolated. The *N*-methyl derivative **3** was submitted to cyclization in the presence of CsF,[9] affording 4-methoxymethyl-1,6,8-trimethylfuro[2,3-*h*]quinolin-2(1*H*)-one (**4**). As already described,[3,8] intermediate **4** was reacted with hydrobromic acid, obtaining the 4-bromomethyl analogue **5**, which by treatment with acetic anhydride gave the 4-acetoxymethyl derivative **6**, then hydrolyzed in alkaline conditions to give the desired 4-hydroxymethyl-6,8-dimethylfuro[2,3-*h*]quinolin-2(1*H*)-one (HOFQ, **7**). Using this new synthetic pathway the yield for the common intermediate **4** was much higher (17%) than that previously reported (6%).

The furoquinolinone bearing a formyl group in the 4 position was prepared as described in the Scheme 2. In the starting 4-formyl-7-acetoxy-1,6-dimethylquinolin-2(1*H*)-one[10] (**8**) the formyl group was protected by reaction with trimethyl orthoformate to give 4-dimethoxymethyl-7-hydroxy-1,6-dimethylquinolin-2(1*H*)-one (**9**): in this reaction hydrolysis of the 7-acetoxy group also occurred. Compound **9** was condensed with propargyl bromide, giving the 7-*O*-propargyl ether **10**, which was submitted to cyclization in the presence of CsF,[9] affording 4-dimethoxymethyl-1,6,8-trimethylfuro[2,3-*h*]quinolin-2(1*H*)-one (**11**). This compound was deprotected in acid conditions to give the desired 4-formyl-1,6,8-trimethylfuro[2,3-*h*]quinolin-2(1*H*)-one (**12**).

Scheme 2[a]



[a] Reagents and conditions: (i) trimethyl orthoformate, *p*-toluenesulfonic acid, MeOH, reflux, 63%; (ii) propargyl bromide, acetone, K_2CO_3, reflux, 81%; (iii) *N*,*N*-diethylaniline, CsF, 210 °C, 61%; (iv) HCl, dioxane, room temperature, 89%.

Biology

The initial aim of this study was to determine whether the furoquinolinone derivative HOFQ caused cell death in Jurkat cells in the dark and investigate the underlying mechanism(s). Figure 2A shows that apoptosis was by far the prevailing form of cell death, while necrosis was negligible. The assay was performed by evaluating chromatin condensation with Hoechst 33258 as a sign of apoptosis and nuclear staining with propidium iodide (PI) as a marker of necrosis (as shown in Figure 2B).

As it is widely accepted that the caspases play a key role in the execution of the programmed cell death, we incubated Jurkat cells exposed to HOFQ with a broad caspase inhibitor, namely, Z-VAD-fmk. Apoptosis was completely prevented when Z-VAD-fmk was added to the cells prior to HOFQ incubation, as reported in Figure 3.

This result highlights the key role of caspase activation in HOFQ-induced cell death in the execution of the programmed cell death induced by HOFQ.

To investigate the mechanism(s) activated by HOFQ and responsible for apoptosis, we focused our attention on mitochondria, since the dysfunction of these organelles is considered to be a crucial step in the commitment of the cell to apoptosis.[11]

To assess the changes in mitochondrial function, Jurkat cells were loaded with tetramethylrhodamine methyl ester (TMRM), a lipophilic cation that accumulates within polarized mitochondria and is commonly used for assessment of the mitochondrial membrane potential ($\Delta\psi_m$).[12] To exclude artifacts due to the different loading capacity of the various cells which can

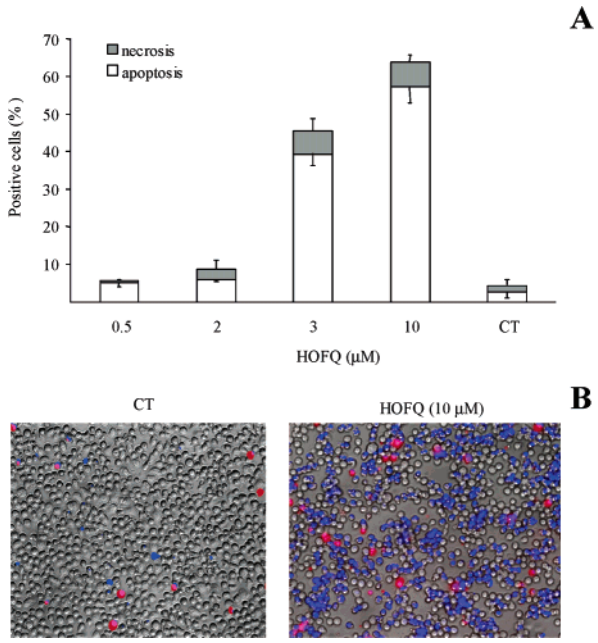


Figure 2. Loss of cell viability induced by HOFQ. (A) Jurkat cells (10^6 mL^{-1}) were incubated for 16 h at 37 °C in the absence (control, CT) or presence of increasing concentration of HOFQ (0.5−10 μM). Necrosis was assessed by the staining of nuclei with PI, whereas apoptosis was visualized by the appropriate changes of nuclei stained with Hoechst 33258. Values are mean ± SD of at least three experiments and are normalized as described in the Experimental Section. (B) The effect of HOFQ (10 μM) is visualized by the blue staining with Hoechst 33258 and the red staining with PI (and it is compared to the control, CT).

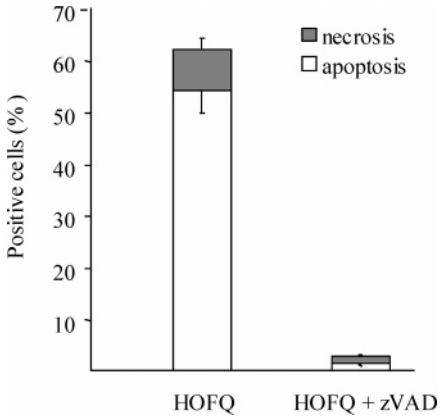


Figure 3. HOFQ-induced apoptosis was blocked by Z-VAD-fmk. Jurkat cells (10^6 mL^{-1}) were incubated for 16 h at 37 °C with HOFQ (10 μM) in the absence (HOFQ) or presence (HOFQ + Z-VAD) of Z-VAD-fmk (50 μM), a broad caspase inhibitor. Necrosis and apoptosis were assessed as described in Figure 2.

be erroneously interpreted as $\Delta\psi_m$ differences, after each treatment the cells were added with an uncoupler of oxidative phosphorylation, namely, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP), which abolishes $\Delta\psi_m$[13] as shown in Figure 4**A.** Thus, in each cell the difference of fluorescence intensities obtained before and after FCCP provided a reliable assessment of $\Delta\psi_m$. At 2 h after cell incubation with HOFQ a significant decrease of $\Delta\psi_m$ could be already detected, as documented in Figure 4B. Of note, the chromatin condensation used as apoptotic marker was not detectable after 2 h of incubation with HOFQ (not shown). This result documented that mitochondrial depolarization was a causative event in HOFQ-induced apoptosis.

To evaluate whether HOFQ directly exerted its activity on mitochondria, we performed the swelling assay in the isolated organelle, commonly used to assess the opening of the permeability transition pore (PTP).[14] Surprisingly, the molecule was found to be completely inactive (as shown in Figure 5A), and this result suggested that HOFQ required a metabolic transformation, namely, oxidation. Our working hypothesis was that the hydroxymethyl group at the 4 position of the furoquinolinone nucleus in HOFQ could be oxidized. To this aim we incubated isolated mitochondria with HOFQ in the presence of ADH and NAD^+. Figure 5B shows a dramatic swelling, thus indicating that HOFQ oxidation was required to induce the opening of PTP. In parallel experiments isolated mitochondria were incubated with the furoquinolinone analogue FQ (Figure 1), which bears a methyl group at the 4 position, in the presence of ADH and NAD^+. No effect was detectable as shown in Figure 5B, thus suggesting that the hydroxymethyl group at the 4 position of the furocoumarin nucleus was the target of oxidation by ADH.

Figure 6 illustrates the in vitro enzymatic assays performed to analyze whether HOFQ was a substrate of ADH. Indeed, HOFQ, but not FQ, was oxidized by the purified enzyme as shown by NADH(H^+) formation. HOFQ oxidation was prevented by 4-MP, an ADH inhibitor.

On the basis of the evidence collected from in vitro experiments, we investigated whether HOFQ oxidation is required for its apoptotic effect by using three different approaches. First, FQ was much less apoptogenic compared to HOFQ, thus highlighting the relevance of the hydroxymethyl group (Figure 7).

Second, we evaluated whether HOFQ required an intracellular metabolic activation before exerting its apoptotic potency. To this aim we incubated Jurkat cells with the ADH inhibitor 4-MP before HOFQ addition to the medium. The results shown in Figure 8A demonstrated that 4-MP completely prevented the loss of cell viability induced by HOFQ. 4-MP was unable to prevent staurosporine-induced apoptosis, thus indicating that this molecule is not an aspecific antiapoptotic agent (Figure 8B). Of note, staurosporine is devoid of oxidizable hydroxyl moieties.

Third, with the aim of directly assessing the apoptotic potency of the putative oxidative product of HOFQ, we carried out the synthesis of 4-formyl-1,6,8-trimethylfuro-[2,3-*h*]quinolin-2(1*H*)-one (CHOFQ), a furoquinolinone bearing a formyl group at the 4 position of the furoquinolinone nucleus. Figure 9A illustrated that CHOFQ was a molecule highly apoptogenic, similar to HOFQ. Furthermore, CHOFQ caused the opening of the PTP (Figure 9B) in isolated mitochondria and caused mitochondrial depolarization in intact cells (Figure 9C). Figure 9B also showed that cyclosporin A (CsA), a specific inhibitor of the PTP, completely prevented the PTP opening.

Conclusions

The present results demonstrated that mitochondria play a key role in HOFQ-induced cell death and that the ADH-catalyzed oxidation of HOFQ is necessary for its cytotoxic effect in the dark.

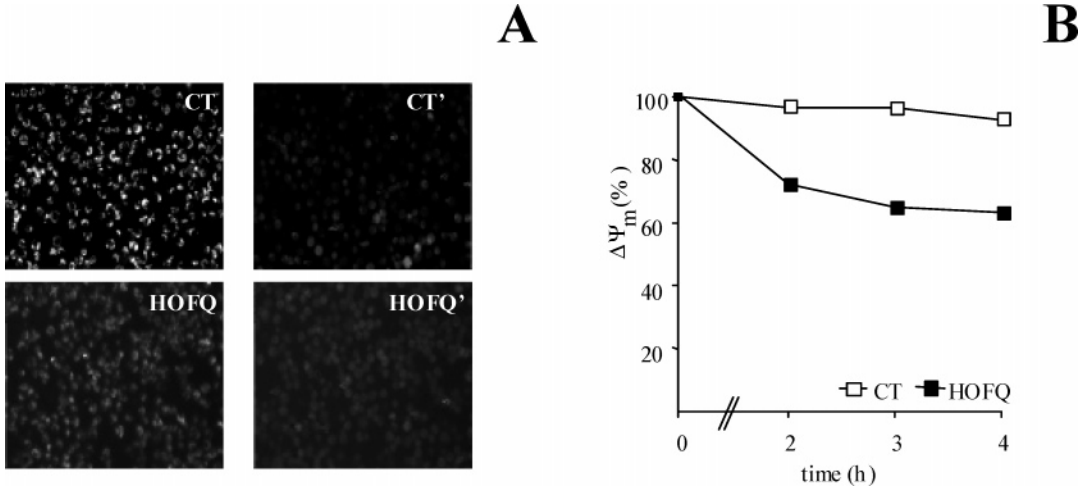


Figure 4. $\Delta\psi_m$ decrease induced by HOFQ. Jurkat cells (10^6 mL^{-1}) were incubated for increasing time (0−4 h) in the absence (CT) or presence of 10 μM HOFQ. After incubation, cells were stained with 25 nM TMRM and 1.6 μM cyclosporin H. The images were acquired before (CT, HOFQ) and after (CT′, HOFQ′) addition of 2 μM FCCP (A). The differences between the fluorescence values of each cell before and after the uncoupler (rather than the absolute intensity before FCCP addition) reflect the actual magnitude of $\Delta\psi_m$. (B) Time course of mitochondrial depolarization. $\Delta\psi_m$ values were normalized as described in the Experimental Section.

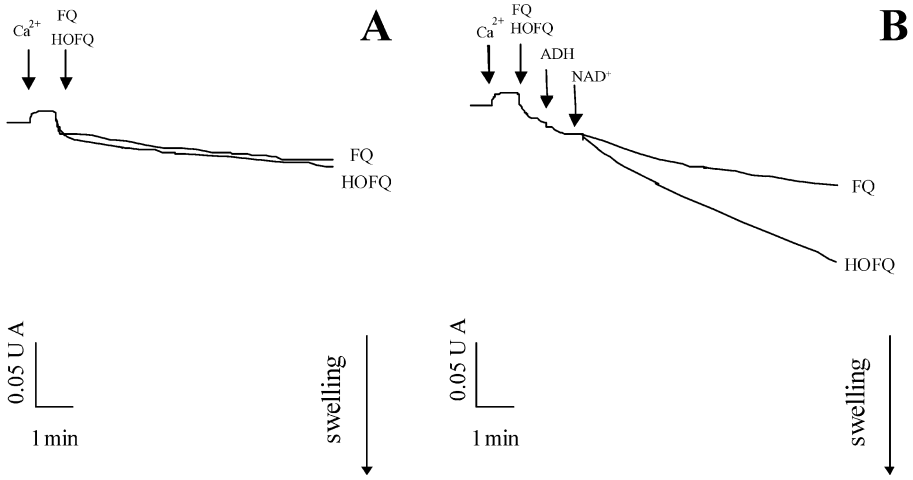


Figure 5. ADH-catalyzed HOFQ oxidation is required for PTP opening in isolated mitochondria. Mitochondrial swelling was monitored as the decrease in light scattering at 540 nm. Rat liver mitochondria (0.5 mg·mL^{-1}) were incubated with an equimolar concentration of HOFQ or FQ (10 μM) in the absence (A) or presence (B) of 300 U·mL^{-1} ADH and 0.1 mM NAD^+. PTP opening was induced by HOFQ only in the presence of ADH, indicating its oxidation as a prerequisite for affecting mitochondrial function as shown in B.

Previous studies showed that a few furoquinolinone compounds exert antiproliferative effects in the dark.[15,16] In addition, insertion of a hydroxymethyl group at the 4 position has been shown to be relevant for the antiproliferative effects of several compounds.[2] These findings prompted us to hypothesize that the biological activity of HOFQ might not depend on the native molecules but could require the formation of metabolite(s) by means of oxidative bioactivation. From a theoretical standpoint, this hypothesis was supported by previous reports showing that various alcohols require metabolic activation to cause cell death. Relevant examples are perillyl alcohol[17] and allyl alcohol.[18] Moreover, acetaldehyde was found to account for ethanol cytotoxicity.[19] Common to these studies is the demonstration that the cellular effects are completely prevented by 4-MP, an inhibitor of alcohol dehydrogenase.[20] Notably, the active metabolite of the different alcohols was identified as the aldehyde derivative, namely, perillaldehyde and acrolein in the case of perillyl and allyl alcohol, respectively. In this respect, the Michael addition involving nonsaturated α,β-aldehydes appears to be especially important.[21] However, the reactions with intracellular components and the targets responsible for cytotoxicity have not been elucidated yet. Of note, the effects on a given cell of aldehydes or oxidizable alcohols are not easily predictable. In fact, not all the aldehydes are cytototoxic since for some of them a protective effect has been reported.[22] Therefore, the present results elucidating the determining role of PTP in causing mitochondrial dysfunction and cell death appear to be especially relevant, providing an intracellular target to the oxidative products of HOFQ. The present findings support the established role of mitochondrial dysfunction, especially of PTP, in the apoptotic cascade.[11] Previous work of our laboratory showed that this mechanism underlies the cytotoxic effects by PUVA, namely, the combination of psoralen addition and UVA irradiation.[13] In that case, we have shown that apoptosis could be also induced in the dark

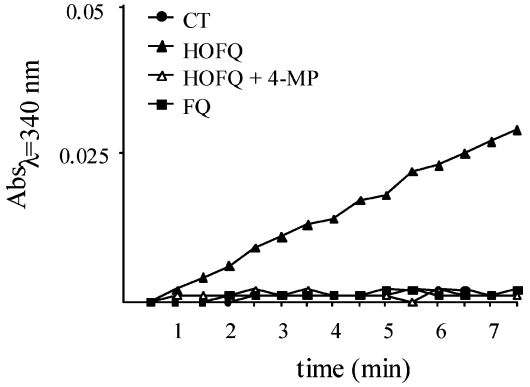


Figure 6. HOFQ is a substrate for ADH. The activity of 300 $U \cdot mL^{-1}$ of ADH was assessed as the formation of $NADH(H^+)$ in a solution containing 0.1 M NaH_2PO_4 (pH 8.8), 0.9 M semicarbazide, and 0.1 mM NAD^+. The reactions were started by addition of HOFQ (10 μM, ▲), FQ (10 μM, ■), or an equal volume amount of the solvent (DMSO) as a control, CT (●). When ADH inhibition was required, 4-MP (10 mM, △) was preincubated before adding HOFQ.

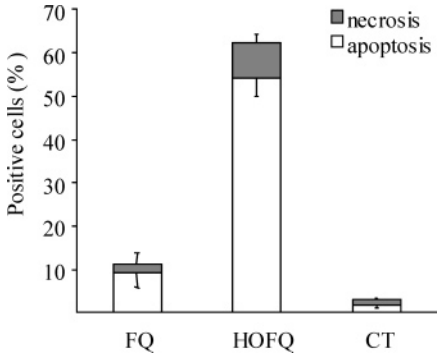


Figure 7. Apoptogenic activity of HOFQ and FQ. Jurkat cells (10^6 mL^{-1}) were incubated for 16 h at 37 °C with an equimolar concentration of HOFQ or FQ (10 μM) or with an equal volume amount of DMSO as a control, CT. After incubation, cells were stained with PI and Hoechst 33258 as described in Figure 2. The hydroxymethyl group at the C-4 position is required for HOFQ apoptotic activity.

adding the cells with the photooxidized products of psoralens. Thus, it is likely that the molecules responsible for the effects of PUVA are those generated by photodynamic mechanisms within the cells. A similar process also has been reported for merocyanins,[23,24] indicating the central role of irradiation, either in vitro or in situ, for the generation of cytotoxic compounds. The present results provide a mitochondria-centered cytotoxic mechanism that is totally independent of irradiation. To our knowledge, this is the first demonstration of such a mechanism for a photosensitizer in general and for a quinolinone compound in particular.

The present findings might potentially shift the attention on the mechanism of cytotoxicity of photosensitizers in general and HOFQ in particular that are generally related to DNA lesions. Beyond doubt, DNA damage can be a critical factor for the action of anticancer drugs. It is important to recognize, however, that it represents only one aspect of the pleiotropic reactivities of nominally DNA-reactive drugs.[25] Several lines of evidence suggest that DNA damage does not fully account for the pro-apoptotic effect of at least some nominally DNA-reactive drugs. Also, protein damage can, in principle, promote apoptosis since it distorts the cell redox homeostasis, which facilitates apoptosis execution. Indeed, a recent study demonstrated that protein damage is sufficient to trigger the apoptotic pathway since the SH-reactant 1,1′-azobis(*N*,*N*-dimethylformamide), commonly referred as diamide, reacts only with proteins and is apoptogenic.[26] On the other hand, there are other drugs, namely, bizelesin, which induce DNA damage and stimulate antiproliferative activity but do not cause apoptosis.[27] The limited relevance of binding to DNA is supported by the present results, which highlight the pivotal role of mitochondria in ensuing apoptosis. Indeed, both FQ[1] and HOFQ[3] display DNA-binding and antiproliferative properties, yet only HOFQ is apoptogenic. The present results clearly relate apoptosis to mitochondrial dysfunction since HOFQ induces (i) $\Delta\psi$m collapse in intact cells and (ii) PTP opening in isolated mitochondria incubated with ADH and NAD^+ to promote HOFQ oxidation. In particular, the latter finding demonstrates the direct action of HOFQ on mitochondria. The induction of apoptosis is currently considered to be the main mechanism underlying the therapeutic efficacy of anticancer agents. Therefore, the present results suggest that HOFQ might successfully be exploited to target tumor cells. In this respect the direct action on mitochondria has two major advantages: (i) apoptosis is obtained without interfering with the majority of cellular sites or processes that eventually require the involvement of mitochondria for ensuing apoptosis; this mode of action might reduce the occurrence of collateral and/or toxic effects improving the therapeutic efficacy; (ii) higher degrees of apoptosis should be obtained in malignant tissues that generally display an abnormally elevated mitochondrial content.

The observed differences between FQ and HOFQ unambiguously suggest that structural alterations of furoquinolinones result in remarkable changes of their biological activities. Therefore, we are currently investigating the possibility that the apoptogenic efficacy of HOFQ might be increased by selective modifications of its structure. In addition, it appears to be worth using furoquinolinone or its analogues as tools in ligand-binding studies to identify which mitochondrial proteins are involved in apoptosis.

Experimental Section

Chemistry. Melting points were determined on a Gallenkamp MFB-595-010M melting point apparatus and are uncorrected. Analytical TLC was performed on precoated 60 F_{254} silica gel plates (0.25 mm; Merck) developing with a $CHCl_3$/MeOH mixture (9:1) unless otherwise indicated. Preparative column chromatography was performed using silica gel 60 (0.063−0.100 mm; Merck). ^1H NMR spectra were recorded on a Bruker AMX300 spectrometer with TMS as internal standard. Elemental analyses were obtained on all intermediates, and final compounds and are within ±0.4% of theoretical values (see Supporting Information). Compounds **1**[8] and **8**[10] were prepared as previously reported.

4-Methoxymethyl-6-methyl-7-propargyloxyquinolin-2(1*H*)-one (2). A mixture of 7-hydroxy-4-methoxymethyl-6-methylquinolin-2(1*H*)-one[8] (**1**) (1.5 g, 6.9 mmol), propargyl bromide (1.3 g, 1.0 mL, 10.9 mmol), and anhydrous K_2CO_3 (5.0 g) in acetone (100 mL) was refluxed until **1** disappeared (48 h, TLC: AcOEt/cyclohexane, 7/3). After cooling, the solid was filtered off and washed with fresh acetone. The solvent was evaporated under reduced pressure from the combined filtrate and washings. The residue was crystallized from acetone to give **2** (0.83 g, 47%): mp 198 °C; ^1H NMR (DMSO-d_6) 11.91

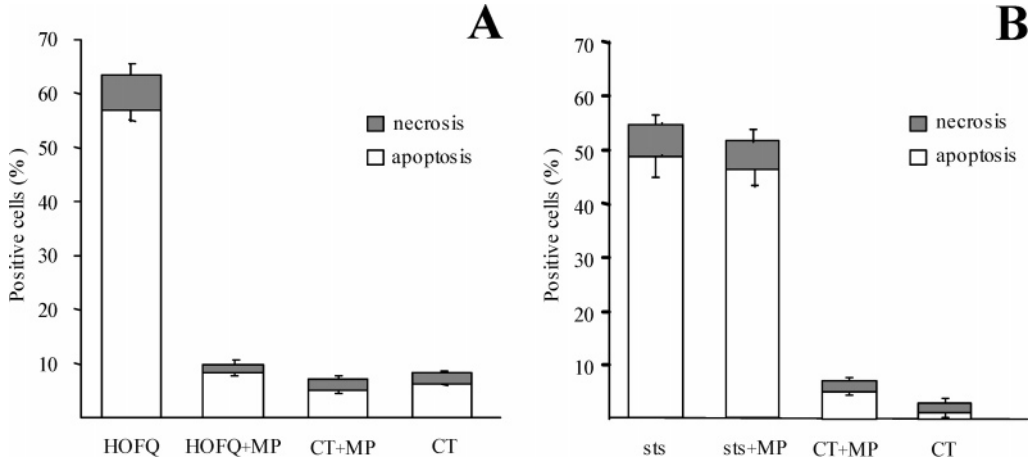


Figure 8. Effect of 4-methylpyrazole on the loss of cell viability induced by HOFQ (A) and staurosporine (B). Jurkat cells (10^6 mL^{-1}) were incubated with 10 μM HOFQ (A) or 0.6 μM staurosporine (sts) (B) in the presence and absence of 4-MP (MP) (10 mM). Duration of incubation and concentrations of apoptotic inducers were chosen to yield the same apoptotic response. CT, control, i.e., incubation without HOFQ or sts. After incubation, cells were stained as described in Figure 2.

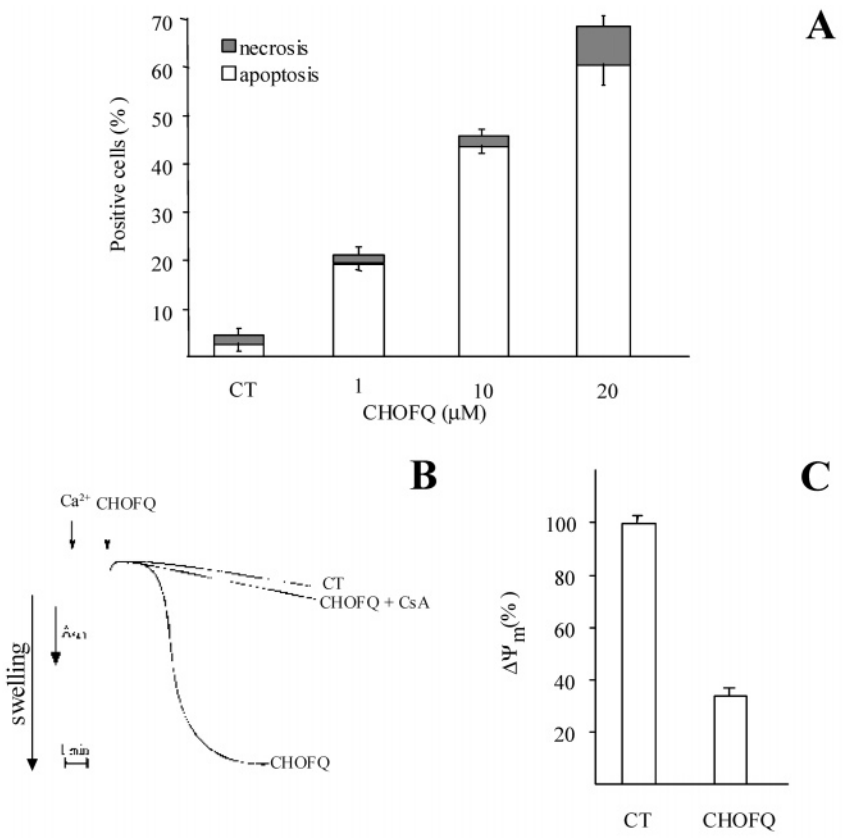


Figure 9. CHOFQ induces apoptosis, opening of the PTP, and mitochondrial dysfunction. (A) Jurkat cells (10^6 mL^{-1}) were incubated for 16 h in the absence (CT) or presence of increasing concentrations of CHOFQ (1−20 μM). Necrosis and apoptosis were assessed as detailed in Figure 2. (B) Mitochondrial swelling was monitored as the decrease in light scattering at 540 nm. Rat liver mitochondria (0.5 mg·mL^{-1}) were incubated with CHOFQ (10 μM) in the absence or presence of the PTP inhibitor cyclosporin A (CsA, 1 μM). CT, control, i.e., incubation without CHOFQ. (C) Jurkat cells (10^6 mL^{-1}) were incubated for 4 h in the absence (control, CT) or presence of 10 μM CHOFQ. After incubation, cells were stained as described in Figure 4.

(s, 1H, −NH), 7.34 (s, 1H, 5-H), 6.79 (s, 1H, 8-H), 6.55 (br s, 1H, 3-H), 4.87 (br s, 2H, 4-CH_2OMe), 4.78 (d, 2H, J = 2.1 Hz, 1′-H), 3.60 (s, 3H, 4-CH_2O*Me*), 2.60 (t, 1H, J = 2.1 Hz, 3′-H), 2.18 (s, 3H, 6-Me). Anal. ($C_{15}H_{15}NO_3$): C, H, N.

4-Methoxymethyl-1,6-dimethyl-7-propargyloxyquinolin-2(1*H*)-one (3). A mixture of **2** (0.83 g, 3.2 mmol), dimethyl sulfate (0.57 g, 0.43 mL, 4.5 mmol), and anhydrous K_2CO_3 (10.0 g) in acetone (300 mL) was refluxed until **2** disappeared (24 h, TLC: AcOEt). After cooling, the solid was filtered off and washed with fresh acetone. The solvent was evaporated under reduced pressure from the combined filtrate and washings. The residue was chromatographed on a silica gel column eluting with AcOEt to give **3** (0.37 g, 42%): mp 122 °C; ^{1}H NMR ($CDCl_3$) 7.31 (s, 1H, 5-H), 6.77 (s, 1H, 8-H), 6.55 (br s, 1H, 3-H), 4.85 (br s, 2H, 4-CH_2OMe), 4.76 (d, 2H, J = 2.2 HJz, 1′-H), 3.58 (s, 3H, 4-CH_2O*Me*), 3.37 (s, 3H, −NMe), 2.59 (t, 1H, J = 2.2 Hz, 3′-H), 2.19 (s, 3H, 6-Me). Anal. ($C_{16}H_{17}NO_3$) C, H, N.

4-Methoxymethyl-1,6,8-trimethylfuro[2,3-*h*]quinolin-2(1*H*)-one (4). A mixture of **3** (0.36 g, 1.3 mmol) and CsF (0.20 g, 1.3 mmol) in *N*,*N*-diethylaniline (10 mL) was heated at 210 °C until **3** disappeared (2 h, TLC: AcOEt/cyclohexane, 7/3).

After cooling, the precipitate was filtered off. The filtrate was diluted with EtOAc (10 mL), washed with 2 N HCl (4 × 10 mL), dried with Na_2SO_4, and evaporated under reduced pressure to give **4** (0.31 g, 86%): mp 171 °C (crude product); ^{1}H NMR ($CDCl_3$) δ 7.35 (q, 1H, J = 0.9 Hz, 5-H), 6.92 (q, 1H, J = 1.1 Hz, 9-H), 6.77 (t, 1H, J = 1.0 Hz, 3-H), 4.70 (d, 2H, J = 1.0 Hz, 4-*CH*$_2$OMe), 4.03 (s, 3H, −NMe), 3.47 (s, 3H, 4-CH$_2$O*Me*), 2.54 (d, 3H, J = 0.9 Hz, 6-Me), 2.52 (d, 3H, J = 1.1 Hz, 8-Me). Anal. ($C_{16}H_{17}NO_3$) C, H, N.

4-Bromomethyl-1,6,8-trimethylfuro[2,3-*h*]quinolin-2(1*H*)-one (5). A mixture of **4** (0.31 g, 1.1 mmol), HBr 48% (1 mL), and acetic acid (10 mL) was refluxed until starting product disappeared (10 h, TLC). The mixture was poured into cold water (50 mL), and the precipitate was collected, washed with water, and crystallized from EtOAc to give **5** (0.20 g, 55%); mp 229 °C; ^{1}H NMR ($CDCl_3$) 7.46 (br s, 1H, 5-H), 6.92 (q, 1H, J = 1.0 Hz, 9-H), 6.77 (br s, 1H, 3-H), 4.61 (br s, 2H, 4-CH$_2$-Br), 4.02 (s, 3H, −NMe), 2.58 (br s, 3H, 6-Me), 2.53 (d, 3H, J = 1.0 Hz, 8-Me). Anal. ($C_{15}H_{14}BrNO_2$) C, H, N, Br.

4-Acetoxymethyl-1,6,8-trimethylfuro[2,3-*h*]quinolin-2(1*H*)-one (6). A mixture of **5** (0.20 g, 0.62 mmol) and anhydrous AcONa (0.5 g) in acetic anhydride (10 mL) was refluxed for 1 h. The mixture was cautiously diluted with water (10 mL) and poured into cold water (100 mL). The precipitate obtained was filtered, washed with water, and crystallized from AcOEt to give **6** (0.16 g, 86%); mp 171 °C; ^{1}H NMR ($CDCl_3$) 7.20 (q, 1H, J = 0.9 Hz, 5-H), 6.93 (q, 1H, J = 1.1 Hz, 9-H), 6.78 (t, 1H, J = 1.3 Hz, 3-H), 5.39 (t, 2H, J = 1.3 Hz, 4-*CH*$_2$OAc), 4.03 (s, 3H, −NMe), 2.54 (d, 3H, J = 1.1 Hz, 8-Me), 2.53 (d, 3H, J = 0.9 Hz, 6-Me), 2.21 (s, 3H, −OAc). Anal. ($C_{17}H_{17}NO_4$) C, H, N.

4-Hydroxymethyl-1,6,8-trimethylfuro[2,3-*h*]quinolin-2(1*H*)-one (7). To a methanolic solution (10 mL) of **6** (0.12 g, 0.4 mmol) a 5% methanolic potassium hydroxide solution (10 mL) was added, and the mixture was refluxed for 1 h. The solution was cooled, diluted with cold water (50 mL), and acidified with diluted HCl. The precipitate obtained was collected and crystallized from MeOH to give **7** (0.08 g, 76%); mp 302 °C; ^{1}H NMR (DMSO-d_6) 7.40 (br s, 1H, 5-H), 7.24 (s, 1H, 9-H), 6.65 (s, 1H, 3-H), 5.54 (t, 1H, J = 5.5 Hz, 4-CH$_2$*OH*), 4.79 (d, 2H, J = 5.5 Hz, 4-*CH*$_2$OH), 3.90 (s, 3H, −NMe), 2.52 (br s, 3H, 6-Me or 8-Me), 2.49 (br s, 3H, 6-Me or 8-Me); HRMS (ESI) calcd for $C_{15}H_{16}NO_3$ $(M + 1)^+$ 258.1125, found 258.1117. Anal. ($C_{15}H_{15}NO_3$) C, H, N.

7-Hydroxy-4-dimethoxymethyl-1,6-dimethylquinolin-2(1*H*)-one (9). A mixture of 4-formyl-7-acetoxy-1,6-dimethylquinolin-2(1*H*)-one[10] (**8**) (0.70 g, 2.7 mmol), trimethyl orthoformate (1.1 g, 1.1 mL g, 10.0 mmol), and *p*-toluenesulfonic acid (0.25 g) in MeOH (10 mL) was refluxed until starting material disappeared (20 min, TLC). The reaction mixture was made basic with MeONa, diluted with water (50 mL), and extracted with EtOAc (3 × 50 mL). The organic phase was dried with Na_2SO_4, and the solvent was evaporated under reduced pressure. The residue was chromatographed on a silica gel column eluting with AcOEt/cyclohexane mixture (1/1) to give **9** (0.45 g, 63%): mp 239 °C; ^{1}H NMR (DMSO-d_6) 7.64 (s, 1H, 5-H), 6.83 (s, 1H, 8-H), 6.43 (d, 1H, J = 0.8 Hz, 3-H), 5.62 (d, 1H, J = 0.8 Hz, 4-*CH*(OMe)$_2$), 3.51 (s, 3H, −NMe), 3.29 (s, 6H, 4-CH(O*Me*)$_2$), 2.17 (s, 3H, 6-Me). Anal. ($C_{14}H_{17}NO_4$) C, H, N.

4-Dimethoxymethyl-1,6-dimethyl-7-propargyloxyquinolin-2(1*H*)-one (10). A mixture of **9** (0.45 g, 1.7 mmol), propargyl bromide (0.28 g, 0.21 mL, 2.4 mmol), and anhydrous K_2CO_3 (5.0 g) in acetone (50 mL) was refluxed until **9** disappeared (6 h, TLC: AcOEt/cyclohexane, 7/3). After cooling, the solid was filtered off and washed with fresh acetone. The solvent was evaporated under reduced pressure from the combined filtrate and washings. The residue was crystallized from MeOH to give **10** (0.41 g, 81%): mp 119 °C; ^{1}H NMR (acetone-d_6) 7.80 (q, 1H, J = 0.9 Hz, 5-H), 7.12 (s, 1H, 8-H), 6.62 (d, 1H, J = 0.6 Hz, 3-H), 5.65 (d, 1H, J = 0.6 Hz, 4-*CH*(OMe)$_2$), 5.02 (d, 2H, J = 2.5 Hz, 1′-H), 3.67 (s, 3H, −NMe), 3.35 (s, 6H, 4-CH(O*Me*)$_2$), 3.17 (t, 1H, J = 2.5 Hz, 3′-H), 2.25 (d, 3H, J = 0.9 Hz, 6-Me). Anal. ($C_{17}H_{19}NO_4$) C, H, N.

4-Dimethoxymethyl-1,6,8-trimethylfuro[2,3-*h*]quinolin-2(1*H*)-one (11). A mixture of **10** (0.39 g, 1.3 mmol) and CsF (0.20 g, 1.3 mmoli) in *N*,*N*-diethylaniline (12 mL) was heated at 210 °C until **10** disappeared (4 h, TLC: AcOEt/cyclohexane, 7/3). After cooling, the obtained precipitate was filtered off. The filtrate was diluted with EtOAc (15 mL), washed with 2 N HCl (4 × 20 mL), dried with Na_2SO_4, and evaporated under reduced pressure to give **11** (0.24 g, 61%): mp 166 °C (crude product); ^{1}H NMR (acetone-d_6) 7.73 (q, 1H, J = 0.8 Hz, 5-H), 7.21 (q, 1H, J = 1.1 Hz, 9-H), 6.75 (d, 1H, J = 0.5 Hz, 3-H), 5.72 (d, 1H, J = 0.5 Hz, 4-*CH*(OMe)$_2$), 3.98 (s, 3H, −NMe), 3.37 (s, 6H, 4-CH(O*Me*)$_2$), 2.53 (d, 3H, J = 0.8 Hz, 6-Me), 2.51 (d, 3H, J = 1.1 Hz, 8-Me). Anal. ($C_{17}H_{19}NO_4$) C, H, N.

4-Formyl-1,6,8-trimethylfuro[2,3-*h*]quinolin-2(1*H*)-one (12). A mixture of **11** (0.24 g, 0.79 mmol) and HCl (concentrated, 10 mL) in dioxane (50 mL) was stirred at room temperature until **11** disappeared (10 min, TLC: AcOEt/cyclohexane, 7/3). The precipitate was filtered, suspended in water (20 mL), neutralized with 10% $NaHCO_3$, and extracted with EtOAc (3 × 20 mL). The organic phase was dried with Na_2SO_4, the solvent was evaporated under reduced pressure, and the residue was crystallized from EtOAc to give **12** (0.18 g, 89%): mp 281 °C; ^{1}H NMR ($CDCl_3$) 10.17 (s, 1H, CHO), 8.52 (q, 1H, J = 0.8 Hz, 5-H), 7.11 (s, 1H, 3-H), 6.93 (q, 1H, J = 1.0 Hz, 9-H), 4.07 (s, 3H, −NMe), 2.57 (d, 3H, J = 0.8 Hz, 6-Me), 2.54 (d, 3H, J = 1.0 Hz, 8-Me); ^{13}C NMR ($CDCl_3$) 193.35 (CHO), 162.13 (C-2), 155.97 (C-6a), 154.61 (C-8), 140.77 (C-4), 134.55 (C-9b), 128.39 (C-3), 121.49 (C-5), 18.41 (C-6), 116.22 (C-9a), 112.05 (C-4a), 104.24 (C-9), 33.56 (C−NMe), 15.02 (C-6Me), 13.94 (C-8Me); HRMS (ESI) calcd for $C_{15}H_{14}NO_3$ $(M + 1)^+$ 256.0968, found 256.1104. Anal. ($C_{15}H_{13}NO_3$) C, H, N.

Biology. Cell Culture. The human T-lymphoblastoid Jurkat cell line was cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU·mL^{-1} penicillin, and 100 μg·mL^{-1} streptomycin (Life Technologies, Paisley, Scotland) in 5% CO_2−95% air at 37 °C in a humidified atmosphere.

Reagents. TMRM was purchased by Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma.

TMRM Staining and Imaging. At the end of the incubation with HOFQ or CHOFQ, Jurkat cells were washed with Hanks' balanced salt solution supplemented with 10 mM Hepes, pH 7.4 (HBSS), and then incubated for 15 min at 37 °C with 25 nM TMRM. Cyclosporin H (1.6 μM) was also added to inhibit multidrug-resistance pumps, which can affect TMRM loading.[11] Cellular fluorescence images were acquired with an Olympus IMT-2 inverted microscope, equipped with a xenon lamp and a 12-bit digital cooled CCD camera (Micromax, Princeton Instruments) as previously described.[12] For detection of TMRM fluorescence, 568 ± 25 nm excitation and 585 nm long-pass emission filter settings were used. Data were acquired and analyzed using Metamorph software (Universal Imaging). Mitochondria were identified as regions of interest (ROI), and at least 30 ROIs were considered for each experiment. The decrease in fluorescence intensities induced by FCCP was expressed as the difference of the values obtained before and after addition of the uncoupler. The values of these differences obtained in the treated cells were normalized to the difference values of the untreated cells (control).

Assessment of Cell Viability. Jurkat cells (10^6 cells·mL^{-1}) were stained with 10 μM Hoechst 33258 and 1 μM PI for 5 min.[13] Cells were then washed with HBSS and visualized with the fluorescence microscope using excitation/emission cubes of 340/(440 ± 25) and 568/(585 ± 25) nm long-pass filter for Hoechst 33258 and PI, respectively. Three randomly selected fields were acquired from each treatment. The corresponding bright-field images were also acquired, and the three channels were overlaid using the appropriate function of the Metamorph software.

Mitochondrial Isolation and Swelling Assay. Rat liver mitochondria from albino Wistar rats weighing about 300 g were prepared by standard centrifugation techniques as described previously.[28] Swelling was monitored as the changes in absorbance at 540 nm as previously described.[29] Incubations

were carried out at 25 °C with 0.5 mg of mitochondrial protein·mL^{-1} in the medium containing 0.2 M sucrose, 10 mM Tris-Mops, 10 μM EGTA-Tris, 1 mM Pi-Tris, 5 mM succinate-Tris, 2 μM rotenone, 0.5 μg·mL^{-1} oligomycin, pH 7.4, in the presence or absence of 300 U·mL^{-1} ADH and 0.1 mM NAD^+. PTP opening was facilitated by the addition of 80 μM Ca^{2+}.[30]

ADH Assay. ADH-catalyzed oxidation of HOFQ was assessed by following the absorbance increase at 340 nm. Increasing concentrations of HOFQ or FQ were added in 1 mL of a medium containing 0.9 M semicarbazide, ADH 300 U·mL^{-1}, 0.1 mM NAD^+, and 0.1 M NaH_2PO_4 (pH 8.8). 4-MP (10 mM) was incubated for 10 min prior HOFQ addition where indicated.

Statistical Analysis. Data are reported as means ± SD. The Student *t*-test was used, and results were considered to be significant if $p < 0.01$.

Supporting Information Available: Elemental analysis data for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Rodighiero, P.; Guiotto, A.; Chilin, A.; Bordin, F.; Baccichetti, F.; Carlassare, F.; Vedaldi, D.; Caffieri, S.; Pozzan, A.; Dall'Acqua, F. Angular furoquinolinones, psoralen analogs: novel antiproliferative agents for skin diseases. Synthesis, biological activity, mechanism of action, and computer-aided studies. *J. Med. Chem.* **1996**, *39*, 1293−1302.

(2) Chilin, A.; Marzano, C.; Guiotto, A.; Manzini, P.; Baccichetti, F.; Carlassare, F.; Bordin, F. Synthesis and biological activity of (hydroxymethyl)- and (diethylaminomethyl)benzopsoralens. *J. Med. Chem.* **1999**, *42*, 2936−2945.

(3) Chilin, A.; Marzano, C.; Baccichetti, F.; Simonato, M.; Guiotto, A. 4-Hydroxymethyl- and 4-methoxymethylfuro[2,3-h]quinolin-2(1*H*)-ones: synthesis and biological properties. *Bioorg. Med. Chem.* **2003**, *11*, 1311−1318.

(4) Gottlieb, R. A. Cytochrome P450: major player in reperfusion injury. *Arch. Biochem. Biophys.* **2003**, *420*, 262−267.

(5) Galter, D.; Carmine, A.; Buervenich, S.; Duester, G.; Olson, L. Distribution of class I, III, and IV alcohol dehydrogenase mRNAs in the adult rat, mouse and human brain. *Eur. J. Biochem.* **2003**, *270*, 1316−1326.

(6) Druteika, D. P.; Zed, P. J.; Ensom, M. H. Role of fomepizole in the management of ethylene glycol toxicity. *Pharmacotherapy* **2002**, *22*, 365−372.

(7) Brent, J.; McMartin, K.; Phillips, S.; Aaron, C.; Kulig, K. Fomepizole for the treatment of methanol poisoning. *N. Engl. J. Med.* **2001**, *344*, 424−429.

(8) Chilin, A.; Marzano, C.; Guiotto, A.; Baccichetti, F.; Carlassare, F.; Bordin, F. Synthesis and biological evaluation of a new furo-[2,3-*h*]quinolin-2(1*H*)-one. *J. Med. Chem.* **2002**, *45*, 1146−1149.

(9) Ishii, H.; Hishikawa, T.; Takeda, S.; Ueki, S.; Suzuki, M.; Harayama, T. Cesium fluoride-mediated Claisen rearrangement of aryl propargyl ether and its application to the synthesis of chelerythrine. *Chem. Pharm. Bull.* **1990**, *38*, 1775−1777.

(10) Chilin, A.; Manzini, P.; Confente, A.; Pastorini, G.; Guiotto, A. Synthesis of some benzo[*c*][2,6]naphthyridin-5-ones and new tetracyclic benzofuro[4,5-*c*]-2,6-naphthyridin-5(6*H*)-ones. *Tetrahedron* **2002**, *58*, 9959−9964.

(11) Bernardi, P.; Petronilli, V.; Di Lisa, F.; Forte, M. A mitochondrial perspective on cell death. *Trends Biochem. Sci.* **2001**, *26*, 112−117.

(12) Petronilli, V.; Miotto, G.; Canton, M.; Brini, M.; Colonna, R.; Bernardi, P.; Di Lisa, F. Transient and long-lasting openings of the mitochondrial permeability transition pore can be monitored directly in intact cells by changes in mitochondrial calcein fluorescence. *Biophys. J.* **1999**, *76*, 725−734.

(13) Canton, M.; Caffieri, S.; Dall'Acqua, F.; Di Lisa, F. PUVA-induced apoptosis involves mitochondrial dysfunction caused by the opening of the permeability transition pore. *FEBS Lett.* **2002**, *522*, 168−172.

(14) Bernardi, P.; Scorrano, L.; Colonna, R.; Petronilli, V.; Di Lisa, F. Mitochondria and cell death. Mechanistic aspects and methodological issues. *Eur. J. Biochem.* **1999**, *264*, 687−701.

(15) Bordin, F.; Marzano, C.; Carlassare, F.; Rodighiero, P.; Guiotto, A.; Caffieri, S.; Baccichetti, F. Photobiological properties of a new tetramethylfuroquinolinone, *J. Photochem. Photobiol., B: Biol.* **1996**, *34*, 159−168.

(16) Marzano, C.; Chilin, A.; Guiotto, A.; Baccichetti, F.; Carlassare, F.; Bordin, F. Photobiological properties of 1-(3′-hydroxypropyl)-4,6,8-trimethylfuro[2,3-*h*]quinolin-2(1*H*)-one, a new furocoumarin analogue. *Farmaco* **2000**, *55*, 650−658.

(17) Boon, P. J.; van der, B. D.; Mulder, G. J. Cytotoxicity and biotransformation of the anticancer drug perillyl alcohol in PC12 cells and in the rat. *Toxicol. Appl. Pharmacol.* **2000**, *167*, 55−62.

(18) Burcham, P. C.; Fontaine, F. Extensive protein carbonylation precedes acrolein-mediated cell death in mouse hepatocytes. *J Biochem. Mol. Toxicol.* **2001**, *15*, 309−316.

(19) Holownia, A.; Ledig, M.; Braszko, J. J.; Menez, J. F. Acetaldehyde cytotoxicity in cultured rat astrocytes. *Brain Res.* **1999**, *833*, 202−208.

(20) Popescu, G.; Napoli, J. L. Analysis of rat cytosolic 9-cis-retinol dehydrogenase activity and enzymatic characterization of rat ADHII. *Biochim. Biophys. Acta* **2000**, *1476*, 43−52.

(21) Fontaine, F. R.; Dunlop, R. A.; Petersen, D. R.; Burcham, P. C. Oxidative bioactivation of crotyl alcohol to the toxic endogenous aldehyde crotonaldehyde: association of protein carbonylation with toxicity in mouse hepatocytes. *Chem. Res. Toxicol.* **2002**, *15*, 1051−1058.

(22) Irwin, W. A.; Gaspers, L. D.; Thomas, J. A. Inhibition of the mitochondrial permeability transition by aldehydes. *Biochem. Biophys. Res. Commun.* **2002**, *291*, 215−219.

(23) Pervaiz, S. Reactive oxygen-dependent production of novel photochemotherapeutic agents. *FASEB J.* **2001**, *15*, 612−617.

(24) Hirpara, J. L.; Clement, M. V.; Pervaiz, S. Intracellular acidification triggered by mitochondrial-derived hydrogen peroxide is an effector mechanism for drug-induced apoptosis in tumor cells. *J. Biol. Chem.* **2001**, *276*, 514−521.

(25) Woynarowska, B. A.; Woynarowski, J. M. Preferential targeting of apoptosis in tumor versus normal cells. *Biochim. Biophys. Acta* **2002**, *1587*, 309−317.

(26) Zamzami, N.; Marzo, I.; Susin, S. A.; Brenner, C.; Larochette, N.; Marchetti, P.; Reed, J.; Kofler, R.; Kroemer, G. The thiol crosslinking agent diamide overcomes the apoptosis-inhibitory effect of Bcl-2 by enforcing mitochondrial permeability transition. *Oncogene* **1998**, *16*, 1055−1063.

(27) Woynarowski, J. M.; McHugh, M. M.; Gawron, L. S.; Beerman, T. A. Effects of bizelesin (U-77779), a bifunctional alkylating minor groove agent, on genomic and simian virus 40 DNA. *Biochemistry* **1995**, *34*, 13042−13050.

(28) Costantini, P.; Petronilli, V.; Colonna, R.; Bernardi, P. On the effects of paraquat on isolated mitochondria. Evidence that paraquat causes opening of the cyclosporin A-sensitive permeability transition pore synergistically with nitric oxide. *Toxicology* **1995**, *99*, 77−88.

(29) Scorrano, L.; Petronilli, V.; Di Lisa, F.; Bernardi, P. Commitment to apoptosis by GD3 ganglioside is mediated by selective opening of the mitochondrial permeability transition pore. *J. Biol. Chem.* **1999**, *274*, 22581−22585.

(30) Petronilli, V.; Cola, C.; Massari, S.; Colonna, R.; Bernardi, P. Physiological effectors modify voltage sensing by the cyclosporin A-sensitive permeability transition pore of mitochondria. *J. Biol. Chem.* **1993**, *268*, 21939−21945.

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