# Fluoride curcumin derivatives: new mitochondrial uncoupling agents

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**Abstract The mitochondrial effects of two fluoride curcumin derivatives were studied. They induced the collapse of mitochondrial membrane potential ($\Delta\psi$), increased mitochondrial respiration, and decreased $O_2^{\cdot-}$ production and promoted $Ca^{2+}$ release. These effects were reversed by the recoupling agent 6-Ketocholestanol, but not by cyclosporin A, an inhibitor of the permeability transition pore (PTP), suggesting that these compounds act as uncoupling agents. This idea was reinforced by the analysis of the physico-chemical properties of the compounds indicating, that they are mainly in the anionic form in the mitochondrial membrane. Moreover, they are able to induce PTP opening by promoting the oxidation of thiol groups and the release of cytochrome *c*, making these two molecules potential candidates for induction of apoptosis.**


*Keywords:* Curcumin; Uncoupler; Mitochondrial membrane potential; Permeability transition pore; 6-Ketocholestanol; Swelling

## 1. Introduction

It is now widely accepted that several mitochondrial events control the apoptosis process [1].

One of them is the mitochondrial permeability transition; permeability transition is characterized by a sudden increase in the permeability of the inner mitochondrial membrane to solutes with molecular mass below 1500 Da [2]. Permeability transition appears to be due to the opening of a pore, called the permeability transition pore (PTP), which promotes membrane depolarization, uncoupling of oxygen consumption and synthesis of ATP, and large amplitude mitochondrial swelling. The consequence is a loss of the outer membrane integrity, leading to translocation of pro-apoptotic proteins from the intermembrane space to the cytosol, with subsequent activation of caspases [3].

Thus, PTP may constitute a target to protect cells against cellular injuries encountered during some pathologic situations (such as ischemia-reperfusion [4]); the induction of PTP opening may provide a strategy to induce cell death in tumor cells [5]. This property was shown to be involved in the chemotherapeutic effect of certain anticancer drugs [6,7].

Different reagents have been shown to induce pore opening. They include adenine translocator reagents, oxidizing or transmembrane potential reducing agents such as uncouplers [8]. In a recent work, we demonstrated that a natural yellow pigment originally isolated from *Curcuma longa* L, curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), which possesses a wide range of pharmacological properties [9], was able to open the PTP [10]. This effect was attributed to the ability of curcumin to promote the oxidation of mitochondrial membrane thiol functions; we postulated that this may be part of the mechanism by which curcumin induces apoptosis of tumor cells [11,12] and thus exerts its anti-proliferation activity [13].

In order to search for new antitumor agents acting on mitochondria, we synthetized various analogs of curcumin, which were compared to the well-known uncoupler agent carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). We present evidence that two of these agents obtained by chemical substitution of curcumin ([14]; Fig. 1) combine both the prooxidant PTP inducing properties of the parent drug with potent mitochondrial uncoupling properties.

## 2. Materials and methods

Fluoride curcumin derivatives 1,7-bis(4-hydroxy-3-fluorophenyl)-1,6-heptadiene-3,5-dione (Cu12) and 2,6-bis(4-hydroxy-3-fluorobenzylidene)cyclohexanone (Cy12) were synthetized according to the methods described by [15]. The benzaldehyde used for synthesis of Cu12 and Cy12 was 3-fluoro-4-hydroxybenzaldehyde. The powder obtained was purified by recrystallization. The structures of the compounds were confirmed by microanalysis, $^1H$ magnetic resonance and measurement of melting points.

Rat liver mitochondria were isolated from male Wistar rats as described by Elimadi et al. [16]. Mitochondrial membrane potential ($\Delta\psi$) was monitored by means of the fluorescent dye rhodamine 123 at the excitation and emission wavelengths of 503 and 527 nm, respectively, [16]. $Ca^{2+}$ flux and mitochondrial respiration were simultaneously measured in a thermostat-controlled reaction chamber (4 ml) at 25 °C as described previously [10]. Mitochondrial respiration was measured using a Clark-type oxygen microelectrode fitted to an oxygen

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*Abbreviations:* Cu12, 1,7-bis(4-hydroxy-3-fluorophenyl)-1,6-heptadiene-3,5-dione; Cy12, 2,6-bis(4-hydroxy-3-fluorobenzylidene)cyclohexanone; CsA, cyclosporin A; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; PTP, permeability transition pore; $\Delta\psi$, mitochondrial membrane potential; 6-KCh, 6-ketocholestanol; *t*-BH, *tert*-butylhydroperoxide

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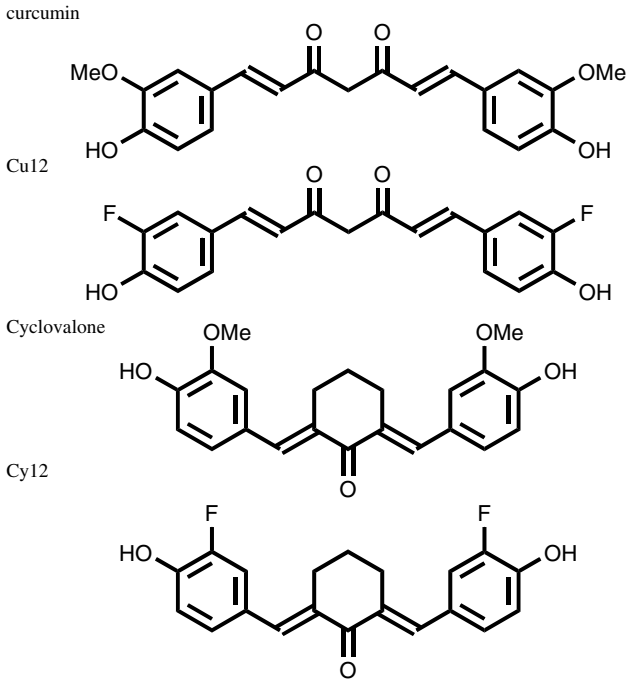


Fig. 1. Chemical structures of fluoride curcumin derivatives.

monitoring system (Hansatech®). The concentration of $Ca^{2+}$ in the extramitochondrial medium was determined by means of a specific $Ca^{2+}$ electrode (Orion®) connected to the auxiliary output of the oxygen monitoring system via a 720 A Orion ionometer. Swelling was assessed by measuring the decrease in absorbance at 540 nm of a suspension of energized or deenergized mitochondria according to [17] and [18], respectively. The same incubation conditions were used to study the release of cytochrome *c*. After incubation, the mitochondrial suspension was centrifuged at $15\,000 \times g$ for 10 min at 4 °C and cytochrome *c* levels in the supernatant were determined by western immunoblot analysis. The generation of $O_2^{\cdot -}$ was measured at 560 nm using the reduction reaction of nitro blue tetrazolium into monoformazan [19]. Briefly, mitochondria (1 mg/ml) were incubated for 1 min before the addition of 6 mM succinate which induced the production of $O_2^{\cdot -}$. For this particular experiment, 1 μM cyclosporin A (CsA) was added to the incubation medium to inhibit mitochondrial swelling which slightly interfered with the spectroscopic detection of the reduction reaction. Protein thiol content was measured according to [15].

The ionization constants of solutes were measured by potentiometric titrations using the GlpKa instrument (Sirius Analytical Instruments, Forrest Row, UK). The pH-metric titrations were carried out in water containing 0.15 M KCl to adjust ionic strength. Since the four compounds are lipophilic, methanol was used as co-solvent. The apparent $pK_a$ values were extrapolated to zero cosolvent using the Yasuda–Shedlovsky procedure [20].

Titrations in the presence of different volumes of organic solvent (*n*-octanol or *o*-nitrophenyl octyl ether (NPOE)) were carried out. Addition of organic solvent induces a shift in the titration curve, which is related to the $pK_a$ and the lipophilicity of the solute. The $\log P$ values were estimated from difference Bjerrum plots and refined by a non-linear least squares procedure by including previously determined $pK_a$ values as unrefined contributions. The detailed experimental procedures can be found elsewhere [21,22]. All titration measurements were carried out at $25 \pm 1$ °C under an argon atmosphere.

## 3. Results

### 3.1. Fluoride curcumin derivatives induced mitochondrial uncoupling

In the first series of experiments, mitochondria (1 mg/ml) were incubated in 1 ml of phosphate buffer supplemented with 2 μM rotenone and 1 μM CsA, and respiration was induced by the addition of 6 mM succinate. Cu12 and Cy12 induced a concentration-dependent stimulation of respiration which became obvious at about 5 μM (Fig. 2A, lines a and b). Stimulation of respiration was associated with a concentration-dependent decrease in the membrane potential monitored by means of rhodamine 123 (Fig. 2B). Identical results were observed with the well-known uncoupling agent CCCP.

Parallel measurements were performed in the presence of 25 μM $Ca^{2+}$. $Ca^{2+}$ was taken up and retained by mitochondria until Cu12 or Cy12 was added (Fig. 2C). The two compounds induced $Ca^{2+}$ release; this process was insensitive to CsA, indicating that it was not mediated by PTP. Similar results were found after addition of CCCP (data not shown).

These data suggest that both Cu12 and Cy12 act as uncouplers.

Their effect on superoxide radical ($O_2^{\cdot -}$) generation reinforced this hypothesis. Fig. 2D shows that pre-incubation of mitochondria with either 20 μM Cu12 or Cy12 totally abolished $O_2^{\cdot -}$ production. The same result was obtained in the presence of 1 μM CCCP.

### 3.2. 6-Ketocholestanol reversed mitochondrial uncoupling induced by fluoride curcumin derivatives

The same set of experiments were performed in the presence of different recoupling agents. Fig. 3A shows that increasing concentrations of 6-Ketocholestanol (6-KCh) restored the membrane potential abolished by maximal uncoupling concentrations of Cy12 while other recouplers, i.e., progesterone, testosterone, atractylate or carboxyatractylate, which were soluble in the incubation medium at the concentrations used, were ineffective. Similar results were obtained with Cu12 (data not shown). Similarly, Fig. 2A (lines c and d) and Fig. 3B show that the increase in the respiration rate induced by Cu12, Cy12 or CCCP was inhibited by 6-KCh.

### 3.3. Physico-chemical properties of fluoride curcumin derivatives

The physico-chemical parameters measured are reported in Table 1. The replacement of methoxy groups by fluorine atoms induced three significant physico-chemical changes, namely an enhancement of the phenolic acidity, a large increase in partition coefficient in *n*-octanol/water biphasic system, and a large increase in $\Delta \log P^{N}_{oct-npoe}$ parameter. If the latter change was associated only with the formation of an intramolecular hydrogen bond in the neutral form of methoxy derivatives, the higher lipophilicity and the higher acidity of fluoride derivatives suggested that this substitution increased the concentration of the anionic forms in organic media. However, measurements of free energy associated with interfacial transfer of anionic forms in order to characterize their lipophilicity [23] were not possible due to non-optimal conditions for voltammetry experiments.

### 3.4. Effect of fluoride curcumin derivatives on mitochondrial swelling

Mitochondrial swelling was first measured in energized mitochondria. In a first series of experiments, the protocol of Bernardi et al. [17] was used. Mitochondria were loaded with 25 μM $Ca^{2+}$ and then the swelling was promoted by the addition of either Cu12, Cy12 or CCCP. The three compounds

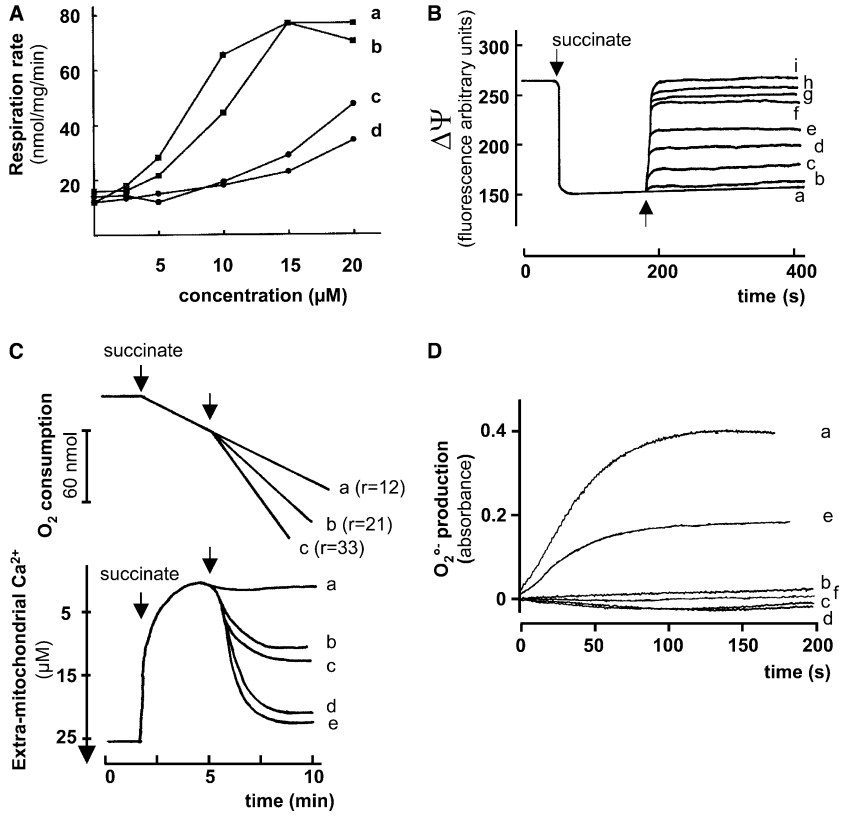


Fig. 2. Effect of Cu12 and Cy12 on mitochondrial respiration, membrane potential, $Ca^{2+}$ fluxes and $O_2^{\cdot-}$ production. Panel A: Liver mitochondria (1 mg/ml) were incubated in 1 ml of phosphate buffer containing 0.25 M sucrose and 5 mM $KH_2PO_4$ supplemented with 2 μM rotenone, pH 7.2, at 25 °C. Respiration was initiated by the addition of 6 mM succinate in the presence of increasing concentrations of either Cu12 (lines a, c) or Cy12 (lines b and d). These experiments were performed in the absence (lines a and b) or in the presence of 400 μM 6-KCh (lines c and d). Panel B: Mitochondria (0.5 mg/ml) were added to 1.8 ml of phosphate buffer supplemented with 2 μM rotenone, 0.3 μM rhodamine 123 and 1 μM CsA. $\Delta\psi$ was measured after the addition of 6 mM succinate, followed (arrow) by increasing concentrations of Cy12 (2.5 μM, line b; 5 μM, line c; 6 μM, line d; 7.5 M, line e; 10 μM, line f; 20 μM, line h), 10 μM Cu12 (line g) or 1 μM CCCP (line i). Line a, no addition. Panel C: Mitochondria (4 mg) were added to 4 ml of phosphate buffer supplemented with 2 μM rotenone, 6 mM succinate and 1 μM CsA and loaded with 25 μM $Ca^{2+}$. Addition (arrow) of either 20 μM Cu12 (line b) or 20 μM Cy12 (lines c) induced a simultaneous increase in mitochondrial respiration and $Ca^{2+}$ release. A total $Ca^{2+}$ release was observed after addition of either 30 μM Cu12 (line d) or 30 μM Cy12 (line e). Line a, control. "*r*" values correspond to the respiration rates expressed in nmol/min/mg protein. Panel D: Mitochondria (1 mg/ml) were incubated in 1.2 ml of phosphate buffer supplemented with 2 μM rotenone, 1 μM CsA and 100 μM nitro blue tetrazolium. $O_2^{\cdot-}$ production was initiated by addition of 6 mM succinate (line a) and inhibited in the presence of either 1 μM CCCP (line b), 20 μM Cu12 (line c), or 20 μM Cy12 (line d). Superoxide dismutase (1000 units; line e) and α-tocopherol (300 μM; line f) were used as reference compounds.

induced a large amplitude swelling associated with cytochrome *c* release, which was fully sensitive to CsA and therefore mediated by the PTP (Fig. 4A). Given that, under these assay conditions, the effect of CCCP on the pore was attributed to the depolarization of the mitochondrial membrane [24], we hypothesized that the same mechanism of action (i.e., uncoupling effect) could be considered for curcumin derivatives. This assumption was reinforced by the fact that 500 μM 6-KCh completely abolished the effect of Cu12 and Cy12 (Fig. 4A).

In a second series of experiments, mitochondria were incubated in phosphate buffer and swelling was obtained by the addition of $Ca^{2+}$ (Fig. 4B). Preincubation of mitochondria for 2 min with either CCCP, Cu12 or Cy12 inhibited mitochondrial swelling, confirming that these drugs acted as uncouplers. This effect was concentration-dependent with $IC_{50}$ values of 0.01, 5.5 and 11.5 μM, respectively (not shown). Indeed, the elimination of the transmembrane potential by the drugs prevented $Ca^{2+}$ uptake and thus PTP opening (Fig. 4B).

We then measured mitochondrial swelling in de-energized mitochondria. Under these particular experimental conditions, respiration and membrane potential are eliminated and mitochondrial swelling cannot be ascribed to mitochondrial uncoupling as confirmed by the fact that CCCP was unable to induce swelling. Conversely, Cu12 and Cy12 caused mitochondrial swelling and this effect was due to PTP opening as attested by the inhibition effect of CsA (Fig. 4C).

### 3.5. Fluoride curcumin derivatives promoted thiol oxidation

It is now well-established that PTP opening is related to membrane protein thiol status [25], and we recently described that curcumin is able to oxidize mitochondrial membrane thiol functions [15]. Therefore, it was logical to assume that mitochondrial membrane thiol oxidation might be the trigger of PTP opening when it is unrelated to membrane depolarization. Indeed, Table 2 demonstrates that Cu12 and Cy12 promoted membrane thiol oxidation and Fig. 4C shows that the thiol substitution compound *N*-ethylmaleimide completely prevented Cu12 and Cy12-induced swelling, suggesting that a thiol group is involved in swelling induction. Thus, this effect appears to be independent of the uncoupling properties of the drugs but related to their prooxidant properties.

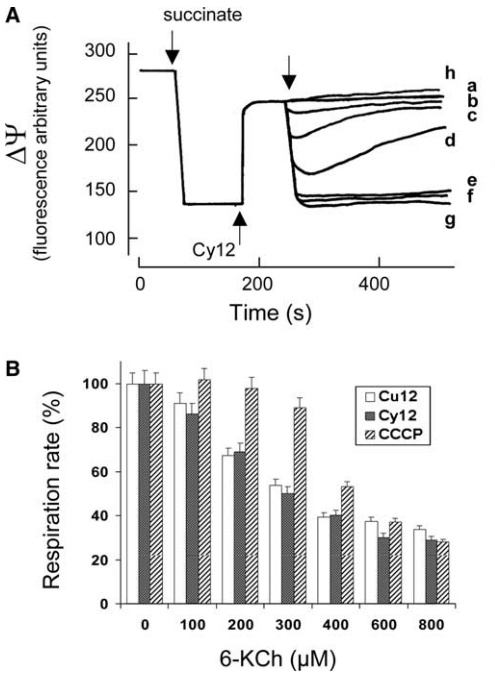


Fig. 3. Effect of 6-KCh on Cu12 and Cy12-induced uncoupling. Panel A: experimental conditions were similar to Fig. 2B. The collapse of $\Delta\psi$ was induced by the addition of 20 μM Cy12 (line a). The effect of Cy12 was reversed by increasing concentrations of 6-KCh: 25 μM (line b), 50 μM (line c), 100 μM (line d), 200 μM (line e), 300 μM (line f) and 600 μM (line g). Other recouplers, i.e, atractylate (3 μM), carboxyatractylate (3 μM), testosterone (500 μM) and progesterone (500 μM) were without effect. Line h shows the result obtained after addition of carboxyatractylate. Panel B: respiration rate was measured as described in Fig. 2A. The presence of either 15 μM Cu12, 15 μM Cy12 or 1 μM CCCP induced a maximal increase in respiration rate (100%) that is inhibited by 6-KCh in a concentration-dependent manner.

## 4. Discussion

The results of the present study show that incubation of rat liver mitochondria with micromolar concentrations of the curcumin derivatives Cu12 and Cy12 induced an increase in the mitochondrial respiratory rate and a dose-dependant collapse of $\Delta\psi$. These molecules also promoted the release of $Ca^{2+}$ from $Ca^{2+}$ pre-loaded mitochondria but prevented $Ca^{2+}$-induced swelling. These results suggest that Cu12 and Cy12 act as uncoupling agents, since these events have been associated with the effects of several uncouplers, e.g., SF6847 [26], pinacidil and diazoxide [27], CCCP, 2,4-dinitrophenol, and palmitate [28].

This idea is reinforced by the observation that the two compounds were also able to inhibit the production of $O_2^{\cdot-}$. Indeed, Korshunov et al. [29] have demonstrated that the rate of $H_2O_2$ production (generated after $O_2^{\cdot-}$ dismutation) in rat heart mitochondria was inhibited by a protonophorous uncoupler. The mechanism would involve a decrease in the lifetime of the ubisemiquinone anion and some other electron transport intermediates competent in the reduction of $O_2$ to $O_2^{\cdot-}$ [30] caused by the lowering of $\Delta\psi$.

The mechanism of action of uncouplers is not yet well-established. Uncouplers translocate protons from the intermembrane space to the matrice when they cross the membrane in their protonated form. This is followed by the translocation of their deprotonated form to the outer side of the membrane to bind another proton. The action of low concentrations of several uncouplers can be suppressed by specific inhibitors, suggesting that uncoupling is protein facilitated [26–28,31]. These proteins localized in the membrane seem to be necessary for the translocation of the uncoupler [26,32].

Therefore, to investigate the mechanism of action of Cu12 and Cy12, we analyzed their physico-chemical properties and tested the effects of well-known recoupling agents. Restoration of mitochondrial function appeared neither in the presence of steroids hormones (probably because of the absence of bovine serum albumin [26,31]), nor in the presence of atractylate or carboxyatraclylate, two inhibitors of the ADP/ATP antiporter. This last observation allowed us to exclude a possible role of the ADP/ATP transporter in Cu12 or Cy12-mediated uncoupling. On the other hand, the effects of the two curcumin-derivatives were reversed in the presence of 6-KCh, the 3-keto-derivative of cholesterol. The mechanism of action of 6-KCh is not well understood but it was suggested to incorporate into the outer leaflet of the membrane and to induce a strong asymmetrical increase in the membrane dipole potential and therefore a deceleration of the protein-mediated movement of the uncoupler agent in the membrane [26]. However, it should be noted that to date this protein is unknown. 6-KCh was shown to inhibit the action of uncouplers which cross the membrane in the form of the anion, $A^-$ as SF6847 or CCCP but was ineffective in inhibiting uncoupling mediated by compounds which cross the membrane in the form of a dimer of anionic and protonated species $HA_2^-$ as dinitrophenol [28]. These results are in accordance with our physico-chemical data, which suggest that Cu12 and Cy12 can exist as $A^-$ anionic form in lipophilic media such as mitochondrial membranes. Even if it was not possible to measure the lipophilicity of anionic forms by cyclic voltammetry, the accumulation of anions in organic phase can be seen qualitatively in partition experiments by changes in organic phase coloration. It was also clearly observed that the presence of $Li^+$ in partition experiments enhanced the organic phase coloration and, thus, the concentration of anion in organic phase. These effects are markedly important for fluorine derivatives at pH around pH

Table 1
Physico-chemical parameters of curcumin and curcumin derivatives

| Compound | $pK_{a1}$ | $pK_{a2}$ | $\log P_{oct}^{N}$ [a] | $\log P_{npoe}^{N}$ [a] | $\Delta \log P_{oct-npoe}^{N}$ [b] |
|---|---|---|---|---|---|
| Curcumin | 9.39 | 7.97 | 3.24 | 3.57 | −0.33 |
| Cu12 | 7.60 | 6.53 | 5.11 | 3.75 | 1.36 |
| Cyclovalone | 9.52 | 8.00 | 3.67 | 3.56 | 0.11 |
| Cy12 | 7.87 | 6.78 | 4.97 | 3.41 | 1.56 |

[a] $\log P$ of the neutral form measured by potentiometry.
[b] $\log P_{oct-npoe}^{N} = \log P_{oct}^{N} - \log P_{npoe}^{N}$.

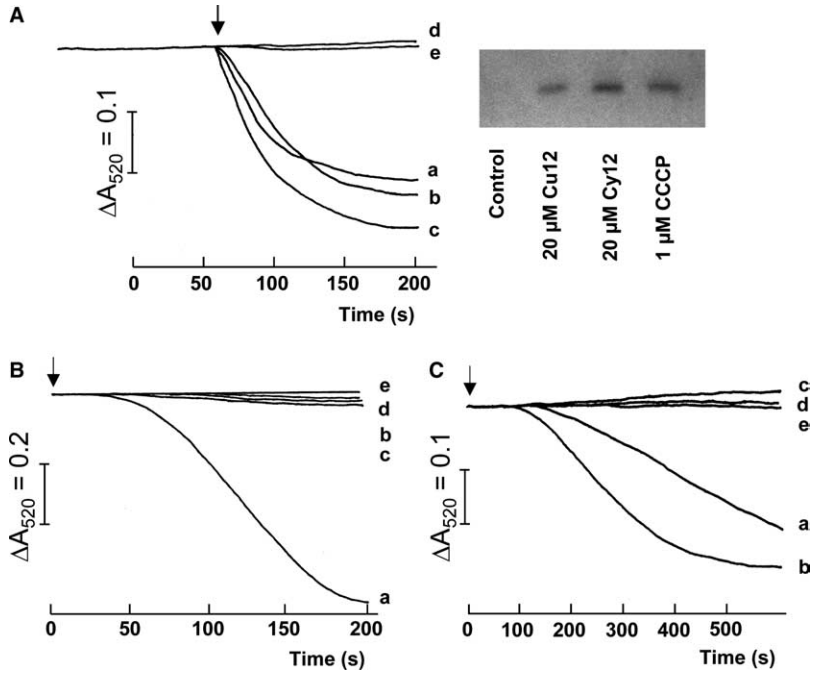


Fig. 4. Comparison of the effect of Cu12, Cy12 and CCCP on mitochondrial swelling in energized and deenergized conditions. Panel A: Rat liver mitochondria (0.5 mg/ml), energized with succinate (5 mM), were incubated in a medium containing 0.2 M sucrose, 10 mM Tris, pH 7.4, 1 mM $KH_2PO_4$, 20 μM EGTA–Tris, 2 μM rotenone and 1 μg/ml oligomycin. After 1 min incubation, a pulse of 25 μM $Ca^{2+}$ was added. Two min later, swelling was induced (arrow) by either 20 μM Cu12 (line a), 20 μM Cy12 (line b) or 1 μM CCCP (line c). 1 μM CsA (line d) and 500 μM 6-KCh (line e) inhibited the effect of Cy12. Identical results were found with Cu12 but for clarity reasons the curves were not introduced in the figure. In the same conditions, mitochondria were incubated for 20 min at room temperature. The release of cytochrome *c* was observed after addition of 1 μM CCCP, 20 μM Cu12 or 20 μM Cy12. Panel B: Liver mitochondria were preincubated for 3 min in the buffer supplemented with 6 mM succinate and 2 μM rotenone, pH 7.2, at 25 °C in the absence (line a) or in the presence of 50 μM Cy12 (line b), 50 μM Cu12 (line d), 1 μM CCCP (line c) or 1 μM CsA (line e). Then, swelling was induced (arrow) by the addition of 25 μM $Ca^{2+}$. Panel C: Mitochondria were preincubated for 3 min in a buffer containing 0.15 M sucrose, 5 mM Tris–HCl, 2 μM rotenone, and 1 μM antimycin A, pH 7.4, at 25 °C. Swelling was induced (arrow) by either 40 μM Cu12 (line a), 40 μM Cy12 (line b) or 1 μM CCCP (line c). The effect of Cy12 was inhibited in the presence of 1 μM CsA (line d) or 50 M *N*-ethylmaleimide (line e).

Table 2
Cu12 and Cy12-induced oxidation of membrane protein thiols

| Conditions | Thiol groups (%) |
|---|---|
| Control | 100.0 ± 1.8 |
| *t*-BH 1 mM | 66.1 ± 4.1[a] |
| Cu12 20 μM | 65.8 ± 3.9[a] |
| Cy12 20 μM | 74.4 ± 3.2[a] |
| CCCP 1 mM | 100.0 ± 4.8 |

Control value, expressed as 100%, corresponds to 255 nmol of thiol groups/mg proteins.
Values represent the means ± S.D. of five experiments in triplicate.
[a] $P < 0.01$ compared to the control value.

7.0 underlining that not only high lipophilicity but also high acidity and high complexation power may be responsible for the increase in uncoupler effects of Cu12 and Cy12.

All these results indicate that Cu12 and Cy12 act as uncoupling agents. Moreover, like curcumin, they induce protein thiol groups oxidation. These properties confer on Cu12 and Cy12 the ability to promote mitochondrial swelling and release of cytochrome *c*, events associated with induction of apoptosis by PTP opening. It was recently shown that treatment with uncouplers can induce apoptotic cell death in tumor lines [33]. Therefore, Cu12 and Cy12 may represent interesting antitumor agents, but this possibility remains to be investigated.

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