Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 65 (2003) 1979–1989
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Toxic effects of copper-based antineoplastic drugs (Casiopeinas®) on mitochondrial functions

Alvaro Marín-Hernández[a], Isabel Gracia-Mora[b], Lena Ruiz-Ramírez[b], Rafael Moreno-Sánchez[a,c,*]

[a]*Departamento de Bioquímica, Instituto Nacional de Cardiología, Juan Badiano # 1, Col. Sección XVI, Tlalpan, México D.F. 14080, Mexico*
[b]*Departamento de Química Inorgánica y Nuclear, Facultad de Química, UNAM, México D.F. 04510, Mexico*
[c]*Unidad de Biomedicina, Facultad de Estudios Superiores-Iztacala, UNAM, México D.F. Mexico*

Accepted 28 March 2003

Abstract

To elucidate some of the subcellular and biochemical mechanisms of toxicity of metal-based antineoplastic drugs, mitochondria and cells were exposed to Casiopeinas®, a new class of copper-based compounds with high antineoplastic activity. The rates of respiration and swelling, the H^+ gradient, and the activities of succinate (SDH) and 2-oxoglutarate dehydrogenases (2-OGDH) and ATPase were measured in mitochondria isolated from rat liver, kidney, heart, and hepatoma AS-30D. Also, oligomycin-sensitive respiration and ATP content in hepatoma AS-30D cells were determined. Casiopeinas® (CS) II-gly and III-i inhibited the rates of state 3 and uncoupled respiration in mitochondria. CS II was 10 times more potent than CS III. The sensitivity to CS II was 4–5-fold higher in mitochondria incubated with 2-OG than with succinate. Thus, at low concentrations ($\leq$10 nmol (mg protein)$^{-1}$; 10 μM), CS II disturbed mitochondrial functions only when 2-OG was present, due to a specific inhibition of 2-OGDH. At high concentrations ($\geq$15 nmol (mg protein)$^{-1}$), CS II-induced stimulation of basal respiration, followed by a strong inhibition, which correlated with K^+-dependent swelling and cytochrome *c* release, respectively; K^+-channel openers induce a similar mitochondrial response. Mitochondria from liver, kidney and hepatoma showed a similar sensitivity towards CS II, whereas heart mitochondria were more resistant. Oxidative phosphorylation and ATP content were also decreased in tumor cells by CS II. The data suggested that CS affected several different mitochondrial sites, bringing about inhibition of respiration and ATP synthesis, which could compromise energy-dependent processes such as cellular duplication.

Keywords: O_2 uptake; Membrane potential; Swelling; 2-Oxoglutarate dehydrogenase; K^+-channel openers; Cytochrome *c* release

1. Introduction

The success of cisplatin in the treatment of several solid tumors, particularly testicular cancer [1–3], which was unfortunately accompanied by severe side effects [4–6], prompted the search and development of new metal-based antineoplastic drugs with diminished toxic effects.

The predominant cisplatin toxic effect in both humans and laboratory animals involves renal tubular dysfunction, degenerative changes, and necrosis (reviewed by Goldstein and Mayor [7]). At the subcellular level, several morphological alterations including mitochondrial swelling, chromatin condensation, and microvilli loss, develop in kidney after cisplatin administration [4,8]. Kidney tubular epithelial cells contain a large number of mitochondria, and thus some of the cisplatin toxic effects seem to involve a direct interaction with these organelles. Indeed, the cisplatin concentration in renal cortical slices is at least five times higher than that in plasma [9]. Intracellularly, cisplatin is located in all subcellular fractions, but it is mainly concentrated in cytosol, microsomes and mitochondria [9–11]. This last observation indicates an active uptake of the cisplatin cation by cellular energy-dependent processes, which generate negative-inside transmembrane electric gradients.

* Corresponding author. Tel.: +52-555-573-2911x298; fax: +52-555-573-0926.
E-mail address: rmorenos@campus.iztacala.unam.mx (R. Moreno-Sánchez).

Abbreviations: CS II, casiopeina II-gly; CS III, casiopeina III-i; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; cisplatin, cisdichlorodiamine platinum(II); EGTA, ethylene glycol bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; Mops, 3-(*N*-morpholino)propane sulfonic acid; 2-OG, 2-oxoglutarate; 2-OGDH, 2-oxoglutarate dehydrogenase complex; SDH, succinate dehydrogenase.

0006-2952/03/$ – see front matter
doi:10.1016/S0006-2952(03)00212-0

Effects of cisplatin on the functions of kidney mitochondria, either isolated or inside intact cells, comprise collapse of membrane potential, disturbance of Ca^{2+} homeostasis, inhibition of ADP-stimulated (state 3) respiration, and enhancement in the formation of reactive oxygen species [8,12,13]. Tumor mitochondria also undergo similar alterations induced by cisplatin [14–16]. However, it has been proposed that mitochondria might not be a primary target for cisplatin and derivatives [17], since mitochondria are only marginally impaired by micromolar cisplatin concentrations, which are in the range of concentrations used in patients.

Despite this controversy, the observations on alterations of mitochondrial function may have clinical relevance. Mitochondria seem involved in tumorigenesis, through mutagenesis by transfer and insertion of mitochondrial DNA into nuclear DNA, or altered expression and mutation of mitochondrial DNA-encoded proteins. Mitochondria also seem involved in the maintenance of the malignant phenotype by still unknown mechanisms, perhaps related to ATP production [18,19]. Several different drugs, including polycyclic aromatic compounds [20,21], aflatoxin [22], cisplatin [23,24], and perhaps bleomycin [25], preferentially bind to mitochondrial DNA than to nuclear DNA. The naked (histone-lacking) mitochondrial DNA structure facilitates the access of drugs, and its limited repair mechanisms enhance mutation rate and permanent damage induced by drugs and oxidative stress [19,26]. The development of resistance to cisplatin in many tumor cell lines also seems related to mitochondrial alterations [27].

Seeking less toxic metal-based antineoplastic drugs, it was thought that the design and synthesis of mixed chelate copper-based drugs should bring about such a result, considering that copper is an essential metal ion [28]. Some of these copper-based drugs called Casiopeinas® have exhibited greater antineoplastic potency than cisplatin in *in vitro* and *in vivo* studies of a variety of tumor cell lines [28,29]. In addition, Casiopeinas® also show superoxide dismutase-like activity [30] and a low potency to induce genomic instability through intrachromosomal recombination [31]; these features suggest that these drugs have diminished undesirable side effects. However, the toxicological effects of Casiopeinas® on mitochondrial functions have not been evaluated as yet.

2. Material and methods

2.1. Chemicals

The following compounds were acquired from Sigma: sucrose, EGTA, protease Nagarse, digitonin 50%, safranin O, dithiothreitol (DTT), thiamine pyrophosphate, 2,6-dichlorophenol indophenol (DCPIP), phenazine methosulfate (PMS), Triton X-100, CCCP, pyruvate, 2-OG, succinate, glutamate, malate, glucose-6-phosphate dehydrogenase,

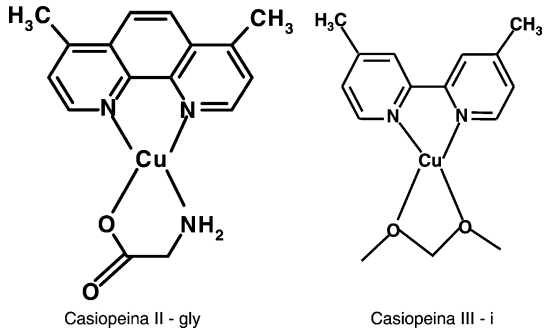

Fig. 1. Chemical structure of Casiopeinas®.

rotenone, antimycin, and oligomycin. NADH, NAD^+, $NADP^+$, ADP, ATP, coenzyme A, hexokinase, and valinomycin were from Boehringer. Mops and Hepes were from Research Organics. Horse heart cytochrome *c* was from ICN. [^{3}H]-Tetraphenylphosphonio ([^{3}H]-TPP^+) was from New England Nuclear. Pyranine was from Molecular Probes. All other chemicals of analytical grade were from J.T. Baker or Merck.

Casiopeinas® II-gly [(4,7-dimethyl-1,10-phenanthroline) (glycinate) copper(II) nitrate] and III-i [(4,4-dimethyl-2,2-bipyridine) (acetylacetonate) copper(II) nitrate] (Fig. 1), synthesized as described before [32], were dissolved in distilled water to 1 mg mL^{-1} weekly, stored at room temperature and kept in the dark. Cisplatin acquired from ABIC was freshly prepared to a concentration of 10 mM in distilled water.

2.2. Mitochondrial and cellular preparations

Mitochondria from liver [33], kidney [34], heart [35], and AS-30D hepatoma [36], liver submitochondrial particles (SMPs) [37] as well as AS-30D hepatoma cells [36] were isolated from Wistar rats as previously described. Protein was determined by the biuret method in the presence of 0.1% (w/v) deoxycholate, using bovine serum albumin as standard.

2.3. Mitochondrial functions

Respiration of mitochondria was measured by using a Clark-type O_2 electrode in an air-saturated medium that contained 120 mM KCl, 20 mM Mops, 0.5 mM EGTA, 5 mM K-phosphate, pH 7.2 (KMEPi buffer) at 30°. Respiration of SMP was measured in 250 mM sucrose, 10 mM Hepes, 1 mM EGTA, of pH 7.3 (SHE buffer) at 30°.

Tumor cells (40 mg protein/1.5 mL) were incubated in Ringer–Krebs medium (120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM KH_2PO_4, 1.4 mM $CaCl_2$, 25 mM Hepes, pH 7.4) at 37° under orbital shaking (150 rpm). At predetermined times, aliquots (2 mg protein mL^{-1}) were transferred to the oxymeter chamber for determination of cellular respiration at 37°. The O_2 solubility at 2240 m altitude was 400 and 390 ng oxygen atoms mL^{-1} at 30° in

KMEPi and SHE buffers, respectively, and 380 ng oxygen atoms mL^{-1} at 37° in Ringer–Krebs buffer. The cellular ATP content was determined in cells preincubated as described above. The cell samples were treated with 3% (v/v) perchloric acid and centrifuged; the supernatant was neutralized with KOH/Tris and used for determination of ATP by the standard enzymatic method with hexokinase and glucose-6-phosphate dehydrogenase.

Membrane potential was measured qualitatively by following the fluorescence signal of 5 μM safranin O incubated with mitochondria (0.5 mg protein mL^{-1}) in KMEPi buffer plus 10 mM succinate and 1 μM rotenone at 30°; the excitation and emission wavelengths were 495 and 586 nm [38]. The H^+ gradient was also quantitatively determined by measuring the [^{3}H]-TPP^+ distribution across the inner mitochondrial membrane as described elsewhere [39]. Swelling was measured at 25–28° by following the optical dispersion of mitochondrial suspensions (0.5 mg protein mL^{-1}) at 540 nm. ATP hydrolysis was determined from the scalar proton release measured with the fluorescent probe pyranine as described before [40].

2.4. Enzyme activities

The 2-OGDH activity was determined at 30° by following the generation of NADH at 340 nm. Mitochondria (0.5 mg protein mL^{-1}) were incubated in KME medium that contained 0.025% (v/v) Triton X-100, 0.85 mM thiamin pyrophosphate, 1 mM NAD^+, 10 mM 2-OG, and 15 mM $MgCl_2$; the reaction was started by addition of 0.1 mM fresh CoA [41]. The SDH activity was determined at 25–28° from the rate of reduction of 0.2 mM DCPIP at 600 nm by mitochondria incubated in 0.02% Triton X-100, 0.2 mM PMS, and 1 μM antimycin, in KME buffer; the reaction was started by addition of 10 mM succinate. The *b*–*c*1 cytochrome complex activity was determined from the rate of ferricyanide reduction, which was measured by the change in the absorbance difference at 440 − 490 nm in a dual wavelength spectrophotometer and using an extinction coefficient 0.59 mM^{-1} cm^{-1} [42]. The reaction mixture contained KME buffer plus 0.5 mg protein mL^{-1}, 0.6 mM KCN, 1 μM rotenone, and 1.5 mM sodium ferricyanide; the reaction was started by addition of 5 mM succinate.

2.5. Cytochrome *c* release

Liver mitochondria (10 mg protein) were added to 3 mL (final volume) of KME or SHE buffer that contained 10 mM succinate, 5 mM Pi, 2 μM rotenone and different CS II concentrations. At predetermined times, the mitochondrial suspension was centrifuged at 14,000 *g* for 3 min at 4°. The supernatant was used to determine the release of cytochrome *c* by measuring the absorbance difference spectrum of dithionite-reduced minus persulfate-oxidized samples; for calculations the extinction coefficient used was 19 mM^{-1} cm^{-1} for 550 − 540 nm [43].

3. Results

3.1. Respiration and membrane potential

ADP-stimulated (state 3) respiration was inhibited by both CS II or III in a time-dependent fashion, in mitochondria incubated with 2-OG (Fig. 2A) or succinate (not shown) as substrate. Similarly, membrane potential collapsed in the presence of CS in a time- and concentration-dependent manner (Fig. 2B). To establish the sensitivity of liver and hepatoma mitochondria towards CS, respiration was measured after 4 min of preincubation with CS II using two different oxidizable substrates. The rates of state 3 (Fig. 3A) and uncoupled respiration (Fig. 3C) with 2-OG were four to five times more sensitive to CS II than the respective respiration with succinate (+rotenone). There was not an apparent difference in respiratory sensitivity towards CS II between liver and hepatoma mitochondria. Under the same experimental conditions, cisplatin at a concentration of 75 μM did not significantly affect state 3 or uncoupled respiration in liver mitochondria (data not shown).

At the range of concentrations that inhibited state 3 respiration supported by 2-OG (<10 nmol (mg protein)$^{-1}$), CS II did not affect basal (state 4) respiration. At higher concentrations, CS II prompted stimulation of basal respiration with succinate, and inhibition with 2-OG (Fig. 3A, inset).

The use of safranin O allows the continuous monitoring of changes in membrane potential, but it may not be a quantitative measurement of this parameter [39,44]. Therefore, membrane potential ($\Delta\psi$) was also quantitatively determined by estimating the distribution of the lipophylic cation TPP^+ across the inner mitochondrial membrane. In the presence of a high Pi concentration (5 mM), most of the H^+ electrochemical gradient across the inner mitochondrial membrane is established by the electrical gradient or $\Delta\psi$, since the chemical gradient or ΔpH is stabilized at a value of approximately 0.1 [39,44]. The sensitivity of $\Delta\psi$ towards CS II (Fig. 3B) was similar to that attained for state 3 or uncoupled respiration, with 2-OG. However, $\Delta\psi$ was more sensitive to CS II than respiration with succinate as oxidizable substrate, suggesting that low concentrations of CS II may have other effects on liver and hepatoma mitochondria.

The force–flux relationship (Fig. 3D), attained with the low range of CS II concentrations (1–10 nmol (mg protein)$^{-1}$), revealed a proportional diminution of both the rate of state 3 respiration and $\Delta\psi$ when mitochondria were incubated with 2-OG. This observation indicated a strong inhibition of the H^+ gradient generating branch of the oxidative phosphorylating pathway. On the other hand,

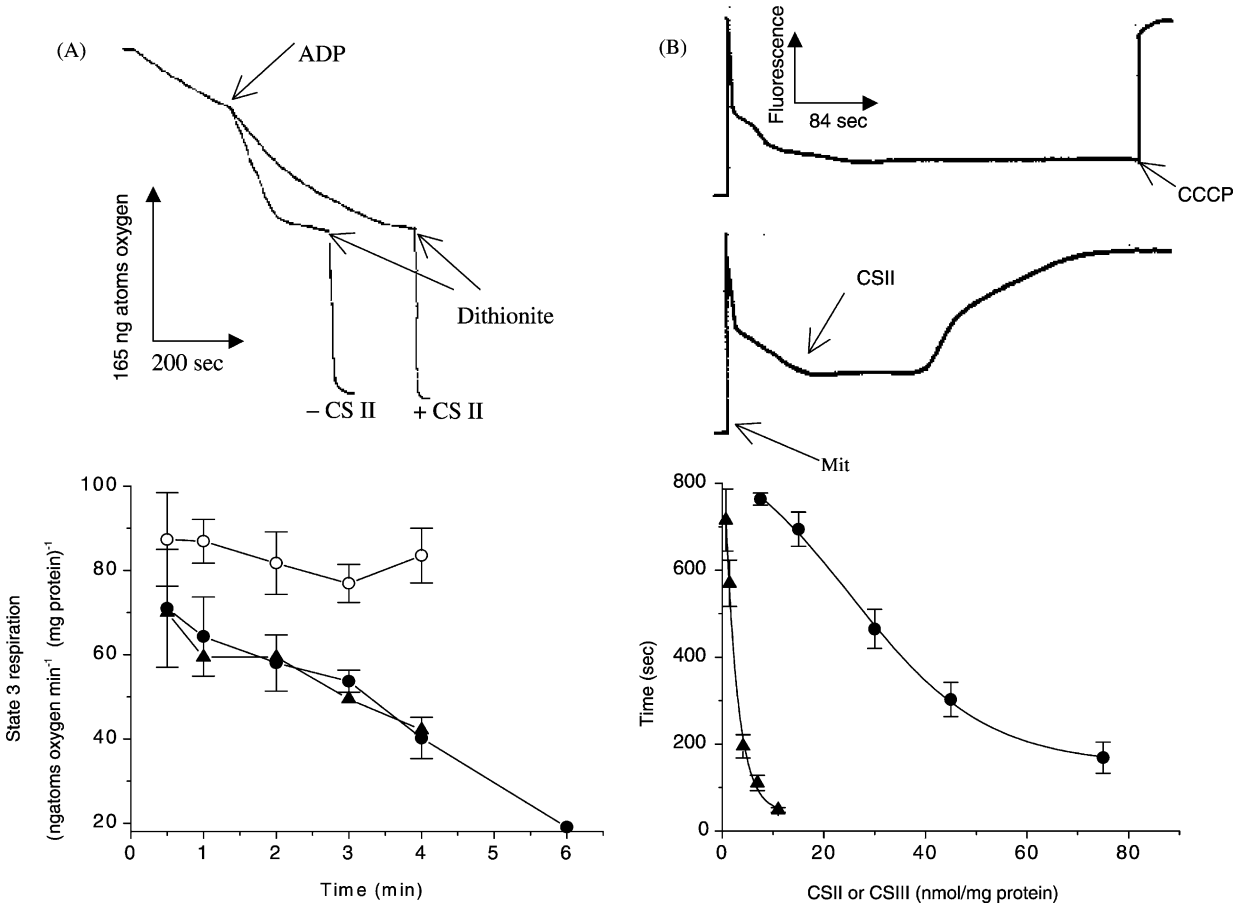

Fig. 2. Effect of casiopeinas II and III on state 3 respiration and membrane potential. (A) Rat liver mitochondria (1 mg protein mL^{-1}) were incubated at 30° in 2 mL of KMEPi buffer with 10 mM 2-OG for the indicated times. Then, 0.6 mM ADP was added to initiate state 3 respiration. Casiopeinas II (8.9 nmol (mg protein)$^{-1}$; 8.9 μM) (triangles) or III (73 nmol (mg protein)$^{-1}$; 73 μM) (full circles) were added at the beginning of the incubation period; mitochondria incubated in the absence of CS II (open circles) were used as control. A few grains of dithionite were added where indicated to obtain the chemical zero of oxygen concentration. The data shown represent the mean ± standard error of the mean (SEM) of three to four different mitochondrial preparations. The vertical scale (165 ng atoms oxygen) applies to the total incubation medium of 2 mL. (B) Rat liver mitochondria (0.5 mg protein mL^{-1}; Mit) were incubated at 30° in 2 mL of KMEPi buffer with 10 mM succinate and 5 μM safranin O. The fluorescence signal of safranin was measured as described under Section 2. Casiopeinas II (triangles) or III (circles) were added as indicated. The time elapsed from the addition of the drug to the initiation of the collapse of the membrane potential was plotted against the casiopeina concentration. CCCP, 4.7 μM. Mean ± SEM; N = 4.

mitochondria incubated with CS II and succinate exhibited a diminution of $\Delta\psi$ with no concomitant lowering of state 3 respiration. In consequence, at the same steady-state value of $\Delta\psi$, mitochondria that oxidized succinate showed higher rates of respiration than those with 2-OG. Thus, CS II apparently uncoupled succinate oxidation.

The half-maximal inhibitory concentrations (IC_{50}) for CS are shown in Table 1. For the parameters measured in liver mitochondria, CS II was up to 10–20 times a more potent inhibitor than CS III. The higher sensitivity to CS II when mitochondria oxidized 2-OG suggested a specific inhibition of either 2-OG transport and dehydrogenation or NADH dehydrogenation, sites that were not active when mitochondria oxidized succinate (+rotenone). Similarly, addition of 75 μM $CuCl_2$ or $Cu(NO_3)_2$ fully inhibited the rate of basal respiration in liver mitochondria incubated with 2-OG in an EGTA-lacking medium (data not shown). Since state 3 and uncoupled respiration exhibited similar IC_{50}, the lower sensitivity to CS II when mitochondria oxidized succinate suggested a second site of inhibition in the respiratory chain, although the phosphorylating system (Pi and ATP/ADP carriers, and ATP synthase) might also be affected. The sensitivity of the liver, kidney and hepatoma mitochondrial functions towards CS II was essentially identical, whereas mitochondria from heart showed a significantly greater resistance (Table 1).

3.2. Mitochondrial sites for CS inhibition

Seeking specific sites of action of CS II, the rates of respiration and ATP hydrolysis, and the H^+ gradient of coupled SMPs were examined. In SMP, the respiratory chain and the ATP synthase do not require a substrate (oxidizable substrate, ADP, or Pi) transport reaction and both enzyme systems are directly accessible for interaction with CS II. However, CS II, up to a concentration of 10 nmol (mg protein)$^{-1}$, did not affect the rate of respiration with

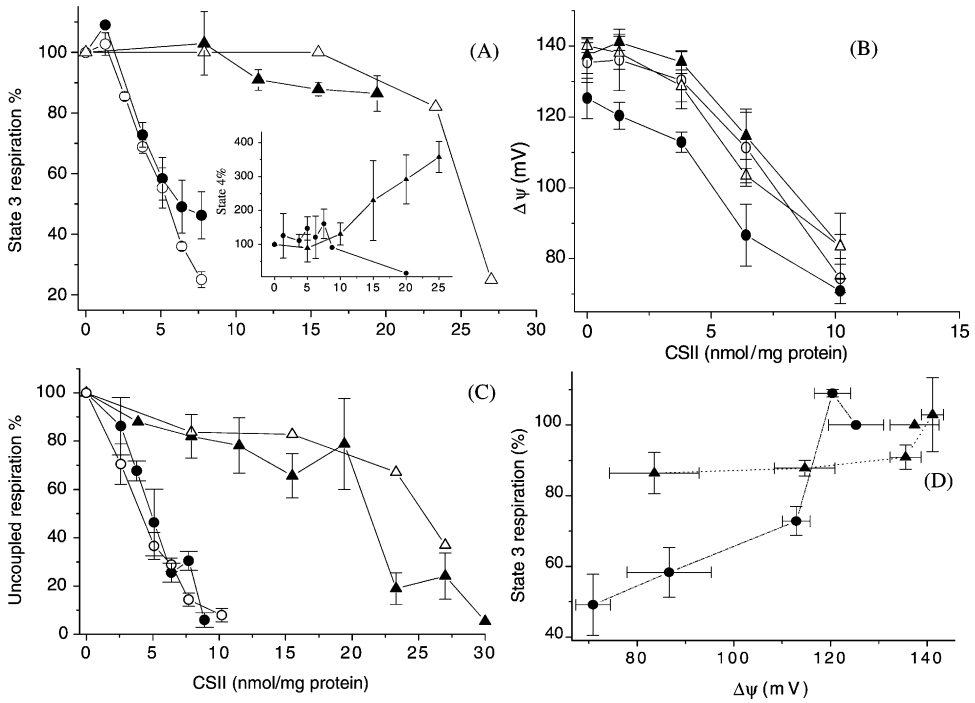

Fig. 3. Sensitivity of coupled respiration, uncoupled respiration and membrane potential towards casiopeina II. Rat liver (●, ▲) or hepatoma AS-30D (○, Δ) mitochondria were incubated as described in the legend to Fig. 2 with the indicated concentrations of casiopeina II and either with 10 mM 2-oxoglutarate (circles) or 10 mM succinate (+1 μM rotenone) (triangles). After 4 min, 0.6 mM ADP or 0.4 μM CCCP was added to start state 3 (A) or uncoupled (C) respiration, respectively. The rates of state 3 and uncoupled respiration in liver mitochondria, in the absence of drugs, were 75 ± 15 (3) ng atoms oxygen min^{-1} (mg protein)$^{-1}$ and 60 ± 13 (3) ng atoms oxygen min^{-1} (mg protein)$^{-1}$ for 2-OG, and 245 ± 59 ng atoms oxygen min^{-1} (mg protein)$^{-1}$ and 313 ± 3 (3) ng atoms oxygen min^{-1} (mg protein)$^{-1}$ for succinate, respectively. In hepatoma mitochondria the rates of state 3 and uncoupled respiration were 113 ± 2 (3) ng atoms oxygen min^{-1} (mg protein)$^{-1}$ and 83 ± 15 (3) ng atoms oxygen min^{-1} (mg protein)$^{-1}$ for 2-OG, and 165 (2) and 106 ± 2 (3) ng atoms oxygen min^{-1} (mg protein)$^{-1}$ for succinate, respectively. The ADP/O ratio values for 2-OG and succinate were 2.7 ± 0.2 (3) and 1.7 ± 0.2 (3) in liver mitochondria, and 2.3 ± 0.2 (3) and 1.4 (2) in hepatoma mitochondria, respectively. The rates of state 4 respiration were 15 ± 3 (3) ng atoms oxygen min^{-1} (mg protein)$^{-1}$ and 23 ± 6 (3) ng atoms oxygen min^{-1} (mg protein)$^{-1}$ in liver mitochondria, and 14 ± 2 (3) and 64 (3) ng atoms oxygen min^{-1} (mg protein)$^{-1}$ in hepatoma mitochondria with 2-OG and succinate, respectively. For determination of Δψ (B, D), mitochondria (1.5–2 mg protein) were incubated in 0.5 mL of KMEPi buffer with 0.8 μM ^{3}H-TPP^{+}. ADP (2 mM) was added after 4 min; 1 min later mitochondria were centrifuged to estimate the electric gradient across the inner mitochondrial membrane as described before [39,44], using the Nernst equation and correcting for unspecific external and internal binding. Data shown are the mean ± SD of four different preparations.

either succinate or NADH as oxidizable substrate, or the rate of ATP hydrolysis (data not shown). These data indicated that CS II inhibited respiration and membrane potential at sites different from the respiratory chain and the ATP synthase. A negligible effect of CS II on the *b–c*1 cytochrome complex activity concurred with the last interpretation. Assay of the cytochrome *c* oxidase activity was not feasible since a high nonenzymatic reaction between ascorbate and CS II evolved.

Because 2-OG oxidation was more sensitive to CS than succinate oxidation, and CS did not affect the respiratory chain, it was thought that a primary target could be the

Table 1
Half-maximal concentrations of casiopeinas (nmol (mg protein)$^{-1}$) for inhibition of mitochondrial functions

	Liver			AS-30D			Kidney	Heart		
	Respiration		Δψ	Respiration		Δψ	Δψ	Respiration		Δψ
Casiopeina II										
Substrate	St 3	unc	St 3	St 3	unc	St 3	St 3	St 3	unc	St 3
2-OG	4.7 ± 0.7 (3)	4.4 ± 0.5 (4)	5.5 ± 0.9 (4)	4.6 ± 0.7 (3)	3.6 ± 0.3 (3)	5.2 ± 0.1 (3)	6.3 (2)	12 ± 2 (3)	10.5 ± 1.8 (3)	9.6 ± 2.3 (3)
Succinate	21 ± 0.7 (3)	16 ± 3 (3)	6.4 ± 0.2 (4)	18 (2)	27.5 (2)	7.4 ± 0.6 (3)				
Casiopeina III										
Succinate	239 ± 12 (3)	330 ± 15 (3)								
2-OG	77 ± 4 (3)	90 ± 12 (3)								

The values shown represent the mean ± standard deviation with the number of different preparations assayed between parentheses. Abbreviations: St 3, state 3 (+ADP) conditions; unc, uncoupled respiration. Half-maximal concentrations were estimated by assuming full CS II inhibition of state 3 and uncoupled respiration. For ψ, the minimal value obtained, of approximately −60 mV, was assumed to be the basal (zero) level.

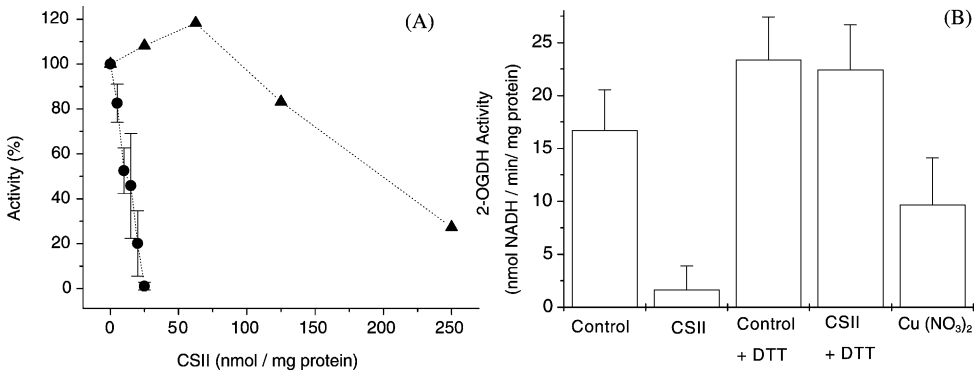

Fig. 4. Inhibition of 2-OGDH and SDH by CS II. (A) Rat liver mitochondria were incubated as described in Section 2 for the determination of the 2-OGDH (circles) and SDH (triangles) activities in the presence of the indicated concentrations of CS II. After 4 min, the reaction was started by addition of either CoA or succinate. The SDH rate in the absence of drug was 100 nmol DCPIP reduced min^{-1} mg^{-1} (2). (B) CS II, 25 nmol (mg protein)$^{-1}$; DTT, 1 mM; $Cu(NO_3)_2$, 75 μM. Adding $CuCl_2$, instead of $Cu(NO_3)_2$, yielded essentially the same result; 7.5 μM Cu did not inhibit. Data with 2-OG are the mean ± SD of three to six independent experiments.

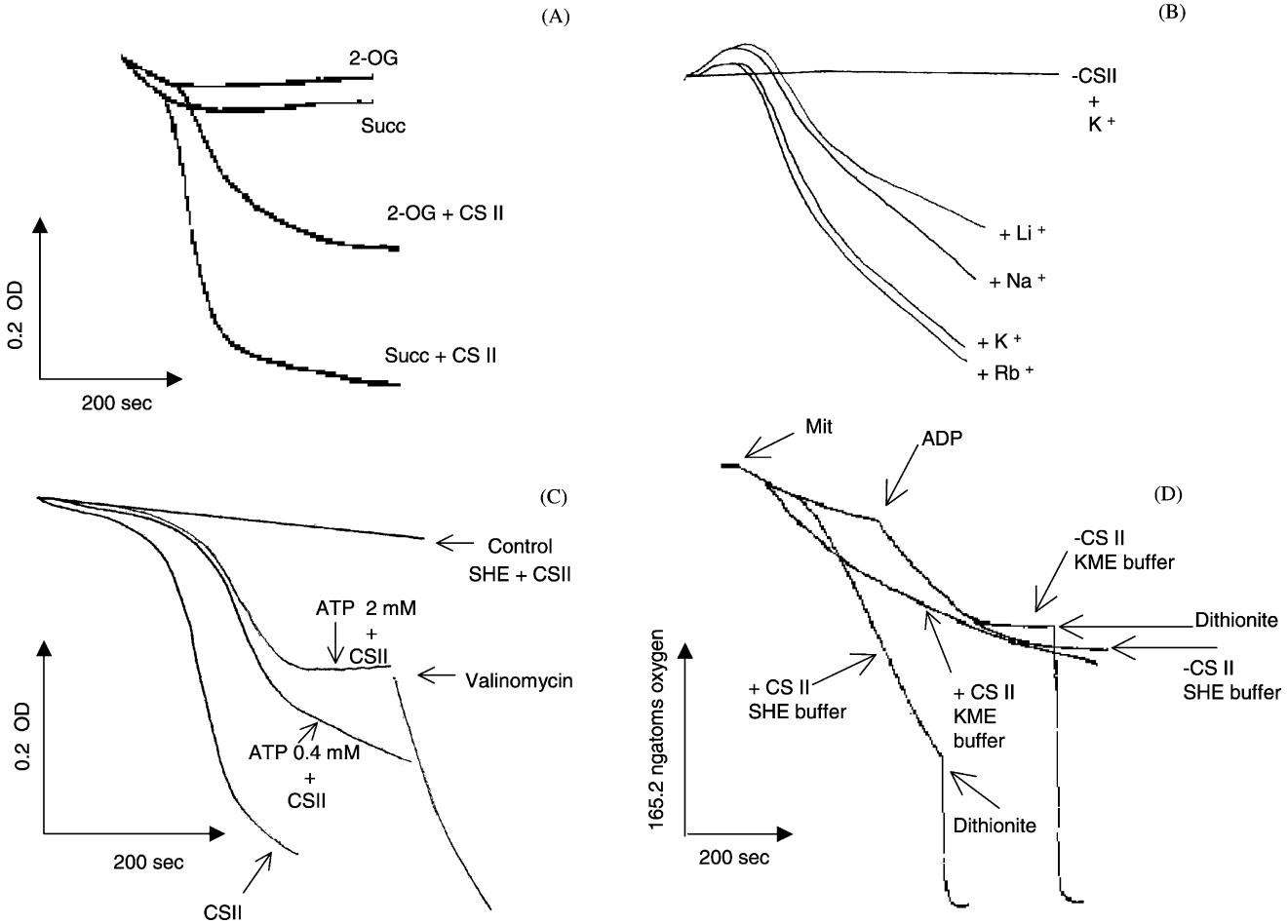

Fig. 5. Ion dependence of the CS II-induced swelling. Rat liver mitochondria (1 mg protein) were added to 2 mL of KME (or SHE buffer where indicated) with 10 mM 2-OG (A) or 10 mM succinate and 1 μM rotenone (A, C), and 25 nmol CS II (mg protein)$^{-1}$. Where indicated, the reaction medium also contained ATP. Valinomycin was 5 nmol. The change in light scattering at 540 nm was recorded after the addition of mitochondria. (B) Mitochondria (1 mg protein) were incubated in 2 mL of 20 mM Mops, 1 mM EGTA, 10 mM succinate + 1 μM rotenone, pH 7.2 with 120 mM of each of the indicated cations (chloride salts) and 25 nmol CS II (mg protein)$^{-1}$. The incubation medium in the absence of added salts contained approximately 10 mM K^+. (D) Mitochondria (1 mg protein) were incubated in 2 mL of either KMEPi or SHEPi media with 10 mM succinate + 1 μM rotenone and, where indicated, 25 nmol CS II (mg protein)$^{-1}$. ADP was 300 nmol.

2-OGDH. The 2-OGDH activity in liver mitochondria was completely inhibited by CS II with an IC_{50} of 10 nmol (mg protein)$^{-1}$ (Fig. 4A). Preincubation of Triton-permeabilized mitochondria with CS II did not significantly increase the inhibition of 2-OGDH, unless the reaction was not started by addition of CoA. This indicated that CS II directly reacted with the CoA thiol group. Protection of the 2-OGDH activity from CS II inhibition by the thiol-reducing agent DTT, and the inhibition by cupric cations (Fig. 4B), supported the notion of a direct reaction of CS II with free thiol groups. SDH, an enzyme with essential thiol groups [45], was also inhibited by CS II, although at much higher concentrations (IC_{50} of 200 nmol (mg protein)$^{-1}$).

3.3. Ion-dependent swelling

Uptake of oxidizable substrate, measured by following the rate and extent of swelling of mitochondrial suspensions, incubated with 0.1 M ammonium salts of either 2-OG or succinate [46], was not affected by CS II, in the presence or in the absence of added Pi (data not shown). This observation indicated that the drug did not act on the substrate carriers. However, mitochondria incubated in an isotonic (KME) medium with 10 mM substrate developed swelling after addition of CS II, being faster and more extensive with succinate than with 2-OG (Fig. 5A).

Further analysis revealed that CS II prompted mitochondrial swelling in K^+-containing (Fig. 5B) but not in sucrose-containing media (Fig. 5C). The selectivity of this CS II-induced ion permeability was $Rb^+ > K^+ > Na^+ > Li^+$ (Fig. 5B). Concentrations of CS II lower than 10 nmol (mg protein)$^{-1}$ also induced swelling, but at slower rates, after longer incubation times and of a shorter extent (not shown). The extent of CS II-induced swelling was similar to that induced by the K^+ ionophore valinomycin (Fig. 5C); the uncoupler CCCP was unable to induce significant swelling (not shown). ATP in the absence (Fig. 5C) or in the presence of 1 mM $MgCl_2$ (not shown) strongly blocked K^+-dependent CS II-induced mitochondrial swelling; cyclosporin A (2 nmol (mg protein)$^{-1}$), an inhibitor of the mitochondrial permeability transition pore, slightly prevented the swelling induced by CS II (not shown).

3.4. Cytochrome c release

The time-courses of K^+-dependent swelling (Fig. 5C) and uncoupling of basal respiration (Fig. 5D), both induced by CS II, correlated well, suggesting a mechanistic relationship in which a massive K^+ entry collapsed membrane potential and, hence, stimulated respiratory activity. It was noted that after the initial burst of stimulated respiration induced by CS II, a secondary phase of

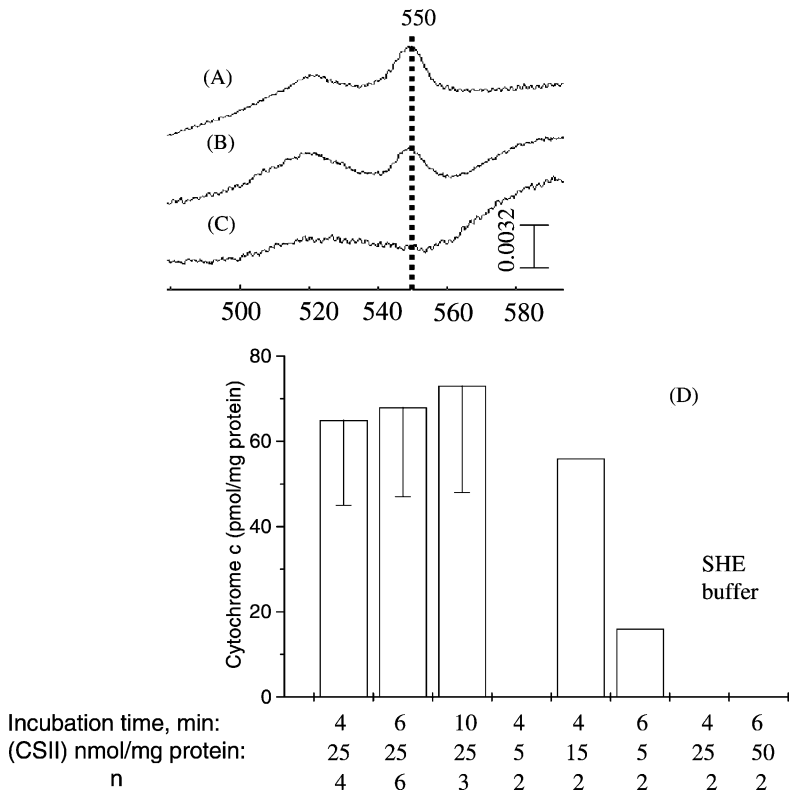

Fig. 6. Release of mitochondrial cytochrome *c* induced by CS II. Mitochondria were incubated in KME (B) or SHE (C) buffer with 25 nmol CS II (mg protein)$^{-1}$ for 4 min as described in Section 2. The absorbance difference spectra shown in B and C were corrected for the spectrum resulting from supernatants of mitochondria incubated in the absence of CS II and also for the spectrum of CS II in solution. The spectrum of 136 pmol horse heart cytochrome *c* is shown in A. Where indicated in D, SHE buffer replaced KME buffer.

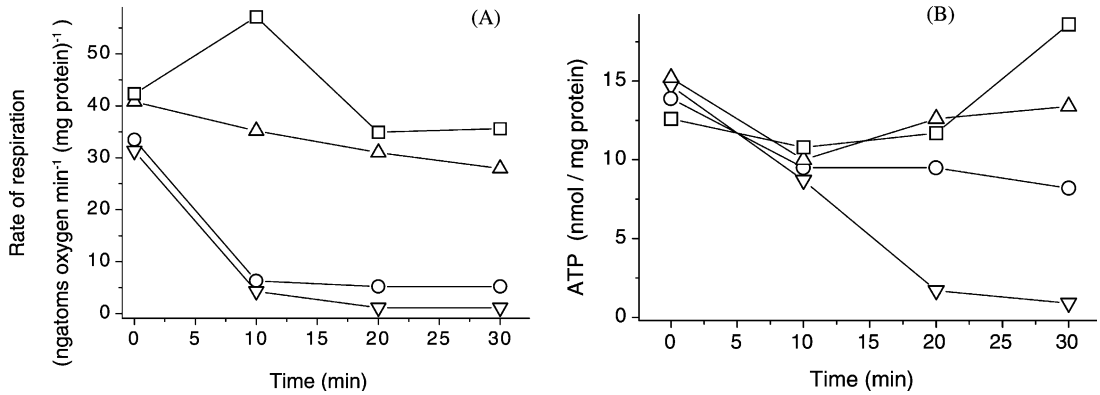

Fig. 7. Inhibition of oxidative phosphorylation in tumor cells by CS II. AS-30D hepatoma cells were preincubated for the indicated times with none (□), 1.5 nmol CS II (mg protein)$^{-1}$ (Δ), 3 nmol CS II (mg protein)$^{-1}$ (○), or 6 nmol CS II (mg protein)$^{-1}$ (▽) as described in Section 2. Then, aliquots were withdrawn for determination of the rate of 2.5 μM oligomycin-sensitive cellular respiration and the ATP content.

respiratory inhibition followed, when KME buffer was used. In turn, this latter phase correlated in a time- and CS II concentration-dependent fashion with the net release of cytochrome *c* (Fig. 6). In sucrose medium, there was also found a strong correlation between the negligible swelling (Fig. 5C) and the null release of cytochrome *c* (Fig. 6) prompted by CS II. The stable stimulatory effect of CS II on respiration in SHE medium, as compared to the transient respiratory stimulation in KME medium (Fig. 5D), suggested that K^+ was not a strict requirement for the uncoupling effect of CS II.

3.5. CS II effect on cellular respiration and ATP content

The rate of oligomycin-sensitive respiration in intact cells, as a measurement of *in situ* oxidative phosphorylation, was inhibited by CS II in a time- and concentration-dependent manner (Fig. 7A). After 4 min of preincubation, an IC_{50} of 2.5 ± 0.06 nmol (mg protein)$^{-1}$ ($N = 3$) was estimated for tumor cells, which was significantly lower than that obtained for isolated mitochondria (see Table 1). Despite full respiratory blockade induced by CS II after 4 min, the intracellular ATP level remained constant for at least 10 min; only after 20 min, incubation with 3 or 6 nmol CS II (mg protein)$^{-1}$ promoted diminution of cellular ATP. Similarly, oligomycin diminished respiration after 2–3 min, whereas the ATP levels started to decrease after 5 min (not shown).

4. Discussion

The data of the present work show that CS may directly interact with mitochondria, isolated or within intact cells, inducing a variety of effects on different sites, which brings about inhibition of oxidative phosphorylation and, eventually, cellular ATP depletion. The most pronounced CS II effect, at concentrations below 10 nmol (mg protein)$^{-1}$, was the strong inhibition of 2-OGDH through a reaction with the CoA thiol group. As the pyruvate dehydrogenase complex also requires free CoA, it is conceivable that inhibition of pyruvate oxidation-dependent reactions by CS II would also be achieved. Indeed, state 3 respiration supported by pyruvate (+malate) showed a sensitivity towards CS II ($IC_{50} = 3.7 \pm 1.5$ nmol (mg protein)$^{-1}$; $N = 3$) similar to that supported by 2-OG (cf Table 1). State 3 respiration with glutamate + malate, a condition which only indirectly depends on CoA availability, was inhibited by CS II ($IC_{50} = 8.2 \pm 2.3$ nmol (mg protein)$^{-1}$; $N = 3$) at concentrations higher than those used with 2-OG, but lower than with succinate. Moreover, CS II very likely interacted with other thiol-containing compounds such as glutathione, but because these other reactions were not directly involved in oxidative phosphorylation, they were not examined.

At concentrations higher than 10 nmol (mg protein)$^{-1}$, the CS II effects were, initially, very probably related to the opening of the ATP-sensitive K^+-channel (recently reviewed by Szewczyk and Wojtczak [47]), which in turn brought about an extensive mitochondrial swelling. A massive K^+ uptake through this channel may induce uncoupling and, hence, collapse of membrane potential, stimulation of basal respiration, and inhibition of oxidative phosphorylation. Shortly after these initial effects, another CS II effect emerged, that of respiratory inhibition by promoting the release of cytochrome *c*. Interestingly, the K^+-channel openers, including cuprous ions, induce a similar pattern of events in mitochondria [48–51]; apoptosis of tumor cells induced by cisplatin [14] may also be mediated by release of cytochrome *c* [52,53]. In addition, the pronounced diminishing effect of CS II on membrane potential with no proportional effect on state 3 respiration, in mitochondria that oxidized succinate, cannot be ascribed to simple uncoupling. This anomalous response to CS II might result from a combination of respiratory inhibition with some uncoupling effect, resulting in collapse of the membrane potential with no significant variation in respiration.

Gordon and Gattone [8] described that a single intraperitoneal (i.p.) injection of 5.5 mg (18.3 μmol) cisplatin kg^{-1}

body weight to rats inhibited both state 4 and state 3 respiration and promoted swelling in kidney mitochondria isolated 3 days later, although it was still ineffective on liver mitochondria. Kharbangar *et al*. [16] reported diminution in the total content of mitochondrial protein 24 hr after a single i.p. injection of 8 mg cisplatin kg^{-1} body weight to mice in kidney and lymphoma, but not in liver, indicating a tissue-specific effect. The lower sensitivity of liver mitochondria towards cisplatin [8,16] might be related to a limitation in its transport, which is not expected to occur with the more hydrophobic CS molecules. Thus, CS II and III induced a similar degree of inhibition on state 3 respiration of both kidney and liver mitochondria (cf Table 1).

Mouse liver mitochondria incubated with 50–100 μM cisplatin (25–50 nmol (mg protein)$^{-1}$) for 45 min exhibited swelling, outer membrane disruption, and diminished respiratory complexes I and II activities [54]. These cisplatin concentrations were comparable to those used in the present work with casiopeinas, although the incubation time was shorter for the copper-based drugs.

Incubation of renal tubular cells with cisplatin (25–100 nmol/10^6cells) for 20 min or longer resulted in a significant decrease in viability, cell ATP, and mitochondrial functions [16]. With whole proximal tubules, higher concentrations of cisplatin and longer incubation times were required to attain 50% inhibition of O_2 uptake (1 mM for 40 min, and 0.1 mM for 4 hr) [13]. By comparison, the mechanical performance and rate of respiration of isolated perfused rat heart were inhibited by CS II concentrations higher than 10 μM, with full blockade of both function achieved with 40 μM after 5 min incubation.[1] CS II also showed a potent effect on tumor cells: 50% inhibition of respiration with 2.5 nmol (mg protein)$^{-1}$ after only 4 min incubation and 50% decline in ATP levels with 6 nmol (mg protein)$^{-1}$ after 15 min incubation (cf Fig. 7). The half-maximal concentrations of CS II to inhibit cell growth after 24 hr incubation in several tumor lines were around 1 μM or 1 nmol/10^6 cells [28]. Data on growth inhibition of normal proliferating cells by casiopeinas remains to be established.

Severe nephrotoxicity in mice appears by the third day after receiving a single i.p. dose of 10 mg cisplatin kg^{-1} [55]. Nephrotoxicity is also caused by multiple low (5 mg kg^{-1}) doses [55], a condition which resembles that of cancer patients receiving low cisplatin doses (2 mg kg^{-1}) for extended periods of time [6]. Furthermore, cisplatin at high (>10 mg kg^{-1}) or low (2 mg kg^{-1}) i.p. doses in mice or rats, respectively, causes death after 1 or 2 days in mice [55], or after 2 or 3 weeks in rats [56], whereas the i.p. and i.v. lethal doses 50 of CS II are 8.9 (22.7 μmol) and 16 mg kg^{-1}, respectively, after 24 hr incubation in mice.[2]

According to the data of the present work, exposure of liver, kidney and ascites AS-30D mitochondria to CS II (1–10 nmol (mg protein)$^{-1}$) should induce similar inhibitory effects on respiration and membrane potential, and hence on ATP synthesis. This may imply a limited therapeutic use of CS II as an antineoplastic drug, although an encouraging lower toxic effect of CS II on heart mitochondria was observed. Moreover, it should be emphasized that the sensitivity of tumor cells [28] and tumor-bearing animals[2] towards Casiopeinas® may differ from that attained in isolated mitochondria.

The greater resistance of heart mitochondria towards CS II may be related to the larger content of respiratory enzymes and transporters involved in respiration and oxidative phosphorylation. Indeed, heart mitochondria exhibit higher respiratory and phosphorylating rates than liver, kidney and AS-30D mitochondria. Alternatively, heart mitochondria may have a higher content of thiol-containing compounds, mainly glutathione.

Current antineoplastic drugs, commonly aimed at nuclear DNA, have achieved only limited success largely due to their lack of specificity for tumor cells, since they also affect normal cells. Many of these drugs damage DNA, triggering the cell death pathways of necrosis and apoptosis. However, tumor cells may develop resistance mechanisms or they may be injured but still be able to recover. Therefore, it is relevant to develop alternative strategies aimed at novel cellular targets that are sufficiently different between normal and tumor cells to enhance the probability of selective tumor cell killing. Lipophilic cation drugs are concentrated by cells into mitochondria because of the large negative-inside electric membrane potential [57–59]. The higher plasma and mitochondrial membrane potentials of tumor cells [60–62] may enhance the selective targeting of this type of drugs into tumor cells and mitochondria. AS-30D hepatoma mitochondria also exhibited higher $\Delta\psi$ values than did mitochondria from liver, the organ from which tumor mitochondria were derived. Indeed, AS-30D and HeLa cells in culture died within 48 hr on exposure to CS II.[3] It is not known whether these tumor cells died because mitochondrial function was specifically impaired by CS II, but at least it certainly was one of the targets since oligomycin-sensitive respiration was inhibited.

One problem that arises from the present findings is that CS were not specific for tumor mitochondria, but it also affected normal mitochondria. Cisplatin also presents a similar problem: its use in patients has shown high antineoplastic potency in testicular, ovarian, and head and neck carcinomas, which is unfortunately accompanied by severe side effects on renal function. Hence, it is conceivable to predict that, despite the exhibited high antineoplastic activity [28,29,31], significant side effects on susceptible tissues and organs at the mitochondrial level will develop by CS administration to animal models carrying carcinomas. Intraperitoneal or intravenous administration of CS to

[1] Carvajal K, unpublished data.
[2] Gracia-Mora I, Ruiz-Ramírez L, unpublished data.
[3] Rodríguez-Enríquez S, Moreno-Sánchez R, unpublished data.

rats results in drug accumulation into several organs, but mainly in liver, kidney and heart; electron microscopy of these organs has shown profound morphological alterations in mitochondria.[2] However, the better understanding of the biochemical basis of the CS toxic effects, as initially described in the present work, may allow to design improved drugs with diminished undesirable secondary effects.

Acknowledgments

This work was partially supported by Grant G 35012-N from CONACyT-México. The authors thank Dr. G. MacCarthy for English corrections.

References

[1] Rosencweig M, Von Hoff DD, Slavik M, Muggia FM. Cisdiamminedichloroplatinum(II). A new anticancer drug. Ann Int Med 1977;86: 803–12.
[2] Williams SD, Einhorn LH. Cisplatinum in the treatment of testicular and other cancers. Adv Int Med 1982;27:531–45.
[3] Ensley JF, Jacobs JR, Weaver A, Kinzie J, Crissman J, Kish JA, Cummings G, Al-Sarraf M. Correlation between response to cisplatinum-combination chemotherapy and subsequent radiotherapy in previously untreated patients with advanced squamous cell cancers of the head and neck. Cancer 1984;54:811–4.
[4] Dobyan DC, Levi J, Jacobs C, Kosek J, Weiner MW. Mechanism of cisplatinum nephrotoxicity. II. Morphologic observations. J Pharmacol Exp Ther 1980;213:551–6.
[5] De Conti RC, Toflness BR, Lange RC, Creasey WA. Clinical and pharmacological studies with cisdiamminedichloroplatinum(II). Cancer Res 1973;33:1310–5.
[6] Porter GA, Bennett WM. Nephrotoxic acute renal failure due to common drugs. Am J Physiol 1981;241:F1–8.
[7] Goldstein RS, Mayor GH. The nephrotoxicity of cisplatin. Life Sci 1983;32:685–90.
[8] Gordon JA, Gattone VH. Mitochondrial alterations in cisplatin-induced acute renal failure. Am J Physiol 1986;250:F991–8.
[9] Safirstein R, Miller P, Guttenplan JB. Uptake and metabolism of cisplatin by rat kidney. Kidney Int 1984;25:753–8.
[10] Levi J, Jacobs SM, Kalman M, McTigue M, Weiner MW. Mechanism of cisplatinum nephrotoxicity. I. Effects of sulfhydryl groups in rat kidneys. J Pharmacol Exp Ther 1980;213:545–50.
[11] Choie DD, del Campo AA, Guarino AM. Subcellular localization of cisdichlorodiammineplatinum(II) in rat kidney and liver. Toxicol Appl Pharmacol 1980;55:245–52.
[12] Kruidering M, Van de Water B, de Heer E, Mulder GJ, Nagelkerke JF. Cisplatin-induced nephrotoxicity in porcine proximal tubular cells: mitochondrial dysfunction by inhibition of complexes I to IV of the respiratory chain. J Pharmacol Exp Ther 1997;280:638–49.
[13] Brady HR, Kone BC, Stromski ME, Zeidel ML, Giebish G, Gullans SR. Mitochondrial injury: an early event in cisplatin toxicity to renal proximal tubules. Am J Physiol 1990;258:F1181–7.
[14] Melendez-Zajgla J, Cruz E, Maldonado V, Espinoza AM. Mitochondrial changes during the apoptotic process of HeLa cells exposed to cisplatin. Biochem Mol Biol Int 1999;47:765–71.
[15] Andrews PA, Albright KD. Mitochondrial defects in cisdiamminedichloroplatinum(II)-resistant human ovarian carcinoma cells. Cancer Res 1992;52:1895–901.
[16] Kharbangar A, Khynriam D, Prasad SB. Effect of cisplatin on mitochondrial protein, glutathione, and succinate dehydrogenase in Dalton lymphoma-bearing mice. Cell Biol Toxicol 2000;16: 363–73.
[17] Kuhlmann MK, Burkhardt G, Kohler H. Insights into potential cellular mechanisms of cisplatin nephrotoxicity and their clinical application. Nephrol Dial Transplant 1997;12:2478–80.
[18] Cavalli LR, Liang BC. Mutagenesis, tumorigenicity, and apoptosis: are the mitochondria involved? Mutat Res 1998;398:19–26.
[19] Penta JS, Johnson FM, Wachsman JT, Copeland WC. Mitochondrial DNA in human malignancy. Mutat Res 2001;488:119–33.
[20] Backer JM, Weinstein IB. Mitochondrial DNA is a major cellular target for a dihydrodiol-epoxide derivative of benzo[a]pyrene. Science 1979;209:297–9.
[21] Allen JA, Coombs MM. Covalent binding of polycyclic aromatic compounds to mitochondrial and nuclear DNA. Nature 1980;287: 244–5.
[22] Niranjan BG, Bhat NK. Preferential attack of mitochondrial DNA by aflatoxin b, during hepatocarcinogenesis. Science 1982;215:73–5.
[23] Murata T, Hibasami H, Maekawa S, Tagawa T, Nakashima K. Preferential binding of cisplatin to mitochondrial dna and suppression of ATP generation in human malignant melanoma cells. Biochem Int 1990;20:949–55.
[24] Olivero OA, Semino C, Kassim A, López-Larraza DM, Poirer MC. Preferential binding of cisplatin to mitochondrial DNA of Chinese hamster ovary cells. Mutat Res 1995;346:221–30.
[25] Lim LO, Neims AH. Mitochondrial DNA damage by bleomycin. Biochem Pharmacol 1987;36:2769–74.
[26] Clayton DA, Doda JN, Friedberg EC. The absence of a pyrimidine dimer repair mechanism in mammalian mitochondria. Proc Natl Acad Sci USA 1974;71:2777–81.
[27] Ara G, Kusumoto T, Korbut TT, Cullere-Luengo F, Teicher BA. Cisdiamminedichloroplatinum(II) resistant human tumor cell lines are collaterally sensitive to $PtCl_4(RH\text{-}123)_2$: evidence for mitochondrial involvement. Cancer Res 1994;54:1497–502.
[28] Gracia-Mora I, Ruiz-Ramírez L, Gómez-Ruiz C, Tinoco-Méndez M, Márquez-Quiñones A, Romero de Lira L, Marín-Hernández A, Macías-Rosales L, Bravo-Gómez ME. Knight's move in the periodic table, from copper to platinum, novel antitumor mixed chelate copper compounds, casiopeinas, evaluated by an *in vitro* human and murine cancer cell line panel. Metal Based Drugs 2001;8: 19–28.
[29] De Vizcaya-Ruiz A, Rivero-Mueller A, Ruiz-Ramírez L, Kass GEN, Kelland LR, Orr RM, Dobrota M. Induction of apoptosis by a novel copper-based anticancer compound, casiopeina II, in L1210 murine leukaemia and CH1 human ovarian carcinoma cells. Toxicol In Vitro 2000;14:1–5.
[30] Ferrer-Sueta G, Ruiz-Ramírez L, Radi R. Ternary copper complexes and manganese(III) tetrakis (4-benzoic acid) porphyrin catalyze peroxynitrite-dependent nitration of aromatics. Chem Res Toxicol 1997; 10:1338–44.
[31] Arnaudeau C, Tenorio-Miranda E, Jenssen D, Helleday T. Inhibition of DNA synthesis is a potent mechanism by which cytostatic drugs induce homologous recombination in mammalian cells. Mutat Res 2000;461:221–8.
[32] Ruíz Azuara L. US Patent Ap. 21 (1992) Number 5, 107, 005. US Patent Re 35, 458, Febeuary 18 (1997). US Patent November 19 (1996) Number 5, 576, 326. 407543 SECOFI; 1993.
[33] Moreno-Sánchez R. Regulation of oxidative phosphorylation in mitochondria by external free Ca^{2+} concentrations. J Biol Chem 1985; 260:4028–34.
[34] Moreno-Sánchez R, Devars S, López-Gómez F, Uribe A, Corona N. Distribution of control of oxidative phosphorylation in mitochondria oxidizing NAD-linked substrates. Biochim Biophys Acta 1991;1060: 284–92.
[35] Moreno-Sánchez R, Hansford RG. Dependence of cardiac mitochondrial pyruvate dehydrogenase activity on intramitochondrial free Ca^{2+} concentration. Biochem J 1988;256:403–12.

[36] López-Gómez F, Torres-Márquez ME, Moreno-Sánchez R. Control of oxidative phosphorylation in AS-30D hepatoma mitochondria. Int J Biochem 1993;25:373–7.
[37] Thayer WS, Rubin E. Effects of chronic ethanol intoxication on oxidative phosphorylation in rat liver submitochondrial particles. J Biol Chem 1979;254:7717–23.
[38] Weickowski MR, Wojtczak L. Fatty acid-induced uncoupling of oxidative phosphorylation is partly due to opening of the mitochondrial permeability transition pore. FEBS Lett 1998;423:339–42.
[39] Moreno-Sánchez R, Bravo C, Vásquez C, Ayala G, Silveira LH, Martínez-Lavín M. Inhibition and uncoupling of oxidative phosphorylation by nonsteroidal antiinflammatory drugs. Biochem Pharmacol 1999;57:743–52.
[40] Pacheco-Moisés F, García JJ, Rodríguez-Zavala JS, Moreno-Sánchez R. Sulfite and membrane energization induce two different active state of the *Paracoccus denitrificans* F_0F_1-ATPase. Eur J Biochem 2000; 267:993–1000.
[41] Rodríguez-Zavala JS, Pardo JP, Moreno-Sánchez R. Modulation of 2-oxoglutarate dehydrogenase complex by inorganic phosphate, Mg^{2+}, and other effectors. Arch Biochem Biophys 2000;379:78–84.
[42] Stoner CD. Determination of the P/$2e^-$ stoichiometries at the individual coupling sites in mitochondrial oxidative phosphorylation. J Biol Chem 1987;262:10445–53.
[43] Inomoto T, Tanaka A, Moris MB, Sato B, Yanabu N, Tokuka A, Kitai T, Ozawa K, Yamaoka Y. Changes in the distribution of the control of the mitochondrial oxidative phosphorylation in regenerating rabbit liver. Biochim Biophys Acta 1994;1188:311–7.
[44] Moreno-Sánchez R, Rodríguez-Enríquez S, Cuéllar A, Corona N. Modulation of 2-oxoglutarate dehydrogenase and oxidative phosphorylation by Ca^{2+} in pancreas and adrenal cortex mitochondria. Arch Biochem Biophys 1995;319:432–44.
[45] Singer TP, Kearney EB, Kenney WC. Succinate dehydrogenase. Adv Enzymol 1973;37:189–271.
[46] Chappell JB, Haarhoff KN. The penetration of the mitochondrial membrane by anions and cations. In: Slater EC, Kanuiga Z, Wojtczak L, editors. Biochemistry of mitochondria. London: Academic Press; 1967. p. 75–91.
[47] Szewczyk A, Wojtczak L. Mitochondria as a pharmacological target. Pharmacol Rev 2002;54:101–27.
[48] Wojtczak L, Nikitina ER, Czyz A, Skulskii IA. Cuprous ions activate glibenclamide-sensitive potassium channel in liver mitochondria. Biochem Biophys Res Commun 1996;223:468–73.
[49] Holmuhamedov EL, Jovanovic S, Dzeja PP, Jovanovic A, Terzic A. Mitochondrial ATP-sensitive K^+-channels modulate cardiac mitochondrial function. Am J Physiol 1998;275:H1567–76.
[50] Kowaltowski AJ, Seetharaman S, Paucek P, Garlid KD. Bioenergetic consequences of opening the ATP-sensitive K^+-channel of heart mitochondria. Am J Physiol 2001;280:H649–57.
[51] Chaindussi E, Petrussa E, Macri F, Vianello A. Modulation of a plantmitochondrial K^+-channel and its involvement in cytochrome *c* release. J Bioeng Biomembr 2002;34:177–84.
[52] Kharbanda S, Pandey P, Schofield L, Israels S, Roncinske R, Yoshida K, Bharti A, Yuan ZM, Saxena S, Weichselbaum R, Nalin C, Kufe D. Role of Bcl-X_L as an inhibitor of cytosolic cytocrome *c* accumulation in DNA damage-induced apoptosis. Proc Natl Acad Sci USA 1997;94:6939–42.
[53] Cai J, Yang J, Jones DP. Mitochondrial control of apoptosis: the role of cytochrome *c*. Biochim Biophys Acta 1998;1366:139–49.
[54] Rosen M, Figlioimeni M, Simpkins H. The interaction of platinum antitumour drugs with mouse liver mitochodria. Int J Exp Path 1992;73:61–74.
[55] Singh G. A possible cellular mechanism of cisplatin-induced nephrotoxicity. Toxicology 1989;58:71–80.
[56] Choie DD, Longnecker DS, del Campo AA. Acute and chronic cisplatin nephrotoxicity in rats. Lab Inv 1981;44:397–402.
[57] Summerhayes IC, Lampidis TJ, Bernal SD, Nadakavukaren JJ, Nadakavukaren KK, Shepherd EL, Chen LB. Unusual retention of rhodamine 123 by mitochondria in muscle and carcinoma cells. Proc Natl Acad Sci USA 1982;79:5292–6.
[58] Nadakavukaren KK, Nadakavukaren JJ, Chen LB. Increased rhodamine 123 uptake by carcinoma cells. Cancer Res 1985;45:6093–9.
[59] Koya K, Li Y, Wang H, Ukai T, Tatsuta N, Kawakami M, Shishido T, Chen LB. MKT-077, a novel rhodacyanine dye in clinical trials, exhibits anticarcinoma activity in preclinical studies based on selective mitochondrial accumulation. Cancer Res 1996;56:538–43.
[60] Davis S, Weiss MJ, Wong JR, Lampidis TJ, Chen LB. Mitochondrial and plasma membrane potentials cause unusual accumulation and retention of rhodamine 123 by human breast adenocarcinoma-derived MCF-7 cells. J Biol Chem 1985;260:13844–50.
[61] Weiss MJ, Wong JR, Ha CS, Bleday R, Salem RR, Steele GD, Chen LB. Dequalinium, a topical antimicrobial agent, displays anticarcinoma activity based on selective mitochondrial accumulation. Proc Natl Acad Sci USA 1987;84:5444–8.
[62] Modica-Napolitano J, Aprille JR. Basis for the selective cytotoxicity of rhodamine 123. Cancer Res 1987;47:4361–5.