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Cruentaren A, a highly cytotoxic benzolactone from *Myxobacteria* is a novel selective inhibitor of mitochondrial F_1-ATPases

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Abstract **Cruentaren A, a new antifungal benzolactone produced by the myxobacterium *Byssovorax cruenta*, proved to be highly cytotoxic against various human cell lines. It inhibited the proliferation of different cancer cell lines including a multidrug-resistant KB line at low nanomolar levels. It arrested human histocytic lymphoma cells (U-937) in $G_{0/1}$ phase, but did not trigger an apoptotic process. Studies to uncover the molecular target of cruentaren A showed that the novel compound, despite its structural similarity to the benzolactone enamides apicularen and salicylihalamide, was no V-ATPase inhibitor. In contrast, cruentaren specifically inhibited mitochondrial F_OF_1-ATPases with IC50 values of 15–30 nM. Although the exact binding site of cruentaren remains undefined, inhibition was shown to occur by interaction with the catalytic F_1 domain. Since mitochondrial ATPases play a crucial role in the pathophysiology of several human disorders including cancer, cruentaren or synthetic derivatives thereof could form the basis of future therapeutic strategies.**

Keywords: Cruentaren; Myxobacteria; Benzolactone class; Cytotoxic activity; Mitochondrial F1-ATPase inhibitor

1. Introduction

The Gram-negative, fruiting body forming myxobacteria have emerged as a particularly rich source of secondary metabolites, which are characterised by a multitude of unrelated structures as well as by different biological activities with interesting mechanisms of action [1,2]. The recently described cruentarens were discovered in extracts of the new myxobacterium *Byssovorax cruenta* strain By c1 because of the high antifungal activity of cruentaren A, and later on cytotoxicity against L929 mouse cells was also observed [3]. Structurally they are characterized by a 12-membered lactone with an allylamine side chain acylated by a 2-hydroxy-4-methoxy benzoic acid in cruentaren A (Fig. 1) and a corresponding 6-membered lactone in cruentaren B [4]. Thus, cruentarens are closely related to the class of benzolactone enamides, including the salicylihalamides and the lobatamides [5,6]. Close structural similarity also exists to apicularen A, produced by several species of the myxobacterial genus *Chondromyces* [7–9]. So far, all members of the benzolactone enamide class have been described as highly cytotoxic compounds, which specifically inhibit V-ATPases [9–11]. Therefore, we speculated that cruentarens might have the same properties. Hitherto, preliminary studies [3] had indicated that cruentaren A interferes with mitochondrial ATPases, complex ubiquitous proteins which are evolutionarily related to V-ATPases, reflected by their similar overall structure consisting of a catalytic F_1 (V_1) domain and a proton translocating, membrane bound F_O (V_O) domain [12–14]. In this article, we report on the growth inhibitory effect of cruentaren A on various human cell lines and deal with its inhibitory capacity on ion transporting ATPases. We show that cruentaren A specifically inhibits mitochondrial F_OF_1-ATPases by interaction with the F_1 part, while other ion transporting ATPases such as V-ATPase or Na^+/K^+-ATPase remain unaffected.

2. Materials and methods

2.1. Natural compounds

Cruentaren A and apicularen A were kindly supplied by members of the former Division of Natural Product Research at the Helmholtz Centre for Infection Research. Oligomycin was purchased from Sigma. Concanamycin A was kindly provided by the Institute of Organic and Biomolecular Chemistry, University of Göttingen.

2.2. Cell culture assays

Cell lines were obtained from the American Type Culture Collection (ATCC) and the German Collection of Microorganisms and Cell Cultures (DSMZ). All cell lines were cultured under conditions recommended by their respective depositors. Growth inhibition was measured in microtiterplates by using the MTT assay as reported previously [15]. In growth kinetic studies with KB-3-1 (DSMZ ACC158) the inoculum was 50000 cells/ml and as parameter of growth, the protein concentration of harvested and washed cells was determined at different times using Bradford reagent (Bio-Rad). Cell culture reagents were purchased from Life Technologies Inc. (Gibco BRL) and plastic ware was obtained from Nunc.

In order to estimate the amount of inhibitor that is bound to cells, KB-3-1 cells (~500000 in 10 ml) were incubated overnight with 20 ng/ml of cruentaren A and apicularen A, respectively. The cells were then harvested by scrapping, centrifuged, washed with 10 ml PBS, and extracted with 0.5 ml methanol. The methanolic extract was evaporated to dryness, suspended in 200 µl of culture medium, and used for a growth inhibition assay with KB-3-1 cells as mentioned above.

2.3. Caspase measurement

U-937 cells (DSMZ ACC5) were seeded in wells of a 384-well plate (2500 cells/well) and incubated with cruentaren A (20 ng/ml) for different periods of time. Activity of caspase 3 and caspase 7 was measured

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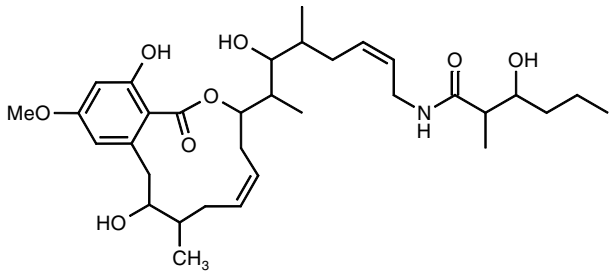

Fig. 1. Structure of the cruentaren A.

by using the Apo-One Homogeneous Caspase-3/7 Assay Kit from Promega.

2.4. Nucleosome quantification

The occurrence of mono- and oligonucleosomes due to apoptotic process was determined in U-937 cells using the Cell Death Detection ELISA kit from Roche Diagnostic.

2.5. Cell cycle analysis

After the appropriate treatment, 10^6 U-937 cells were harvested by centrifugation and then fixed with cold (−20 °C) 80% methanol. After 30 min of incubation on ice, the cells were washed with phosphate buffered saline (PBS) and then treated with 0.1% saponin in PBS (w/v). Finally 500 µl propidium iodide (20 µg/ml) and RNAse (1 mg/ml) were added, and the cells were incubated at 37 °C for 30 min. Samples were analyzed by a FACScan (Becton Dickinson). Results are presented as the number of cells versus the amount of DNA as indicated by fluorescence intensity.

2.6. Enzyme preparations

Yeast mitochondria were obtained as described previously [16]. In some cases the yeast cells mixed with glass beads were only homogenized in a mini-shaker (Ika). Beef heart mitochondria were isolated by differential centrifugation, following the protocol of Smith by using a blender to homogenize the heart mince [17]. The initial homogenisation buffer consisted of 250 mM sucrose, 10 mM KH_2PO_4, 10 mM Tris, 2 mM EGTA, 2 mM $MgCl_2$, pH 7.4, and further isolation procedures were carried out in the same medium without EGTA [18]. Submitochondrial particles (SMP) were obtained by ultrasonic treatment of the mitochondria.

To obtain F_1-ATPase enriched fractions, submitochondrial particles were mixed with half of the volume of chloroform for 15 s at room temperature and immediately centrifuged for 10 min at 6000 rpm to separate the aqueous and organic phases. The upper aqueous phase containing the soluble F_1 part was carefully removed and assayed for ATPase activity as described below. F_1-ATPase in this fraction is relatively unstable and sensitive to low temperatures.

The V-ATPase holoenzyme was purified as published elsewhere [19]. Preparation of highly purified membranes containing Na^+/K^+-ATPase from pig kidney followed the protocol of Jørgensen [20] with three main steps of differential centrifugation, incubation with SDS in the presence of ATP and sucrose density gradient centrifugation in a fixed angle rotor.

2.7. ATPase assays

Unless otherwise noted, ATPase assays were performed in a total volume of 160 µl. The preincubation time with or without additional inhibitor was 5 min and the reactions were stopped at a given time by placing the tubes into liquid nitrogen.

Assays with submitochondrial particles from beef heart and the yeast *Saccharomyces cerevisiae* were carried out at 30 °C and consisted of 4 µg of bovine and 2 µg of yeast protein, respectively, 50 mM Tris–MOPS, pH 8.1, 1 mM $MgCl_2$, 20 mM KCl, and 12.5 mM NaCl. The reaction was started with 1 mM ATP and stopped after 2 min of incubation.

ATPase assays with enriched F_1-ATPase preparations of both beef heart and the yeast *S. cerevisiae* were performed in a final volume of 1 ml and a pH of 8.0 at room temperature The samples contained 6–10 µg of bovine and 14 µg of yeast protein, respectively, 50 mM Tris, 50 mM KCl and 2.5 mM $MgCl_2$. After 5 min of preincubation with or without inhibitors, 5 mM ATP was added, and after an additional incubation time of 15 min the reaction was stopped by the addition of 0.4 ml of 20% TCA.

V-ATPase assays were carried out at a pH of 8.1 and consisted of 3 µg of protein, 50 mM Tris–MOPS, 3 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 20 mM KCl, 0.003% $C_{12}E_{10}$, 20 mM NaCl, and 3 mM Tris–HCl. After preincubation at 30 °C with or without additional inhibitors, 1 mM Tris–ATP was added and after an incubation time of 2 min the reactions were stopped by placing the tubes into liquid nitrogen. Assays using Na^+/K^+-ATPase were performed at pH 7.5 and contained 0.5 µg of protein, 50 mM Tris–MOPS, 5 mM imidazole, 0.2 mM EDTA, 4 mM $MgCl_2$, 20 mM KCl, 3.1% DMSO and 100 mM NaCl. After 5 min of preincubation at 37 °C the reaction was started with 3 mM Tris–ATP and stopped after 2 min of incubation by placing the tubes into liquid nitrogen.

Determination of the inorganic phosphate produced in the assays with enriched F_1 part followed the method of Fiske and Subarrow [21] using ascorbic acid as reducing agent, while the inorganic phosphate produced in the assays of F-ATPase, V-ATPase, and Na^+/K^+-ATPase was measured according the protocol of Wieczorek et al. [22].

2.8. Other procedures

Fifth instar larvae of *Manduca sexta* (Lepidoptera, Sphingidae), weighing 6–8 g, were reared under long day conditions (16 h of light) at 27 °C using a synthetic diet modified according to Bell et al. [23].

3. Results and discussion

3.1. Growth inhibitory effects of cruentaren A on human cancer cell lines

The novel antifungal cruentaren A, showing also cytotoxicity against our standard mouse fibroblast cell L929 [3], was checked for its impact on the growth of a variety of human cancer cell lines from different tissues (Table 1). The IC_{50} values were, comparable with those described for other benzolactones such as apicularen A [7,9], in the nanomolar range, even for the multi-drug-resistant cell line KB-V1.

Fig. 2 shows growth kinetics of KB-3-1 cells in the presence and the absence of cruentaren A. At a concentration of 20 ng/ml cruentaren A, the KB-3-1 cells slowly ceased growing. The protein amount was slightly increasing for about three days. When the culture medium was replaced by fresh medium without cruentaren A after one, two or five days, the cells re-started to propagate transiently, probably due to cellular ATP production via glycolysis, but finally stagnated again after about 2–3 days. This effect of cruentaren A differs from that of the structurally related V-ATPase inhibitor apicularen A, for which in comparable assays full reversibility after inhibitor wash-out had been demonstrated [7].

Table 1
Growth inhibition of different human cancer cell lines by cruentaren A and apicularen A

Cell line	Origin	IC_{50} (ng/ml)	
		Cruentaren A	Apicularen A
KB-3-1	Cervix carcinoma	0.3	1.0
KB-V1	Multi-drug resistant KB line	0.6	10
K-562	Chronic myelogenous leukemia	0.6	2.0
U-937	Histiocytic carcinoma	0.1	1.5
A-549	Lung carcinoma	0.4	0.1
SK-V-3	Ovarian carcinoma	1.0	1.5
A-498	Kidney carcinoma	0.4	0.3

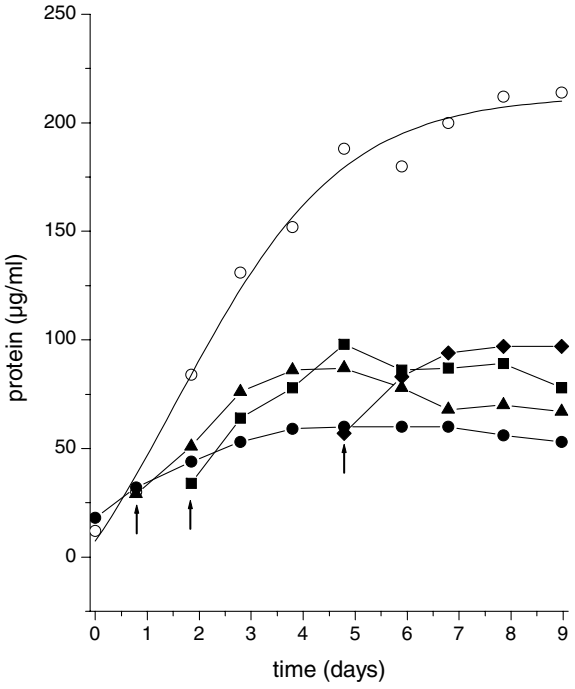

Fig. 2. Kinetics of growth inhibition by cruentaren A in KB-3-1 cells. Control without inhibitor (open circles), with 20 ng/ml of cruentaren A (solid circles), culture medium replaced by fresh medium without inhibitor after 1 day (solid triangles), after 2 days (solid squares) and 5 days (solid diamonds). Growth was determined as protein increase.

One reason for the irreversible effect of cruentaren A could be its influence on the cell cycle. Cell cycle analysis by flow cytometry of U-937 cells showed that the cells treated with cruentaren A arrested in the $G_{0/1}$ phase (Fig. 3). After one day, the percentage of cells in $G_{0/1}$ increased from 31% to 49%, and to 56% after two days of incubation. Since, we did not observe an increased sub-$G_{0/1}$ population, apoptosis induced by cruentaren treatment appears improbable. We also measured caspase activity in U-937 cells that had been incubated with cruentaren A for 1–16 h, but found no increase in activity. Using an ELISA for mono- and oligo-nucleosomes we also found no hints for an apoptosis going on after three days of incubation with cruentaren A, neither with 20 ng/ml nor with an enhanced concentration of 200 ng/ml. From these results we assume that the irreversible effect of cruentaren A is not due to an irreversible induction of apoptosis.

Another reason for the irreversible effect of cruentaren A may be that it just could not be washed out because of its strong binding to the cells. Therefore, we tested methanolic extracts of washed KB-3-1 cells that had been incubated with cruentaren (20 ng/ml) overnight. In a serial dilution assay, we still observed a considerable amount of growth inhibition. The same experiment with apicularen revealed, as expected, no inhibitory activities of the respective extracts. Thus it appears that the effect of cruentaren A is predominantly irreversible because it strongly binds to the target cells.

3.2. Effects of cruentaren A on V- and P-ATPases

Initially, we tested the inhibitory efficacy of cruentaren A on the V-ATPase since we assumed that it would have the comparable inhibitor characteristics as the closely related published benzolactone enamides such as apicularen and salicylihalamide

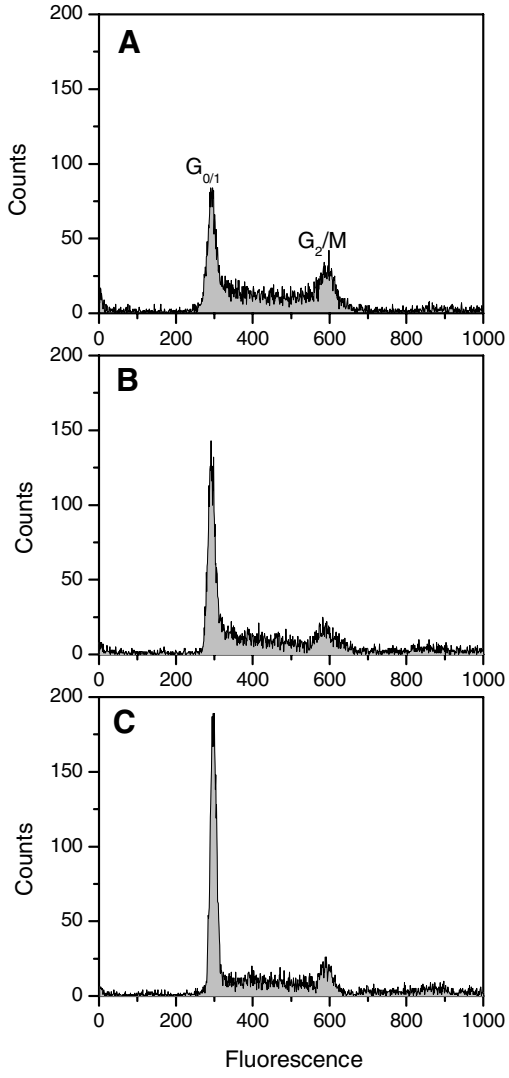

Fig. 3. Histograms of flow cytometry analysis of U-937 cells. In the presence of cruentaren A (20 ng/ml) the number of cells in $G_{0/1}$ phase increased with time. The percentage rose from 31% (A) to 49% after 1 day (B) and 56% after 2 days (C) of incubation.

which exhibit IC_{50} values in the nanomloar range [9–11]. Therefore, we prepared pure V-ATPase holoenzyme from the midgut of the tobacco hornworm and tested if cruentaren A would also block V-ATPase activity. However, even at a concentration of 1 µM which is sufficient for established specific V-ATPase inhibitors such as the plecomacrolides concanamycin and bafilomycin or the above mentioned benzolactone enamides to completely inhibit the V-ATPase, cruentaren A unexpectedly had no effect (Table 2).

To further elucidate the molecular target of the highly cytotoxic cruentaren, we tested its effect on the Na^+/K^+-ATPase. This ion transporting enzyme belongs to the family of P-ATPases and occurs in virtually every animal cell where its main function is the preservation of sodium gradients across the plasma membrane [24]. For our experiments we used purified Na^+/K^+-ATPase containing plasma membranes from pig kidney. As shown in Table 2, cruentaren A had no effect on the activity of the Na^+/K^+-ATPase even at a concentration of 1 µM, which is much higher than the IC_{50} values determined

Table 2
Inhibition of the Na^+/K^+-ATPase and of the V-ATPase by cruentaren A

Compound	Relative activity (%)	
	Na^+/K^+-ATPase	V-ATPase
Control without inhibitors	100[a]	100[a]
Vanadate 1 mM	4 ± 2.6	n.d.
Concanamycin A_1 1 μM	n.d.	3.2 ± 1.3
Cruentaren 1 μM	108 ± 9.8	103 ± 24.7

[a]Values represent the means ± S.E.M. of three independent experiments (n.d. = not determined). The specific ATPase activity without inhibitor was 6.1 ± 0.2 μmol mg^{-1} min^{-1} in Na^+/K^+-ATPase preparations and 3.2 ± 0.7 in V-ATPase preparations.

in growth experiments, whereas the ATPase activity was completely inhibited by the addition of the specific inhibitor vanadate (Table 2).

Taken together the results with V- and P-ATPases strongly indicate that cruentaren A is neither a V-ATPase nor a P-ATPase inhibitor.

3.3. Effects of cruentaren A on F-ATPases

Because cruentaren A did not inhibit V- nor P-ATPases, we consequently investigated as next step the inhibitory efficacy of cruentaren A on F-ATPases which intrinsically could have been suspected from our initial studies with mitochondria [3]. To examine the potency of this effect, we analyzed the dose dependent inhibition of F_OF_1-ATPase activity by cruentaren A in mitochondrial preparations of both beef heart and the yeast *Sacharomyces cerevisiae*. Preliminary studies had shown that about 80% of total ATPase activity in these preparations were sensitive to the specific F_OF_1-ATPase oligomycin [3]. As Fig. 4 reveals, cruentaren A inhibited the F_OF_1-ATPase activity in submitochondrial particles from beef heart and yeast half-maximally at concentrations between 15 and 30 nM. The inhibition curve was rather steep with a Hill coefficient of clearly more than 1, indicating some positive cooperativity in the inhibition process.

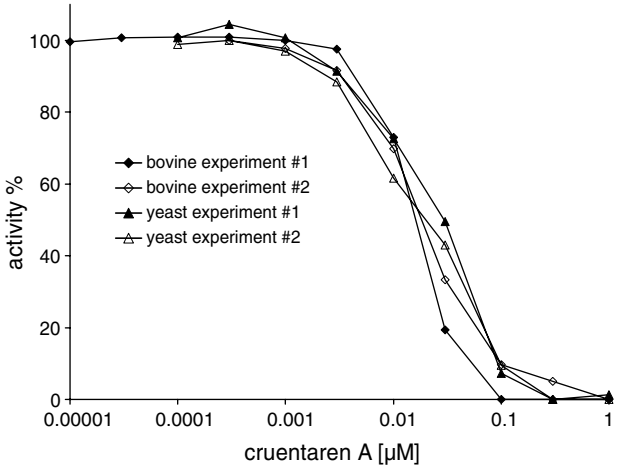

Fig. 4. Dose dependent inhibition of F-ATPase activity in submitochondrial particles of beef heart and *Saccharomyces cerevisiae*. In each case two independent experiments are shown. The specific ATPase activity without inhibitor was 1.5 μmol mg^{-1} min^{-1} in beef heart preparations (solid and open diamonds) and 1.3, respectively 3.9 μmol mg^{-1} min^{-1} in yeast mitochondria (solid and open triangles).

The classification of cruentarens as members of the salicylihalamide/apicularen family on the one hand and the evolutionary relationship of V- and F-ATPases [12] on the other hand imply a possible conserved primary inhibition mechanism or binding site for this group of antibiotics. A comparable common mechanism had already been suggested for the V-ATPase inhibitor bafilomycin and the F-ATPase inhibitor oligomycin [25]. For the benzolactone enamides salicylihalamide and apicularen it has been shown recently that inhibition of the V-ATPase activity is mediated via interaction with the V_O-complex as the ATP hydrolysis catalyzed by the isolated V_1 complex was not effected (salicylihalamide: [26]; apicularen: Huss and Wieczorek, unpublished). Although the nature of this interaction is still unknown, experiments using a radioactively labelled semisynthetic derivative of concanamycin had shown that the binding site for apicularen (and thus evidently also for salicylihalamide) is different from that for the plecomacrolides [9,19].

3.4. Inhibition of the catalytic F_1 domain by cruentaren A

To investigate whether cruentaren A inhibited the F-ATPase activity in a similar way by targeting the F_O-part, we performed assays using samples enriched in F_1-ATPase. Contrary to our expectations, cruentaren A inhibited the activity of F_1-ATPase solubilized from both yeast and beef heart submitochondrial particles (Table 3). The inhibitory effect at the concentrations of 0.1 and 1 μM was nearly identical to that obtained with the F_OF_1-holoenzyme (compare Fig. 4). As expected, the F_O-targeting inhibitor oligomycin did not affect the F_1-ATPase activity. The inhibitory capacity of cruentaren A appears to be limited to eukaryotic F-ATPases, since it has been shown to be completely inactive against a series of Gram-negative bacteria [3], and since it did not inhibit the purified F_1-ATPase from *Escherichia coli* (data not shown).

Based on our results, we conclude that cruentaren A inhibits mitochondrial F-ATPases by targeting the catalytic F_1-domain and not via interaction with the membrane bound F_O-domain. This outcome seems surprising in view of the effect of the related benzolactone enamides which operate on V-ATPases via the V_O-complex. However, in spite of their planar structural similarity, conformation and spatial arrangement of functional groups are quite different, as has been shown by X-ray crystal structure analyses of apicularen A and cruentaren A [4]. From this point of view it is conceivable that these compounds interact with different binding sites in related proteins.

Several natural compounds such as aurovertins or the polyphenolic phyto-chemicals resveratrol and piceatannol are well known inhibitors of mitochondrial F_1-ATPases, for which

Table 3
Inhibition of F_1 ATPase solubilized from submitochondrial particles

Compound	Relative activity (%)	
	Beef heart	*S. cerevisiae*
Control without inhibitors	100[a]	100[a]
Oligomycin 1 μM	99.1 ± 0.1	94.0 ± .2.0
Cruentaren 1 μM	3.3 ± 0.8	3.0 ± 0.7
Cruentaren 0.1 μM	10.0 ± 0.5	10.3 ± 0.7

[a]Values represent the means ± S.E.M. of three independent experiments. The specific ATPase activity without inhibitor was 3.9 ± 0.3 μmol mg^{-1} min^{-1} in F_1 beef heart preparations and 7.5 ± 0.5 in F_1 yeast preparations.

binding sites have been partially characterized [27]. Since we have no evidence for a cruentaren binding site in the F_1-ATPase, further work is urgently required. However, in the light of our finding that cruentaren inhibits the mitochondrial F_1-ATPase from two evolutionarily rather distant eukaryotic organisms such as yeast and mammal but does not inhibit the F_1-ATPase from *Escherichia coli*, it appears tempting to speculate that it binds to the F_1 subunit ε which has no bacterial counterpart [28,29].

Apart from the fact that the inhibitory site needs to be identified, our results substantiate that cruentaren A is, to our knowledge, the most potent inhibitor of mitochondrial F_1-ATPases. A future understanding of the interaction of cruentaren with its binding site may enable the rational development of therapeutic agents for cancer treatment. Although the therapeutic efficacy of cruentaren as anticancer drug has still to be evaluated, its strong binding to cells might be advantageous, provided that it is efficiently delivered to tumor cells. This could possibly be done by tumor targeting via antibody or ligand conjugation [30,31].

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