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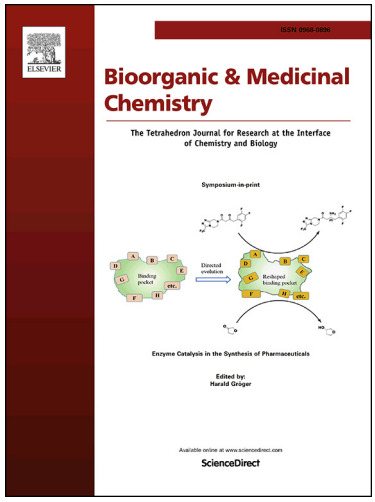

A mitochondria-targeted organic arsenical accelerates mitochondrial metabolic disorder and function injury

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A mitochondria-targeted organic arsenical accelerates mitochondrial metabolic disorder and function injury

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Abstract

Considering the vital role of mitochondria in the anti-cancer mechanism of organic arsenical, the mitochondria-targeted precursor PDT-PAO-TPP was designed and synthesized. PDT-PAO-TPP, as a delocalization lipophilic cation (DLCs) which mainly accumulated in mitochondria, contributed to improve anti-cancer efficacy and selectivity towards NB4 cells. In detail, PDT-PAO-TPP inhibited the activity of PDHC resulting in the suppression of ATP synthesis and thermogenesis disorder. Additionally, the inhibition of respiratory chain complex I and IV by short-time incubation of PDT-PAO-TPP also accelerated the respiration dysfunction and continuous generation of ROS. These results led to the release of cytochrome *c* and activation of caspase family-dependent apoptosis. Different from the mechanism of PDT-PAO in HL-60 cells, it mainly induced the mitochondrial metabolic disturbance resulting in the intrinsic apoptosis via inhibiting the activity of PDHC in NB4 cells, which also implied that the efficacy exertion of organic arsenical was a complex process involved in many aspects of cellular function. This study systematically clarifies the anti-cancer mechanism of mitochondria-targeted organic arsenical PDT-PAO-TPP and confirms the new target PDHC of organic arsenicals, which further supports the organic arsenical as a promising anticancer drug.

Keywords: mitochondria; organic arsenical; PDH; TPP; apoptosis

Introduction

Arsenic trioxide (ATO) has been approved by the U.S. FDA for the successful clinical treatment of acute promyelocytic leukemia [1-4]. Evidence has shown that for the high affinity between arsenic atom and sulfhydryl group, arsenicals have various targets among intracellular proteins and endogenous or exogenous antioxidants, such as thioredoxin, thioredoxinreductase, DNA repair enzymes, hemoglobin, peroxide reductase, glutathione reductase, pyruvate dehydrogenase complex, peroxiredoxin and so on[2-5]. For their better biological compatibility and outstanding designability, arsenicals had aroused much interest and shed light on the antitumor mechanism[6] [7].ATO can give rise to the oligomerization, sumoylation and degradation of PML-RARα fusion protein[8,9]. While, organic arsenicals are generally thought to induce the apoptosis rather than cell re-differentiation, which indicates that organic arsenicals may hold the anti-cancer efficacy towards leukemia and lymphoma, as well as solid tumors[10-12]. Similarly, organic arsenicals can consume the endogenous antioxidants, disturb the intracellular redox balance, impair the mitochondrial function, as well as react with sulfhydryl group in vital active sites[6,7,13-15]. Mitochondrial damage mediated apoptosis induced by organic arsenicals is a common pathway.

Mitochondria as the indispensable organelles can provide the energy in the form of ATP through the process of oxidative phosphorylation, and a mass of metabolic intermediates for the biosynthesis like lipid metabolism[16-18]. Besides, they also participate in regulating multiple signal pathways including cell apoptosis[19]. Compared to the normal mitochondria, the mitochondria in cancer cells have the higher content of ROS, higher level of membrane potential and down-regulated expression of apoptosis-related proteins supporting the rapid cell proliferation and escape from death[20-22] These differences gradually become the potential targets of mitochondria-targeted anti-tumor drugs[22,23]. A report has shown that dichloroacetic acid can inhibit the activity of pyruvate dehydrogenase kinase (PDK) resulting in the disturbance of glycolysis, the burst of ROS and activation of K^+ channel to promote apoptosis and to inhibit cancer growth [24,25]. Besides, there are some drugs, such as

ABT-737, ABT-199 and ATO, which can activate the apoptosis signaling pathways through affecting the activities of related enzymes and regulating the level of intracellular ROS[26,27]. In addition, delocalization lipophilic cation (DLCs) can target cancer cells mitochondria selectively for its higher membrane potential, which puts forward the feasible ideas about the design of mitochondria-targeted drugs[28-31].

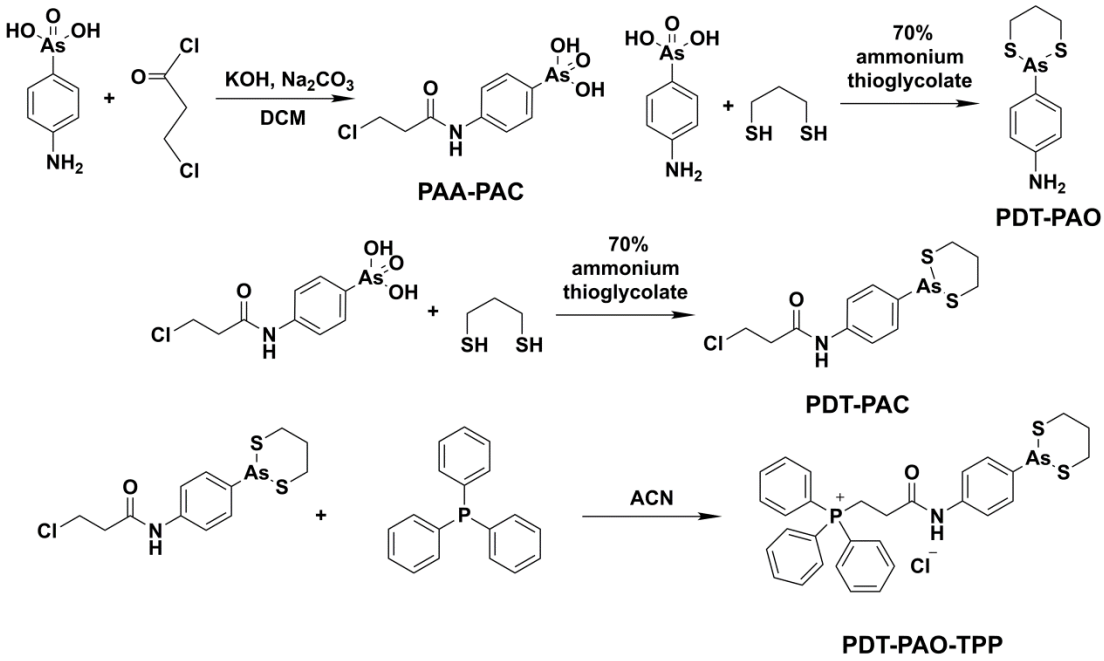
We designed a mitochondria-targeted organic arsenical based on the former finding that mitochondria play the vital role in the anti-cancer mechanism of organic arsenical PDT-PAO, PIM-PAO-PDT and PAM-PAO-PDT. As a type of mitochondria-targeted group[32], TPP was introduced to bind with organic arsenical PDT-PAO to yield mitochondria-targeted precursor PDT-PAO-TPP. In the cytotoxicity screening, organic arsenical PDT-PAO-TPP showed the better efficacy and selectivity towards NB4 cells. After the systematical study, it was confirmed that majority of PDT-PAO-TPP accumulated in mitochondria, which immediately induced the mitochondrial depolarization. What's more, PDT-PAO-TPP showed the dual inhibitions towards pyruvate dehydrogenase complex (PDHC) and respiratory chain complexes, resulting in the release of cytochrome *c* and the activation of caspase-dependent ROS-mediated apoptosis.

Results and Discussion

Synthesis and cytotoxicity

In order to improve the mitochondria-targeted property, TPP was introduced to the organic arsenical PDT-PAO. As shown in Scheme 1, linking the TPP with PDT-PAO via amide bond yielded final product PDT-PAO-TPP. TPP has been reported to enter into the mitochondria selectively with negligible cellular toxicity[33]. The design of PDT-PAO-TPP combined the structural simplicity and better anti-cancer bioactivity. Seen from Table 1, several kinds of cancer cell lines (NB4, HL-60, HeLa, MCF-7, SGC7901) were chosen to assess the killing ability of organic arsenicals by the MTT assay. Comparing with PDT-PAO, compound PDT-PAO-TPP retained the better inhibitory activity towards all of cancer cell lines with half-inhibitory concentrations

(IC_{50}) at below 10 μM. In details, PDT-PAO-TPP displayed the best efficacy towards NB4 cells with IC_{50} value of 0.65 ± 0.05 μM (24 h) and 0.51 ± 0.01 μM (48 h). Thus the newly synthesized compound PDT-PAO-TPP had the better inhibitory capacity.



Scheme 1. The synthetic routes of PDT-PAO and PDT-PAO-TPP.

Table 1. Inhibition effects of PDT-PAO and PDT-PAO-TPP on different cell lines.[a]

Cell lines	PDT-PAO		PDT-PAO-TPP	
	24h	48h	24h	48h
NB4	1.25±0.02	0.82±0.15	0.65±0.05	0.51±0.01
HL-60	1.12±0.02	1.01±0.18	3.58±0.01	3.23±0.32
HeLa	2.51±0.97	1.02±0.24	9.05±2.46	3.91±1.05
SGC7901	6.44±0.31	2.19±0.13	4.63±0.48	0.99±0.09
MCF-7	9.00±2.17	3.36±0.38	4.44±1.46	1.46±0.16
HEK293	6.27±0.73	2.72±0.26	4.63±0.48	2.08±0.35

[a]The data (IC_{50}) are expressedas the mean ± SD of three independent experiments and the unit is μmol·L^{-1}.

Assessment of mitochondria-targeted property

TPP moiety as the mitochondria-targeted group in PDT-PAO-TPP made us to speculate that it can accumulate mainly in the mitochondria. The obvious collapse of mitochondrial membrane potential followed by slight decrease of cell viability was induced by PDT-PAO-TPP after the short-time incubation, whereas there was only slight variation in the intracellular ROS level under the same conditions (Figure 1). However, the addition of PDT-PAO made the negligible difference on membrane potential, minor alterations on ROS content in NB4 cells, as well as slight decline on cell viability. The observations indirectly indicated that compound PDT-PAO-TPP mainly targeted mitochondria to exert its bioactivity via immediately triggering the collapse of the membrane potential.

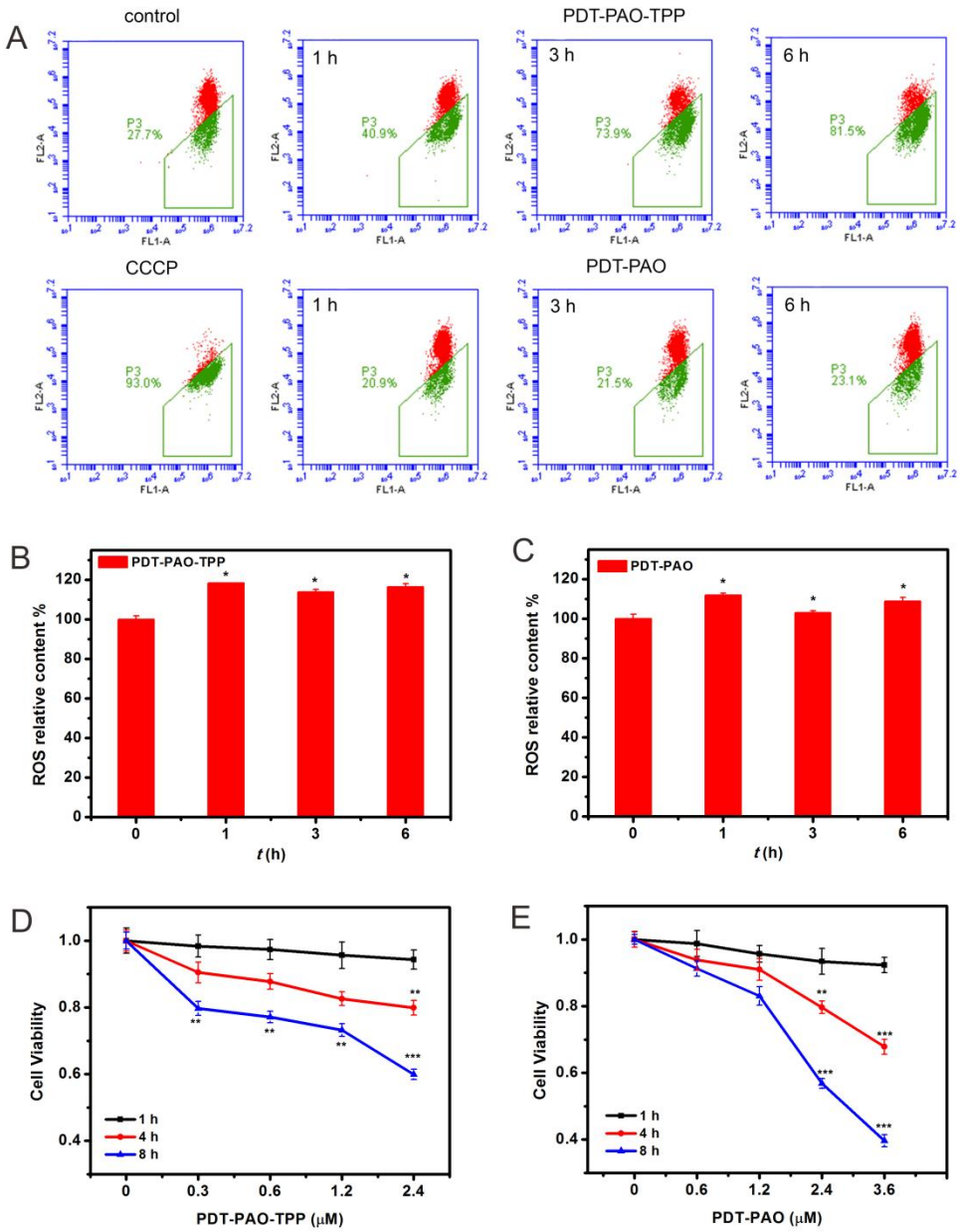


Figure 1. Effects on NB4 cells after short-time incubation. (A) The variation of mitochondrial membrane potential. NB4 cells were incubated with 1.2 μM PDT-PAO or 0.6 μM PDT-PAO-TPP, followed by JC-1 staining. The change of intracellular ROS level by PDT-PAO-TPP (B) or PDT-PAO (C) was shown. NB4 cells were incubated with 1.2 μM PDT-PAO or 0.6 μM PDT-PAO-TPP for 1 h, 3 h and 6 h, followed by DCFH-DA staining. The decrease of cell viability (MTT assay) by PDT-PAO-TPP (D) or PDT-PAO (E) was shown.

Inhibition of pyruvate dehydrogenase complex (PDHC) activity

Pyruvate dehydrogenase complex (PDHC) as a kind of multi-enzyme complex including pyruvate dehydrogenase (E1), dihydrolipoyltransacetylase (E2) and

dihydrolipoamide dehydrogenase (E3), links the glycolysis metabolic pathway to the citric acid cycle and fatty acid synthesis[34,35]. Treatment of PDT-PAO-TPP or PDT-PAO can suppress the PDHC activity by a concentration and time dependent manner (Figure 2A, 2B and Figure S1). Together incubation with LA[37] can protect NB4 cells from death (Figure 2C and Figure S2), which means that PDT-PAO-TPP may disturb the dihydrolipoyltransacetylase (E2) in PDHC via interacting with the sulfydryl in DHLA to prevent the acryl group converted to CoA. The observation (Figure 2D) that the activity of PDHC E2 was inhibited by the treatment of PDT-PAO-TPP and recovered by the dimercapto compounds incubation in advance implied PDHC-targeted of PDT-PAO-TPP. And these dimercapto compounds (DTT, DMSA and DMP) recovered the cell viability to some extent despite of the treatment of PDT-PAO-TPP or PDT-PAO (Figure S3).

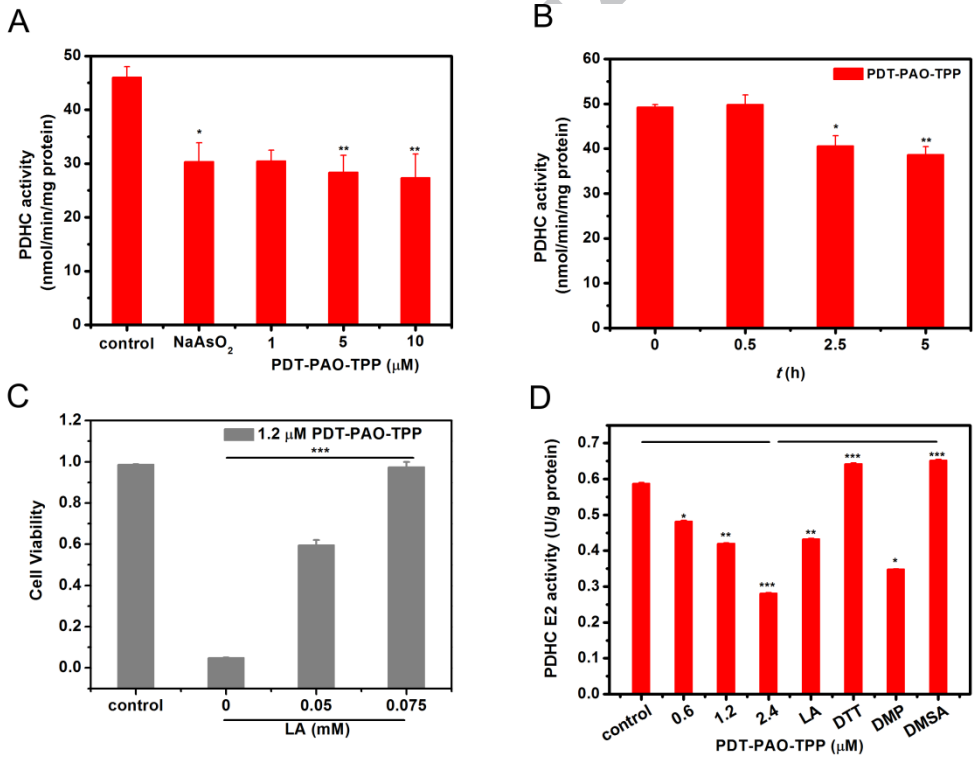


Figure 2. The inhibition of PDHC and E2 activity. The decrease of PDHC activity by a concentration- (A) and time- (B) dependent way. NB4 cells with different concentrations of PDT-PAO-TPP were incubated for 0.5 h, 2.5 h or 5 h, then the cells were sacrificed to assess the PDHC activity. (C) The effect of LA on cell viability (trypan blue staining). (D) The suppression of PDHC E2 activity. NB4 cells with different concentrations of PDT-PAO-TPP and some other

regents (75 μM LA, 500 μM DTT, 300 μM DMP and 150 μM DMSA) were incubated for 3 h for the PDHC E2 activity assessment.

Influence on mitochondrial energy metabolism and thermogenesis

Mitochondria is called the intracellular "energy factory"[36]. As revealed in Figure 3A and 3B, intracellular ATP level declined after the treatment of PDT-PAO-TPP or PDT-PAO, which accorded with the decrease of membrane potential (Figure 3C, 3D and 3E). To avoid interfering from other organelles, isolated mitochondrial thermogenesis was determined with the help of microcalorimeter (Figure 3F and 3G). There are four phases in the thermogenic curve including lag phase, activity recovery phase, stationary increase phase and decline phase. It is the lag phase to adapt to the physiological environment in the initial 20 h. Then mitochondria undergo a short activity recovery phase taking about 10 h and the following duration time of stationary phase lasts approximately 15 h. Finally, because the oxygen and nutrition are exhausted, there is a quick decline phase. Partial metabolic parameters were obtained based on the thermokinetic equation below[37]:

$$\ln P_t = \ln P_0 + k \cdot t \quad (1)$$

where P_t and P_0 are the heat output power at specific time and initial time, respectively. k represents the rate constant of log phase including activity recovery phase (k_1), stationary increase phase (k_2) and decline phase (k_3). Other parameters were seen from the heat output curve. All the parameters were shown in Table 2 and it was suggested that PDT-PAO-TPP or PDT-PAO can slow down the rate constant (k_2 and k_3) and reduce the maximum power [38] resulting in the suppression of the heat output.

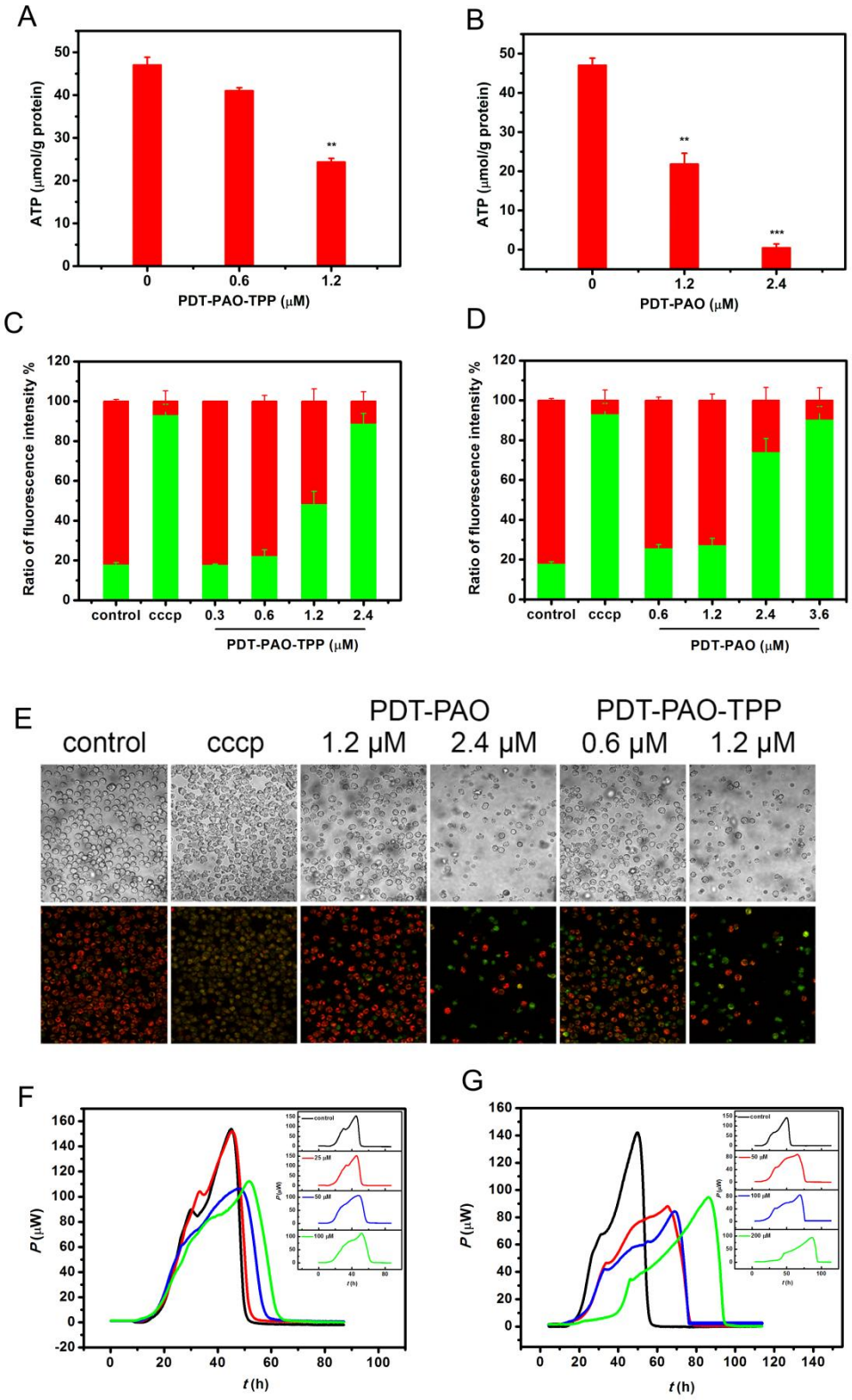


Figure 3. The influence on mitochondrial metabolism. The decrease of intracellular ATP level after 24 h incubation((A) and (B)) (C) (D) The collapse of mitochondrial membrane potential by JC-1 staining. Green: percentage of cells with decreased membrane potential and Red for the normal. (E) Confocal images about mitochondrial membrane potential by JC-1 staining. The

positive sample was treated by 1 μM cccp for 10 min in advance. (F) and (G) The thermogenic curves of isolated mitochondria taking pyruvate as substrate incubated with various concentrations of PDT-PAO-TPP (F) or PDT-PAO (G) for a long time.

Table 2. Influences of PDT-PAO and PDT-PAO-TPP on mitochondrial metabolism parameters.[a]

***c* (μM)**	**PDT-PAO**				**PDT-PAO-TPP**			
	0	50	100	200	0	25	50	100
k_1 (10^{-3} min^{-1})	7.33	4.07	3.08	4.29	5.15	3.82	4.82	3.88
R_1^2	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99
k_2 (10^{-3} min^{-1})	1.18	0.19	0.66	0.62	1.00	0.72	0.46	0.41
R_2^2	0.99	0.98	0.99	0.99	0.99	0.99	0.98	0.99
k_3 (10^{-3} min^{-1})	-24.13	-21.47	-16.17	-16.52	-20.47	-14.05	-9.35	-9.13
R_3^2	0.99	0.99	0.98	0.94	0.99	0.99	0.99	0.99
P_m (W)	141.64	88.2	96.9	94.7	153.92	152.01	106.55	112.34
t_m (h)	49.89	65.18	73.17	86.43	45.07	45.41	47.57	51.77
***Q* (J)**	10.32	11.62	12.23	11.44	10.36	10.82	10.24	11.23

[a]k_1: rate constant in activity recovery phase; k_2: rate constant in stationary increase phase; k_3: rate constant in decline phase; R: correlation coefficient; P_m: maximum power output; t_m: maximum power output time; Q: total heat output.

Destruction on mitochondrial respiration

Making use of Clark Oxygen Electrode, the mitochondrial respiration process was monitored. It has been known that the ideal isolated mitochondria have the relatively low respiratory rate for state 4 characterizing mitochondrial inner membrane integrity and the high respiratory rate for state 3 (about 3-fold than state 4) meaning the normal function of respiratory chain. The exposure of PDT-PAO-TPP brought a decrease in state 3 and an increase in state 4 collectively resulting in a significant decline in respiratory control ratio (RCR = state 3/ state 4)(Figure 4A). However, the short-time

incubation of PDT-PAO made the negligible effect on mitochondrial respiration (Figure S4). Activities of complex I and IV in the mitochondrial respiratory chain had an obvious inhibition (Figure 4B) and only high dose of PDT-PAO-TPP suppressed the activity of complex III, whereas complex II still maintained the initial catalytic activity.

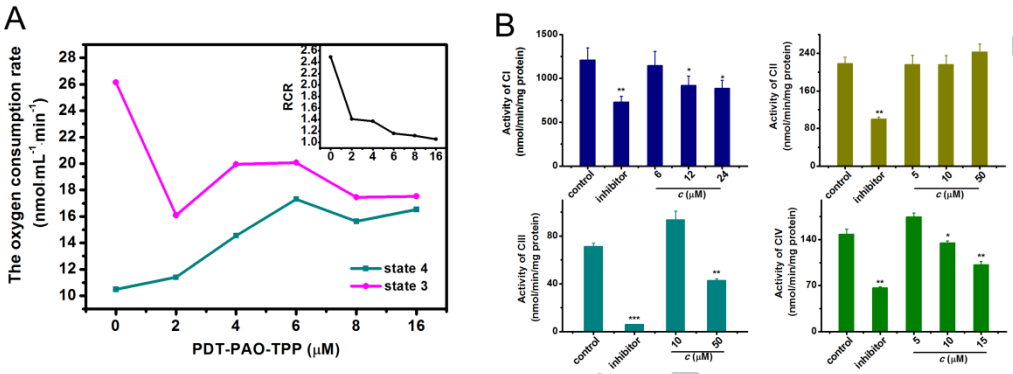


Figure 4. The impairment on mitochondrial respiration. (A) The effect on isolated mitochondrial respiration in the presence of PDT-PAO-TPP. (B) The effect on activities of respiratory chain complex I, II, III and IV by organic arsenical PDT-PAO-TPP. The specific inhibitors added for four complexes are 5 μM rotenone (CI), 10 mM sodium malonate (CII), 4 μM antimycin (CIII), and 3 mM NaN_3 (CIV), respectively. After incubated with compound PDT-PAO-TPP for 3-5 minutes, the absorbance at specific wavelength was monitored for at least 3 minutes.

Assessment of intracellular ROS and thiols level

It has been pointed out that complex I and III are associated with the generation of ROS[39]. As shown in Figure 5A and Figure S5A, the accumulation of ROS was apparent after the long-time incubation with PDT-PAO-TPP or PDT-PAO. Intracellular redox balance is regulated by the variation between free sulfydryl and disulfides in endogenic antioxidants. The generation of excessive ROS and synchronously moderate decrease of total thiols (Figure 5B and Figure S5B) suggested that the redox homeostasis was off and the oxidation status environment in NB4 cells was built. In addition, as the scavenging reagent for intracellular GSH, BSO pre-added can decrease the cellular antioxidant capacity. The cell viability remarkably dropped down even exposed with the nontoxic concentration of

PDT-PAO-TPP or PDT-PAO (Figure 5C and Figure S5C), which confirmed the evidence that reducing inherent GSH level in cancer cells will contribute to enhance the sensitivity for the arsenical drugs.

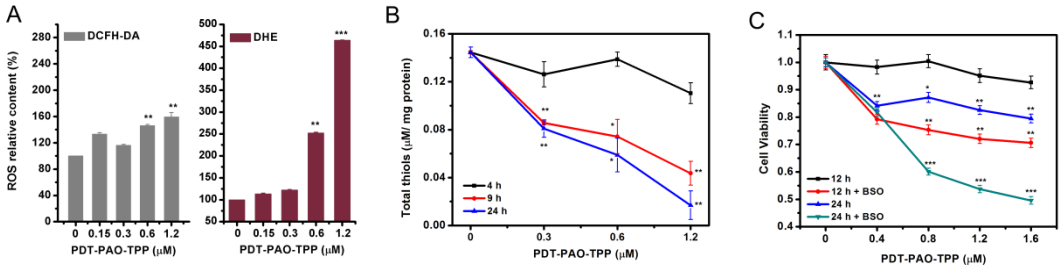


Figure 5. Assessment of intracellular redox state. (A) The variation of intracellular ROS level in the presence of PDT-PAO-TPP by DCFH-DA or DHE staining. (B) The decrease of intracellular total thiols level. (C) The cell viability in the presence of 50 μM BSO or without BSO (MTT assay).

Impairment on mitochondrial membrane and induction of apoptosis

Besides, it is necessary to maintain the mitochondrial membrane integrity for normal function exertion. When exposed with PDT-PAO-TPP, mitochondrial swelling was induced manifesting as the decreased absorbance at 540 nm due to the enhanced permeability to water (Figure 6A), which can be partially deterred by the pre-incubation of CsA, RR and EGTA (Figure S6). Not only that but the membrane permeability to H^+ and K^+ was also enhanced by PDT-PAO-TPP, evidenced by the decline of absorbance at 540 nm (Figure S7). Comparatively, the influences on mitochondrial swelling and permeability by PDT-PAO were slight (Figure S8). Furthermore, the TEM pictures intuitively revealed the declined electron density in mitochondria, the irregular shape and even ruptured membrane after incubated with PDT-PAO-TPP (Figure S9). The defective mitochondrial membrane will prompt some pro-apoptosis proteins to release into cytoplasm, such as cytochrome *c*, pro-caspases and AIF[40,41]. Actually, the release of cytochrome *c* indeed had a remarkable increase via a dose-dependent way (Figure 6B and Figure S10A). Commonly, once cytochrome *c* located in the mitochondria leaks out, the downstream signals like caspase family can be active resulting in cell apoptosis[42]. Just like shown in Figure 6C

and Figure S10B, apoptotic cells proportion (FITC+/PI- and FITC+/PI+) ascended significantly after the addition of PDT-PAO-TPP in a dose- and time-dependent manner, whereas the induction of apoptosis were blocked when the cells pre-cultured with Z-VAD-fmk, a pan-caspase inhibitor[43] (Figure 6D and Figure S10C). Moreover, when the cells underwent apoptosis, the hallmarks including smaller cell size, condensed nuclei, and DNA fragmentation were displayed in Figure S11 evidenced by the blue light point in NB4 cells. Additionally, the death cells were shown as the red light stained by PI.

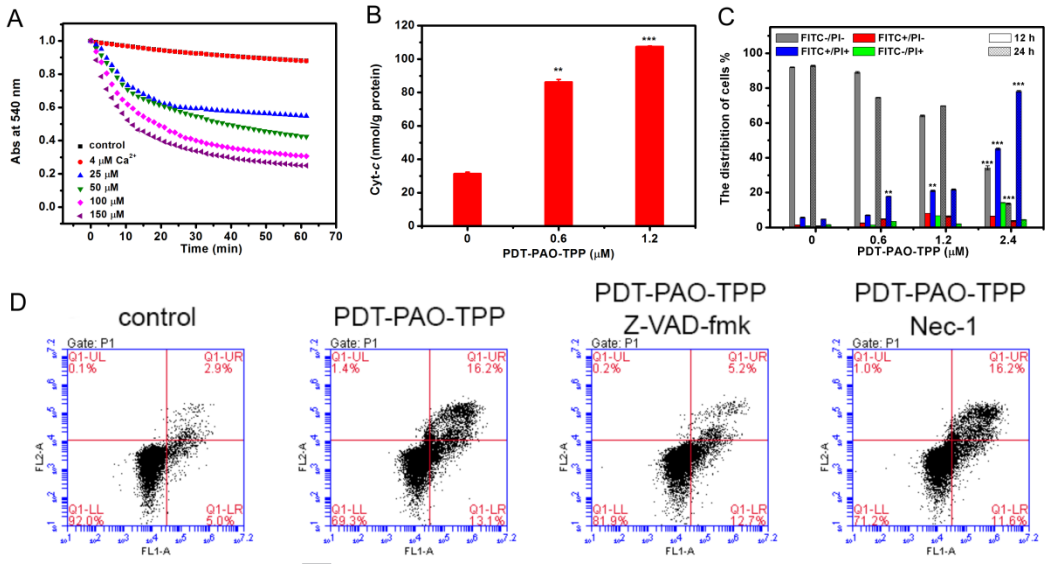


Figure 6. The induction of apoptosis through the mitochondrial pathway. (A) Isolated mitochondrial swelling in the presence of PDT-PAO-TPP in the presence of 4 μM Ca^{2+}. (B) The increase of intracellular Cyt-*c* level for 24 h. (C) Apoptosis induced by PDT-PAO-TPP for 12 h and 24h. (D) The protective effect of 20 μM Z-VAD-fmk or 490 nM Nec-1 on apoptosis.

The newly synthesized organic arsenical PDT-PAO-TPP showed the better inhibitory efficacy to NB4 cells than PDT-PAO, which was attributed to the mitochondria-targeted effect of TPP moity. In the initial 3 h, the majority of PDT-PAO-TPP entered into mitochondria in NB4 cells and immediately affected the membrane potential, which accorded with our assumption about design of PDT-PAO-TPP. It has been reported that PDT-PAO can induce the oxidative stress-mediated apoptosis via targeting thioredoxinreductase in HL-60 cells[6].

Nevertheless, both of PDT-PAO-TPP and PDT-PAO made no apparent differences on intracellular ROS level in the initial 6 h co-culture. Even if the cells were treated with NAC, VE, VC or GSH in advance, the addition of PDT-PAO-TPP or PDT-PAO still induced the decline of cell viability, which indicated the different anti-cancer mechanism.

LA is not only a powerful antioxidant but also a necessary disulfhydryl coenzyme for dihydrolipoyltransacetylase (E2) in pyruvate dehydrogenase complex (PDHC). As expected, whether PDHC or E2 in NB4 cells showed a significant decrease on its catalytic activity after exposure with PDT-PAO-TPP, which supported the result that PDT-PAO-TPP may interact with sulfydryl in DHLA located in the dihydrolipoyltransacetylase (E2) in PDHC to prevent the acryl group converted to CoA in the mitochondria. Moreover, when the As-sulfydryl bond was reduced by the exogenous dimercapto compounds (DTT, DMP and DMSA) to free sulfydryl, the activity of E2 and cell viability of NB4 cells was recovered subsequently. It has been known that tumor cells consume glucose at a dramatically high rate compared to normal cells, which means that they have the priority for aerobic glycolysis for proliferation[44-46]. Although it seems that tumor cells have the less dependency on citric acid cycle to produce ATP, citric acid cycle still plays the key role in cellular proliferation acting as a hub for biosynthesis[46]. The suppression of PDHC activity subsequently inhibited citric acid cycle to provide continuous intermediates, like NADH and FADH, which are associated with the mitochondrial respiratory chain and oxidative phosphorylation process[36,47]. Both PDT-PAO-TPP and PDT-PAO can induce the mitochondrial metabolic disturbance after the long-time exposure via inhibiting the activity of PDHC. Getting rid of other organelles, mitochondria are extracted for further exploration. Based on the assessment of oxygen consumption in isolated mitochondria, the restrained respiration process by PDT-PAO-TPP was revealed clearly. Furthermore, the terminal enzyme complex IV in the respiratory chain passes the electron from cytochrome oxidase to oxygen yielding water, thus it controls the mitochondrial respiration[48]. There is no doubt that the inhibition for complexes by PDT-PAO-TPP was linked tightly to the respiration dysfunction, as

well as the less ATP production through the proton leak[49,50-51]. The proton leak means the escape of electrons from the electron transport chain to randomly hit the nearby molecules and ultimately produce free radicals leading to inevitable ROS. The inhibition of complex I and III by PDT-PAO-TPP induced the leak of electrons and led to the generation of ROS with a higher rate in the mitochondria. Short-time incubation with PDT-PAO-TPP not PDT-PAO can trigger the mitochondrial respiratory depression. Moreover, in the later period of co-incubation with PDT-PAO-TPP or PDT-PAO, the accumulation of ROS and depletion of intracellular thiols can intensify electron escaping, the leakage of protons gradient and disappearance of membrane potential. Besides, enhanced generation of ROS may also attack some reactive cysteine residues located in vital proteins or some important reactions necessary for cellular normal function exertion to trigger a vicious cycle[48].

Mitochondrial membrane permeabilization is thought to be the decisive event delimiting the frontier between cell survival and cell death[50]. Some hallmarks in mitochondrial membrane to indicate cell death appeared after treated by PDT-PAO-TPP. For one thing, it can give rise to mitochondrial swelling as a consequence of increase of IM permeability, which further demonstrated MPTP opening via a Ca^{2+}-dependent way confirmed by the almost complete protection from swelling by CsA, RR and EGTA[52]. Intuitively, this swelling brought about the distension and the disorganization of the cristae as well as the reduction of the electron density of the matrix. For another, the presence of PDT-PAO-TPP enhanced the IM permeability to H^+ and K^+, as well as the interruption of ATP synthesis. Although the effects on mitochondrial membrane by PDT-PAO were relatively slight, it also triggered the mitochondrial dysfunction after the long-time incubation. Additionally, the released cytochrome *c* from mitochondria can promote the formation of "apoptosome" to active the caspase-9, which in turn catalyzes the proteolytic activation of the effector caspases like caspase-3[53]. Based on these observations, both of PDT-PAO-TPP and PDT-PAO induced the mitochondrial dysfunction and imbalance of redox state leading to the caspase family-dependent intrinsic apoptosis via the inhibition towards PDHC, which is different from the

mechanism of PDT-PAO in HL-60 cells due to the different cell properties. The major anti-tumor mechanism difference between PDT-PAO-TPP and PDT-PAO was that organic arsenical PDT-PAO-TPP can work faster with higher efficacy via impairing the mitochondria in the cancer cells as a mitochondria-targeted compound.

Conclusion

In conclusion, a mitochondria-targeted organic arsenical PDT-PAO-TPP was synthesized by the introduction of TPP to PDT-PAO. Compared to PDT-PAO, PDT-PAO-TPP showed the better efficacy towards NB4 cells. The accumulation of PDT-PAO-TPP in mitochondria improved the effective concentration of organic arsenical to exert the influence on mitochondria faster. The dual inhibition of PDHC and respiratory chain complexes accelerated the thermogenesis disorder and respiratory dysfunction, as well as the oxidative stress injury. The further damage on the mitochondrial membrane integrity stimulated the release of cytochrome *c*, which activated the caspase family-dependent mitochondrial apoptosis pathway. These findings provide deep insights into the design and the anti-tumor mechanism of organic arsenical, which indicates the complexity and diversity in the research about organic arsenical as a promising anti-cancer drug.

Experimental

All the details were placed in Supporting Information.

Acknowledgment

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