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Adaphostin and other anticancer drugs quench the fluorescence of mitochondrial potential probes

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Abstract

Fluorescent dyes are widely used to monitor changes in mitochondrial transmembrane potential ($\Delta\Psi_m$). When MitoTracker Red CMXRos, tetramethylrhodamine methyl ester (TMRM), and 3,3′dihexyloxacarbocyanine iodide ($DiOC_6(3)$) were utilized to examine the effects of the experimental anticancer drug adaphostin on intact cells or isolated mitochondria, decreased fluorescence was observed. In contrast, measurement of tetraphenylphosphonium uptake by the mitochondria using an ion-selective microelectrode failed to show any effect of adaphostin on $\Delta\Psi_m$. Instead, further experiments demonstrated that adaphostin quenches the fluorescence of the mitochondrial dyes. Structure–activity analysis revealed that the adamantyl and *p*-aminobenzoic acid moieties of adaphostin are critical for this quenching. Anticancer drugs containing comparable structural motifs, including mitoxantrone, aminoflavone, and amsacrine, also quenched the mitochondrial probes. These results indicate the need for caution when mitochondrial dyes are utilized to examine the effects of xenobiotics on $\Delta\Psi_m$ and suggest that some previously reported direct effects of anticancer drugs on mitochondria might need re-evaluation.

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Abbreviations: $\Delta\Psi_m$, mitochondrial transmembrane potential; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; DNP, 2,4-dinitrophenol; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; PBS, calcium- and magnesium-free Dulbecco's phosphate buffered saline; TMRM, tetramethylrhodamine methyl ester; TTP^+, tetraphenylphosphonium

Introduction

Mitochondrial function is routinely evaluated during a variety of biochemistry, cell biology and cellular pharmacology experiments. For example, changes in mitochondrial function are often monitored in cardiac muscle cells subjected to ischemia/reperfusion injury after cardioprotective treatments[1–3] and in hepatocytes exposed to various toxicants.[4,5] Likewise, mitochondrial integrity is commonly assessed as cells undergo apoptosis.[6] Mitochondrial alterations that occur during this latter process include changes in mitochondrial transmembrane potential ($\Delta\Psi_m$) and ultrastructure[7–10] as well as release of proapoptotic proteins such as cytochrome *c* and Smac/Diablo from the intermembrane space.[11–13]

In normal mitochondria, transport of electrons along the respiratory chain is accompanied by vectorial proton pumping across the inner mitochondrial membrane, thereby creating an electrochemical gradient of hydrogen ions.[14,15] This gradient establishes a transmembrane electrical potential of ~ -220 mV, with the mitochondrial matrix being negatively charged.[16] The measurement of this gradient provides one of the most common ways of assessing mitochondrial function during various processes in living cells.

In living cells and tissues, $\Delta\Psi_m$ is usually assessed using positively charged lipophilic probes. As a result of their positive charges, these probes specifically accumulate within negatively charged mitochondria. Depending on the nature of the probe, this accumulation can be detected by fluorescence (e.g., using MitoTracker Red CMXRos, tetramethylrhodamine methyl ester (TMRM), or 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$)),[6,17] by a selective electrode (tetraphenylphosphonium (TTP^+) chloride)[18] or by scintillation counting (^{3}H-triphenylmethylphosphonium).[17,19,20] For cell biology experiments, the fluorescent probes are commonly used in conjunction with either fluorescence microscopy[21] or, if quantitation is required, flow cytometry.[22] The latter technique permits an assessment of $\Delta\Psi_m$ that is sensitive, quantitative and requires a relatively small number of cells. Using this approach, it has been suggested that changes in $\Delta\Psi_m$ might be an early apoptotic event, particularly after treatment with certain chemotherapeutic agents.[6,23]

As methods for monitoring mitochondrial function have been developed, it has become clear that certain aspects of experimental design can have an important effect on the outcome of experiments. For example, Uren *et al.*[24] recently reported that detection of cytochrome c release from mitochondria during apoptosis is critically

dependent upon the composition of the buffer used to fractionate the apoptotic cells. A number of issues also require careful attention when fluorescent dyes are utilized to measure $\Delta\Psi_m$. In particular, the fluorescence of the probes is dependent on differences in cellular uptake and retention, probe toxicity, photobleaching, signal calibration, and interactions between fluorophores.[17] Inattention to these details has been reported to lead to incorrect interpretation of experimental results.[17]

In the present study, we examined the effects of the investigational anticancer drug adaphostin (NSC 680410) on mitochondrial function using several techniques. Previous studies have demonstrated that adaphostin causes an increase in reactive oxygen species[25] and activation of the mitochondrial apoptotic pathway.[26] In the present study, we observed that adaphostin treatment of cells or isolated mitochondria diminished the fluorescence of mitochondrial fluorescent probes. In contrast, when a TTP^+-sensitive electrode was utilized to assess the effects of treatment, adaphostin had no effect on $\Delta\Psi_m$ or oxidative phosphorylation. Instead, further analysis demonstrated that adaphostin directly quenched the emissions of fluorescent mitochondrial probes. Other anticancer drugs, including aminoflavone (NSC 686288), mitoxantrone, and amsacrine, exhibited a similar effect, suggesting that this quenching of mitochondrial probes is not limited to one investigational agent.

Results

Effect of adaphostin on the fluorescence of cells stained with mitochondrial probes

Recent studies from our laboratory indicated that apoptosis induced by the experimental anticancer drug adaphostin in human leukemia cell lines is preceded by an increase in reactive oxygen species.[25] Since mitochondria are involved in generating reactive oxygen species[27] and in apoptosis,[11–13,28] we investigated the effect of adaphostin on mitochondrial membrane potential.

$\Delta\Psi_m$ was initially measured by staining cells with the widely used mitochondrial probes MitoTracker Red CMXRos, $DiOC_6(3)$, and TMRM. For these experiments, K562 cells were loaded with each mitochondrial probe, then treated with 10 μM adaphostin or solvent (0.1% DMSO) for 30 min. After treatment, cells were subjected to flow microfluorimetry. As shown in Figure 1a–c, adaphostin markedly decreased the fluorescence of K562 cells stained with different probes. Similar results were obtained when other cells, including the human leukemia lines HL-60, Jurkat and JB-6, the murine factor-dependent cell lines 32D and FDCP-1, and freshly isolated rat hepatocytes, were examined in similar experiments (Figure 1d and data not shown) and when cells were labeled with the probe Mitotracker green (data not shown). Identical changes were observed less than 5 min after addition

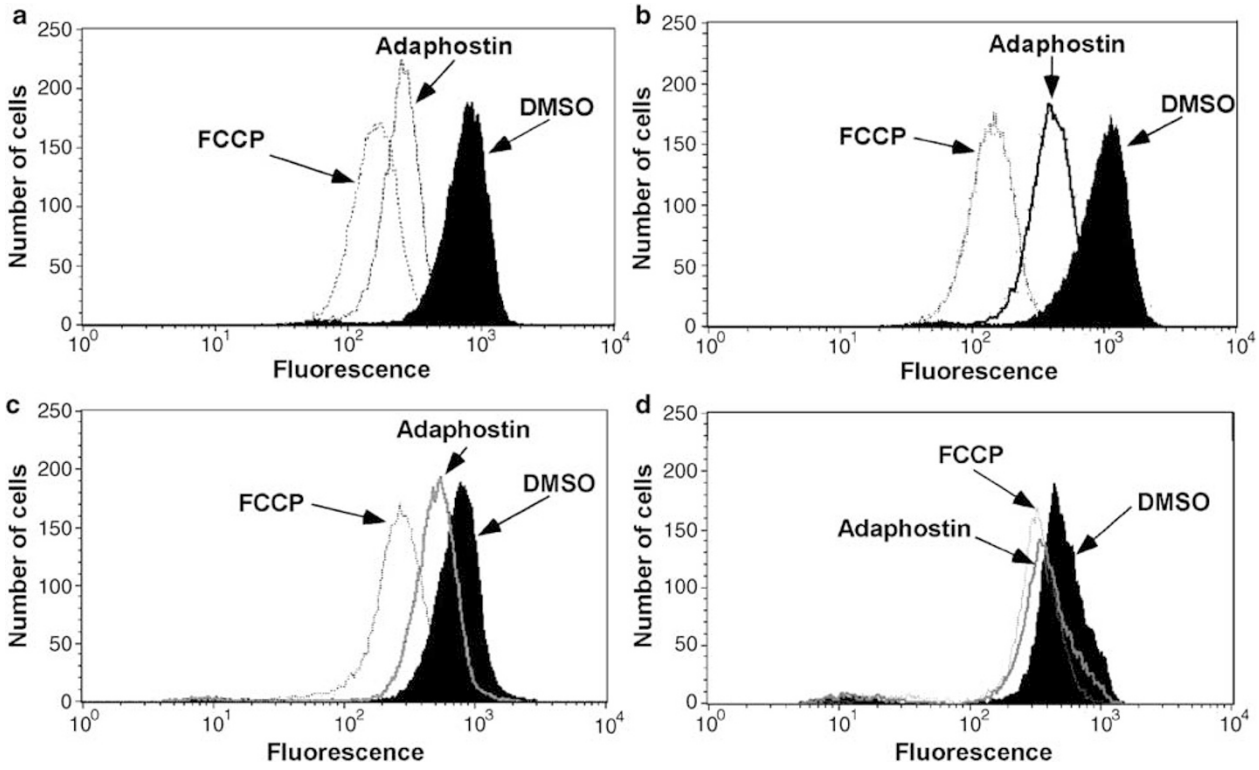


Figure 1 Decreased fluorescence of mitochondrial probes in cells treated with adaphostin. (**a**–**c**), K562 cells stained with Mitotracker Red (**a**), TMRM (**b**), or $DIOC_6(3)$ (**c**) were incubated with 10 μM adaphostin or diluent (0.1% DMSO) at 37°C for 30 min and subjected to flow microfluorimetry. As a positive control for decreased $\Delta\Psi_m$, cells were treated with 10 μM FCCP, a known uncoupler of oxidative phosphorylation, in place of adaphostin. (**d**) Isolated rat hepatocytes stained with TMRM were incubated with 10 μM adaphostin or diluent (0.1% DMSO) at 37°C for 30 min and subjected to flow microfluorimetry. FCCP again served as positive control

of adaphostin to intact cells. These changes were somewhat smaller in magnitude but qualitatively similar to alterations induced by carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), a potent uncoupler of oxidative phosphorylation that induces a drop in mitochondrial potential.[29]

Effect of adaphostin on isolated mitochondria from K562 cells

Since our previous studies demonstrated that adaphostin does not induce caspase activation in K562 cells for 6–8 h,[26] the rapid decrease in mitochondrial fluorescence could not be attributed to some event downstream of caspase activation.[30] Accordingly, further experiments were designed to determine whether adaphostin was directly affecting mitochondria.

Several drugs have recently been reported to induce release of cytochrome *c* and Smac/Diablo from isolated mitochondria *in vitro*.[23] To determine whether adaphostin has a similar effect, mitochondria from K562 cells were treated with 10 μM adaphostin or diluent for 60 min and then sedimented. When the pellet and supernatant were analyzed by immunoblotting using antibodies against cytochrome *c* and Smac/Diablo, adaphostin did not appear to increase leakage of either polypeptide from mitochondria (Figure 2a). These results distinguish adaphostin from peptides that permeabilize the outer mitochondrial membrane.[31] Nonetheless, when K562 mitochondria were stained with TMRM and incubated with 10 μM adaphostin or DMSO (solvent), flow microfluorimetry indicated that adaphostin significantly decreased the fluorescence of isolated mitochondria stained with TMRM (Figure 2b). Similar results were observed using isolated rat liver mitochondria (Figure 2c).

Effect of adaphostin on isolated rat liver mitochondria

To confirm the effect of adaphostin on mitochondrial membrane potential, we next monitored $\Delta\Psi_m$ of isolated mitochondria using an electrode to measure changes in concentration of the mitochondrial specific probe TPP^+.[32] As a result of the electrical membrane potential generated across the inner membrane by oxygen-dependent proton pumps of the mitochondrial respiratory chain,[33] mitochondria incubated in the presence of oxidative substrates and oxygen avidly accumulate TPP^+ from the incubation medium into their matrix.[32] This uptake can be detected as a drop in the free TPP^+ concentration measured using a TPP^+-sensitive electrode. This method requires large amounts of mitochondria but does not depend on fluorescence of the probe.

The steady-state membrane potential of mitochondria oxidizing a mixture of pyruvate and malate reaches -170 to -180 mV (negative inside) within 60 s (Figure 3a). A typical experiment demonstrating the effect of adaphostin on mitochondrial membrane potential monitored using a TPP^+ electrode is shown in Figure 3a. Addition of 10 μM adaphostin did not affect the steady-state mitochondrial membrane potential. In contrast, addition of 2,4-dinitrophenol (DNP), an established protonophoric uncoupler, immediately dissipated

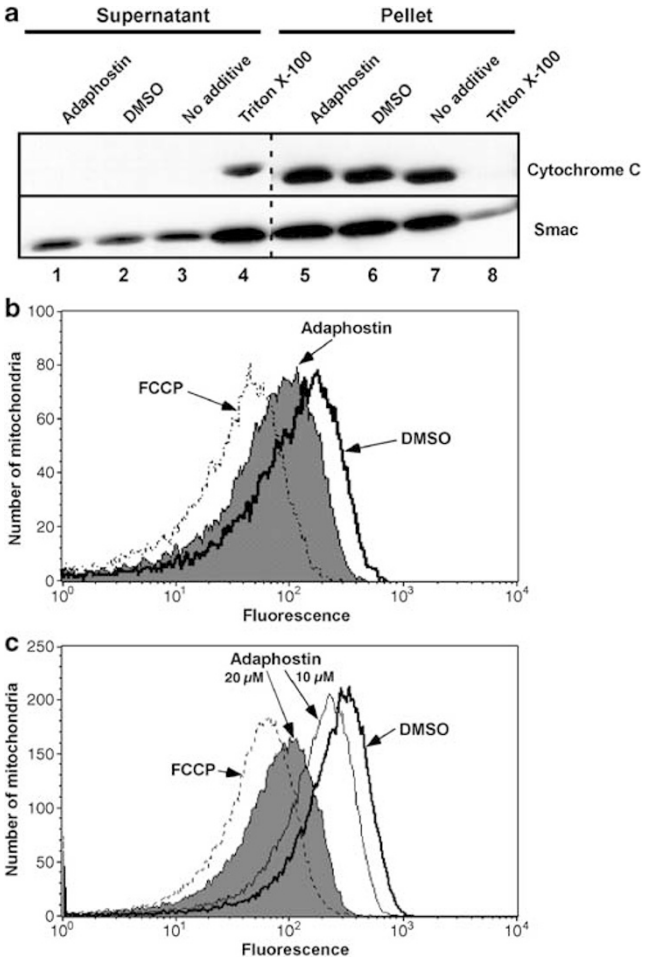


Figure 2 Effects of adaphostin on isolated mitochondria. (**a**) Equal amounts of mitochondria (50 μg total protein) isolated from K562 cells were incubated for 1 h at 30°C with buffer B containing no additives (negative control), 10 μM adaphostin, or 0.1% DMSO. As a positive control for release of mitochondrial intermembrane proteins, 0.1% (w/v) Triton X-100 was substituted for adaphostin. After incubation, mitochondria were sedimented at 12 000 $\times$ *g* for 15 min. The pellet and supernatant were separately subjected to SDS-PAGE followed by blotting with reagents that recognize cytochrome *c* (upper panel) and Smac/Diablo (lower panel). (**b**) Mitochondria isolated from K562 cells were stained with TMRM and then incubated with 10 μM adaphostin or 0.1% (v/v) DMSO in buffer B for 10 min at 30°C prior to flow microfluorimetry. As a positive control for decreased $\Delta\Psi_m$, 10 μM FCCP was substituted for adaphostin. (**c**) Rat liver mitochondria in buffer C were stained with TMRM and then incubated with adaphostin or 0.1% (v/v) DMSO for 10 min at 30°C prior to flow microfluorimetry. As a positive control for decreased $\Delta\Psi_m$, 10 μM FCCP was substituted for adaphostin

$\Delta\Psi_m$, as indicated by a drop in mitochondrial TPP^+ accumulation.

Additional experiments demonstrated that adaphostin (10 μM) also did not affect mitochondrial oxidative phosphorylation stimulated by added ADP (Figure 3b, c). Conversion of added ADP into ATP is associated with dissipation of the proton motive force, mitochondrial depolarization and an increased rate of oxygen consumption (state 3). Once phosphorylation of added ADP is completed, mitochondria return to state 4 (postphosphorylation state) characterized by restoration of the mitochondrial membrane potential and respiration rate.[33] Thus, addition of a defined amount of ADP

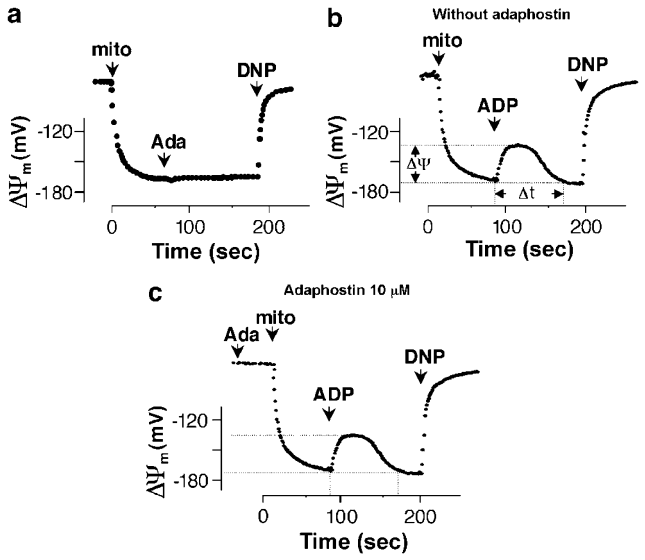


Figure 3 Adaphostin has no effect on $\Delta\Psi_m$ of isolated mitochondria as assessed using a TTP^+ sensitive microelectrode. (**a**) Isolated rat liver microchrondria were added to buffer C containing TTP^+. At the indicated times, 10 μM adaphostin or 10 μM DNP was added. The small alteration in mitochondrial TTP^+ accumulation observed after addition of adaphostin was also seen after addition of diluent. In contrast, dissipation of $\Delta\Psi_m$ with DNP resulted in a marked increase in free TTP^+. (**b**, **c**) Isolated rat liver microchrondria were added to buffer C containing TTP^+ in the absence (**b**) or presence (**c**) of 10 μM adaphostin. At the indicated times, ADP was added. DNP (10 μM) was subsequently added to dissipate the transmembrane proton gradient

into energized mitochondria will result in transient and reversible activation of respiration as well as transient depolarization of the mitochondrial membrane.[32] A typical record of ADP-induced changes in mitochondrial membrane potential, which was monitored using the TPP^+-electrode, is shown in Figure 3b. As shown in Figure 3c, addition of 10 μM adaphostin to the incubation medium before the mitochondria did not affect either the amplitude or the duration of ADP-induced transient depolarization, demonstrating that adaphostin has no effect on mitochondrial oxidative phosphorylation.

Collectively, these results raised the possibility that the decreased fluorescence seen after treatment of intact cells (Figure 1) or mitochondria (Figure 2b, c) with adaphostin might reflect some effect other than an alteration in $\Delta\Psi_m$.

Adaphostin decreases the fluorescence of mitochondrial probes in solution

To assess the possibility that the fluorescence decrease might result from a direct quenching effect of adaphostin on the mitochondrial probes, adaphostin or vehicle was added directly to aqueous solutions of the membrane potential-sensitive fluorescent dyes in the absence of added mitochondria. Adaphostin dramatically attenuated the fluorescence of each of the probes (Figure 4a, b). As indicated in the spectrum in Figure 4a, adaphostin quenched the fluorescence of TMRM without shifting the emission wavelength. Examination of the

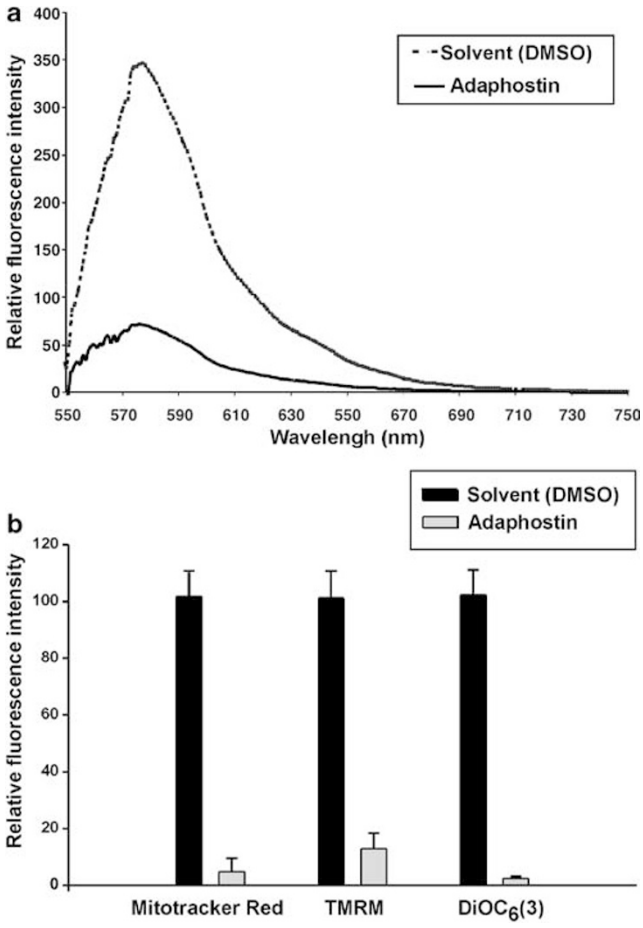


Figure 4 Adaphostin decreases fluorescence of mitochondrial probes in solution. (**a**) Emission spectrum of TMRM (50 nM) excited at 530 nm in PBS in the absence or presence of 10 μM adaphostin. (**b**) Fluorescence emission of each of the indicated dyes in PBS containing 10 μM adaphostin relative to emission in PBS containing 0.1% DMSO. Error bars, mean $\pm$ S.D. of three independent experiments

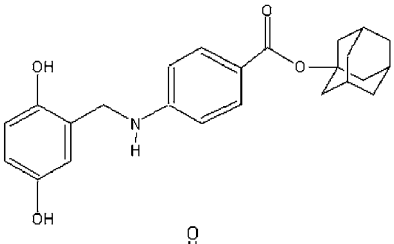
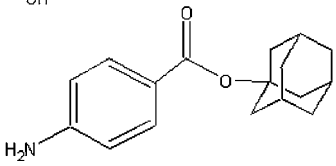
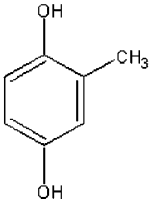
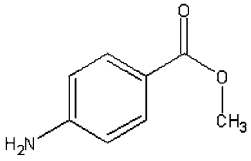
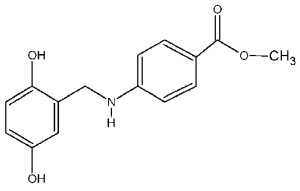
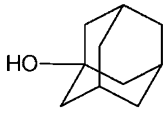
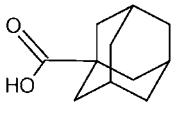
emission spectra of MitoTracker Red CMXRos and $DiOC_6(3)$ revealed similar fluorescence quenching. At the optimal emission wavelength, the fluorescence of MitoTracker Red CMXRos in the presence of 10 μM adaphostin was only $4.9 \pm 4.7\%$ (mean $\pm$ S.D., $n = 3$) of the fluorescence in the absence of adaphostin (Figure 4b). Likewise, adaphostin diminished the fluorescence of the TMRM and $DiOC_6(3)$ probes to 12.9 ± 5.5 and $2.4 \pm 0.8\%$ of control, respectively (Figure 4b).

Functional groups responsible for the quenching effect of adaphostin

To identify the functional group responsible for the quenching effect, the ability of adaphostin analogs to quench the fluorescence of mitochondrial specific dyes was measured as illustrated in Figure 4. Results of this analysis are summarized in Table 1. These experiments led to the following observations:

(1) *The hydroquinone moiety of adaphostin does not contribute much to the quenching ability of adaphostin*. Like

Table 1 Domains of adaphostin responsible for the observed quenching effect

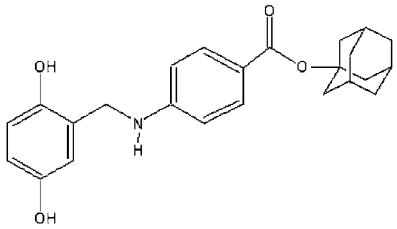
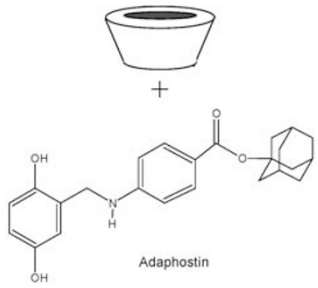
Drug name	Structure	Relative fluorescence		
		TMRM(%)	$DiOC_6(3)$(%)	Mitotraker Red(%)
Adaphostin 10 μM		12.9$\pm$5.5	2.4$\pm$0.8	4.9$\pm$4.7
p-Aminobenzoic acid, adamantyl ester 10 μM		61.4$\pm$1.2	27.0$\pm$1.5	8.2$\pm$1.5
2-methyl hydroquinone 10 μM		100.7$\pm$7.2	91.5$\pm$12.0	63.3$\pm$13.3
Methyl *p*-Aminobenzoate 10 μM		106.0$\pm$12.3	125.9$\pm$16.6	123.8$\pm$5.4
AG957 10 μM		103.3$\pm$6.5	97.1$\pm$6.3	70.7$\pm$4.5
1-adamantanol 10 μM		110$\pm$12	78$\pm$4	110$\pm$4
1-adamantane -carboxylic acid 10 μM		118$\pm$9	95$\pm$7	127$\pm$11

Compounds (final concentration 10 μM) or diluent (0.1% DMSO) were added directly to PBS containing each dye as indicated in the text. Numbers represent the ratio of fluorescence in the presence of adaphostin to fluorescence in the presence of DMSO. Error measurements, mean$\pm$S.D. of three independent experiments

adaphostin, the adamantyl ester of *p*-aminobenzoic acid, a compound that lacks the hydroquinone portion of adaphostin, significantly quenched the fluorescence of all three mitochondrial probes, indicating that the hydroquinone moiety is not necessary for the quenching activity. In contrast, 2-methylhydroquinone did not have significant quenching activity.

(2) *The adamantyl moiety plays an important role in the quenching activity of adaphostin.* AG957, an adaphostin analog that contains a methyl group instead of the adamantyl group, had a much less dramatic effect than adaphostin. Likewise, the adamantyl ester of *p*-aminobenzoic acid quenched the fluorescence of the mitochondrial dyes, but the methyl ester of *p*-aminobenzoic acid did not.

Table 2 β-Cyclodextrin inhibits quenching ability of adaphostin

Drug name	Structure	Relative fluorescence		
		TMRM(%)	$DiOC_6(3)$(%)	Mitotraker Red(%)
Adaphostin 10 μM		12.9$\pm$5.5	2.4$\pm$0.8	4.9$\pm$4.7
β-cyclodextrin 100 μM		101$\pm$2	100$\pm$7	93$\pm$4
adaphostin 10 μM+β-cyclodextrin 100 μM		87$\pm$5	53$\pm$2	51$\pm$3

Experiments were performed as described in the legend to Table 1

(3) *The adamantyl group itself does not quench the fluorescence of mitochondrial specific probes.* Neither 1-adamantanol nor 1-adamantane-carboxylic acid quenched the fluorescence of probes used in the experiment, suggesting that quenching requires both the adamantyl and *p*-aminobenzoic acid moieties.

It has previously been reported that some compounds containing aromatic amino groups can quench the fluorescence of certain fluorophores.[34–36] We speculated that the adamantyl group, due to its lipophilicity, facilitates binding of adaphostin to the relatively hydrophobic surface of fluorescent probes, bringing the aromatic amino group of adaphostin closer to the fluorophore and enhancing the interactions that result in quenching. To access the validity of this model, the possible hydrophobic association between the adamantyl group of adaphostin and fluorescent probes was blocked by addition of β-cyclodextrin. This agent has a hydrophobic cavity that can accommodate the bulky hydrophobic adamantyl group to form an inclusion complex (or host–guest complex)[37–39] surrounded by a hydrophilic dextrin surface that could disrupt the postulated hydrophobic interactions between adamantyl group and fluorophore. As predicted, addition of β-cyclodextrin to adaphostin-containing solutions restored the fluorescence of TMRM, $DiOC_6(3)$, and Mitotracker Red (Table 2).

Quenching effect of other anticancer drugs

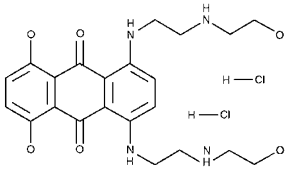
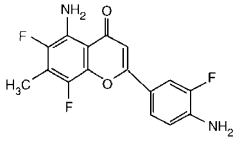
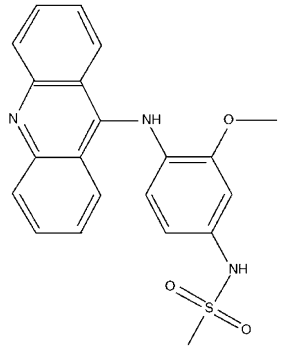
To determine whether the observed quenching effect was unique to adaphostin, we examined the effect of other anticancer drugs containing aromatic amines. Several other anticancer compounds, including aminoflavone, mitoxantrone and amsacrine, also attenuated the fluorescence of probes used to measure $\Delta\Psi_m$ (Table 3). The common motifs in all of these drugs are their hydrophobic groups and their aromatic amines, suggesting that additional drugs containing similar chemical groups might exhibit a similar quenching effect.

Discussion

Experiments in the present study were designed to determine whether adaphostin, like other recently described drugs,[23] exerts a direct effect on mitochondria. When a TTP^+-sensitive electrode was utilized, this analysis failed to demonstrate any effect of 10 μM adaphostin on $\Delta\Psi_m$ or oxidative phosphorylation (Figure 3). On the other hand, the present results also indicated that adaphostin and other drugs containing aromatic amines can interfere with measurement of $\Delta\Psi_m$ when fluorescent dyes are used as mitochondrial probes. This limitation of widely utilized fluorescent mitochondrial probes is important to recognize in order to avoid potentially misleading interpretation of experimental results.

Our initial experiments demonstrated that adaphostin markedly decreases the fluorescence of mitochondrial probes in intact cells (Figure 1) as well as in isolated mitochondria (Figure 2b, c). Although the decrease in fluorescence was similar to that observed when cells or mitochondrial are treated with agents like FCCP, which disrupts $\Delta\Psi_m$, several additional observations argued against the possibility that

Table 3 Quenching effect of selected anticancer drugs

Drug name	Structure	Relative fluorescence		
		TMRM(%)	$DiOC_6(3)$(%)	Mitotraker Red(%)
Mitoxantrone 10 μM		89.3$\pm$3.9	56.9%$\pm$11.5	33.0%$\pm$2.9
Aminoflavone (NSC 686288) 10 μM		66.0$\pm$2.6	45.7%$\pm$1.8	48.5$\pm$2.9
Amsacrine 10 μM		95.2$\pm$0.4	60.7$\pm$10.7	67.8$\pm$4.3

Experiments were performed as described in the legend to Table 1

adaphostin was directly affecting mitochondrial function. First, adaphostin did not cause leakage of cytochrome *c* and Smac/Diablo from mitochondria (Figure 2a). Second, known inhibitors of $\Delta\Psi_m$ loss, such as cyclosporine A and bongkrekic acid, did not prevent the adaphostin-induced decrease in fluorescence intensity (data not shown). Third, when TTP^+ was used to probe $\Delta\Psi_m$ by nonfluorescent methods, no adaphostin-induced decrease in $\Delta\Psi_m$ was detectable (Figure 3a). Based on these results, we turned to the possibility that adaphostin might decrease the fluorescence of the mitochondrial probes through a direct interaction.

Consistent with this hypothesis, we observed that addition of adaphostin to solutions containing mitochondrial dyes reduced their fluorescence (Figure 4 and Table 1). Since other anticancer drugs, including aminoflavone (NSC 686288), mitoxantrone, and amsacrine, also quenched the fluorescence of the mitochondrial probes (Table 3), this effect is clearly not limited to adaphostin. Additional studies are required to determine how widespread this phenomenon is.

Further experiments demonstrated that the adamantyl group is critical for the quenching activity of adaphostin (Table 1). This group itself, however, does not quench the fluorescence of probes, as indicated by the failure of 1-adamantanol and 1-adamantane carboxylic acid to significantly decrease the fluorescence of the probes (Table 1). Accordingly, we hypothesized that the lipophilic nature of the adamantyl group allows a hydrophobic interaction between adaphostin and the lipophilic mitochondrial dyes, allowing closer juxtaposition of the fluorophores with the *p*-aminobenzoic acid moiety of adaphostin that is also crucial for the quenching (Table 1). In agreement with this model, masking the lipophilic surface of adaphostin by β-cyclodextrin inhibited the quenching activity of adaphostin (Table 2).

Despite the widespread use of mitochondrial dyes to measure $\Delta\Psi_m$ during the course of apoptosis[6] and in a variety of other settings,[1–5] we are not aware of any previous publications indicating that measurement of $\Delta\Psi_m$ by fluorescence probes can be affected as a result of direct quenching by chemical compounds. Although a few publications have shown that certain aromatic amines can quench the fluorescence of some fluorescent dyes,[40,41] providing precedence for the current observations, the effect of these amines on biological essays was not investigated. Therefore, we were surprised to observe that anticancer drugs could significantly affect measurement of $\Delta\Psi_m$ with commonly used fluorescent probes. Caution appears to be required when fluorescence probes are used to assess mitochondrial membrane potential changes, as the change in fluorescence readout does not always reflect the true change in potential. It appears that compounds containing two common structural moieties, a highly hydrophobic group (e.g., adamantyl) and a potential electron transfer group (e.g., aromatic amines), are particularly likely to cause this type of quenching.

Materials and Methods

Reagents

Adaphostin (NSC 680410) and the adamantyl ester of *p*-aminobenzoic acid, were synthesized by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Bethesda, MD, USA). Additional agents were purchased from following suppliers: AG957 from CalBiochem (La Jolla, CA, USA); MitoTracker Red CMXRos, $DiOC_6(3)$, and TMRM from Molecular Probes (Eugene, OR, USA); DNP and FCCP from Aldrich (Milwaukee, WI, USA); mouse anticytochrome *c* from Pharmingen (San Diego, CA, USA); and rabbit anti-Smac/Diablo from Biomol (Plymouth Meeting, PA, USA). Aminoflavone (NSC 686288) was kindly provided by Dr. Matthew Ames (Mayo Clinic, MN, USA). Mitoxantrone and amsacrine were from Lederle Laboratories (Hamsphire, England) and Warner-Lambert (Ann Arbor, MI, USA), respectively.

Cells

K562 cells (American Type Culture Collection, Manassas, VA, USA) were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin and 2 mM L-glutamine (medium A). Rat hepatocytes were isolated as described by Bronk and Gores.[42]

Measurement of $\Delta\Psi_m$ in intact cells using flow cytometry

Cells were stained with MitoTracker Red CMXRos (25 nM), $DiOC_6(3)$ (20 nM), or TMRM (50 nM) in medium A at 37°C for 15 min. After staining, cells were treated with anticancer drugs for the indicated length of time, then placed on ice for 5 min and immediately subjected to flow microfluorimetry using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) with a 488 nm laser. The fluorescence emission of MitoTracker Red CMRos, $DiOC_6(3)$, and TMRM was collected through 650 nm/LP, 530 nm/30 and 585/42 nm filters, respectively. Data were accumulated by analyzing 20 000 events using CellQuest software (Verity Software House, Topsham, ME, USA).

Isolation of mitochondria (adapted from Yang *et al.*[43])

All steps were performed at 4°C. About 10^8 K562 cells were harvested by centrifugation at $100 \times g$ for 5 min, washed twice with ice-cold calcium- and magnesium-free Dulbecco's phosphate buffered saline (PBS) and resuspended in 5 ml of buffer B (20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 3 mM $MgSO_4$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, 0.1 mM α-phenylmethylsulfonyl fluoride and 250 mM sucrose). After 20 min on ice, cells were homogenized in a tight-fitting Dounce homogenizer until lysed ($\sim$50 strokes). After two $800 \times g$ spins to remove unbroken cells and nuclei, mitochondria were sedimented at $10\,000 \times g$ for 15 min at 4° C, resuspended in 5 ml ice-cold buffer B and placed on ice for 15–30 min until used. Alternatively, mitochondria were isolated from rat liver as described.[44]

Measurement of $\Delta\Psi_m$ of isolated mitochondria using a tetraphenylphosphonium chloride-sensitive electrode

$\Delta\Psi_m$ was measured in buffer C (200 mM mannitol, 75 mM sucrose, 5 mM KH_2PO_4, 5 mM pyruvate, 5 mM malate, 2 mM $MgCl_2$, 1 mM EGTA, and 10 mM MOPS (pH 7.35)) using a home-made TPP^+-sensitive mini-electrode as described.[32] Briefly, mitochondria (1 mg of protein) were added to the buffer C containing 500 nM TTP chloride; and changes in free TPP^+ concentration in the incubation medium were monitored. $\Delta\Psi$ was calculated from the TPP^+-electrode reading according to the following equation: $\Delta\Psi = 59\log(\nu/\boldsymbol{V}) - 59\log(10^{(E-Eo)/59} - 1)$, where $\Delta\Psi$ is the mitochondrial membrane potential (in mV), ν is the mitochondrial matrix volume (1.6 μl *per* mg mitochondrial protein[45]), $\boldsymbol{V}$ is the volume of the incubation medium (1 ml), and E_0 and E are electrode potentials (in mV) before and after addition of mitochondria, respectively.[46] Adaphostin up to 25 μM and vehicle (DMSO) up to 0.5% did not interfere with the readings of the TPP^+-sensitive electrode. Electrodes that exhibited a Nernstian response were used.

Measurement of $\Delta\Psi_m$ of isolated mitochondria using TMRM and flow cytometry

Isolated mitochondria were suspended in buffer B (for K562 mitochondria) or buffer C (for rat liver mitochondria). TMRM (50 nM) was added to the suspension of isolated mitochondria. After addition of the indicated reagents, mitochondria were incubated at 30°C for the indicated length of time, placed on ice and immediately subjected to flow cytometry as described above for intact cells.

Immunoblotting

Protein samples were separated on SDS-polyacrylamide gels containing 5–15% acrylamide gradients, transferred to nitrocellulose and probed with primary antibodies followed by horseradish peroxidase-coupled secondary antibodies using standard procedures.[47]

Measurement of fluorescence of mitochondrial probes in solution

The mitochondrial probes MitoTracker Red CMXRos, $DiOC_6(3)$, and TMRM were dissolved in PBS at final concentrations 25 nM, 20 nM, and 50 nM, respectively. Compounds of interest were added to each dye containing PBS solution. Aliquots (300 μl volume) were transferred to a Microfluor 2 Black microplate (Thermolabsystem, Franklin, MA, USA) and read on a SpectraMax Gemini EM (Molecular Devices, Menlo Park, CA, USA) fluorescent microplate reader using excitation/emission wavelengths of 544/612, 444/501, and 530/590 nm for MitoTracker Red CMXRos, $DiOC_6(3)$, and TMRM, respectively.

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