Thiol - Dependent Sensitization of Mitochondria and Tumor Cells to Doxorubicin

Nadezhda I Fedotcheva1*, Valeri A Bykov2, Viktor V Banin2, Tatyana A Fedotcheva3, Vladimir M. Rzheznikov1 and Nikolai L Shimanovski3

1Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino, Moscow region, Russia
2All-Russian Research Institute of Medicinal and Aromatic Plants, Moscow, Russia
3Russian State Medical University, Moscow, Russia

Abstract

The problem of multidrug resistance has been intensively studied during the last decade. It is known that some synthetic progestins can chemosensitize tumor cells to cytostatic drugs; however, the mechanisms of the sensitization are not clearly understood. In this study, the effect of synthetic analogue of progesterone (17α-acetoxy-3b-butanoxy-6-methyl-pregna-4, 6-diene-20-on, buterol) on the survival of doxorubicin-resistant MCF-7 cells and the coupling of isolated mitochondria was investigated in comparison with the thiol reagent N-ethylmaleimide (NEM). Both compounds increased the doxorubicin-induced cell death and decreased the oxidative phosphorylation and the coupling in mitochondria; their presence was necessary for the direct action of doxorubicin on the mitochondrial membrane potential. Possible specific targets for NEM and buterol are the SH-groups of mitochondrial ATPase and the nucleotide-binding domain of MDR proteins, owing to which they inhibit the activity of both ATPase and MDR proteins and increase the accumulation of drugs in the cell. In addition, buterol can form hydrogen bonds between its ether carbonyl at carbon C3 and some other thiols, thereby inducing oxidative stress. By contrast to NEM, which irreversibly blocks thiol groups, buterol binds thiol non-covalently, which provides a new useful approach to the enhancement of the anticancer activity of cytostatics in tumor cells.

Introduction

One of the main problems of tumor chemotherapy is a decrease in the efficacy of cytostatics due to the development of resistance to structurally and functionally diverse antitumor drugs. The development of Multidrug Resistance (MDR) occurs even after the first treatment courses. As a result, ever increasing doses of cytostatics are required, which enhances their destructive action on normal cells and tissues.

The main mechanisms of the development of MDR are an increase in the expression of genes encoding antiprototptic proteins and enzymes involved in the metabolism of drugs and antioxidant protection, as well as an increase in the expression of genes responsible for the biosynthesis of membrane ATP-dependent carrier proteins belonging to the class of ABC-transporters, low-sensitivity ATP-dependent cell membrane pumps that remove xenobiotics, including chemotherapeutic drugs, from the cell [1–4]. ABC-transporters (ATP-binding cassettes) are normally present in all eukaryotic cells and use the energy of ATP hydrolysis for the export of various compounds through the cell membrane. A physiological function of this group of ABC-transporters is the protection of the cell against toxic metabolites, including those formed by the cell itself. One of ATP-dependent transporters removing xenobiotics is P-glycoprotein, which belongs to the MDR1 family and is the main MDR-associated protein [4]. Its activation results in the ejection of the cytostatics of the anthracycline series (adriamycin, daunorubicin), vinca alkaloids (vincristine, vinblastine), taxans (taxol, taxotere), mitoxantron, and the topoisomerase inhibitors (etoposide), which diminishes the efficacy of these drugs.

Several ABC-transporters contain redox-sensitive amino acid residues such as cysteine. Cysteine residues enter into intramolecular interactions to form disulfides, which are required for the maintenance of proper conformation for their transport function. Human P-glycoprotein contains seven cysteines; three are located in the transmembrane domains, one is located in the NH2-terminal nucleotide-binding domain, and three are in the COOH-terminal nucleotide-binding domain [5,6]. The presence of thiols affects the sensitivity of ABC transporters to oxidative stress, which induces conformational changes in the transporters, decreases the concentration of cofactors required for the transport function, such as glutathione and thioredoxin, and influences the expression of transporters at the transcriptional and epigenetic levels [5].

A search for new compounds aimed at decreasing the MDR is an urgent problem of modern basic medicine and pharmacology. In recent years, evidence has been reported indicating that progestins can be used as P-glycoprotein inhibitors [7]. Progestins are used in the hormonal therapy of breast, womb, endometrium, and prostate cancers since they possess the antitumor activity [8,9]. However, the mechanisms of their antitumor effect, as well as possible targets of their action in the cell are still not completely understood. One of the possible mechanisms can be connected with the influence on mitochondria and Reactive Oxygen Species (ROS) production.

The key role of mitochondria in cell death allows one to consider them as an important target for anticancer chemotherapy [10,11]. Chemotherapeutic drugs acting on the mitochondrial apoptotic pathway inhibit as a rule the respiratory function or stimulate futile redox cycles, thereby inducing the cell death [12]. Anthracyclines, in particular doxorubicin, act in this way. One of the mechanisms of the cytotoxic action of DOX is its capacity to enter the redox cycle...
via one-electron transfer reactions. The quinone moiety of DOX is reduced by microsomal NADPH reductase or mitochondrial NADH dehydrogenase to semiquinone radicals, generating ROS. The toxic effect of DOX increases in the presence of iron, which forms complexes with DOX [13,14].

Some steroids, in particular estradiol and its derivatives 2-methoxyestradiol and estrone, decrease the membrane potential, activate the production of ROS, and induce cell death [15,16]. Progesterone, allopregnanolone, and dehydroepiandrosterone decrease the Ca²⁺ retention capacity and stimulate the mitochondrial permeability transition pore (MPTP) opening by suppressing the respiratory functions [17,18]. The presence of the butyric ester residue at carbon C3 distinguishes it from progesterone and the known progesterone analogues. A possible mechanism of the direct effect of buterol on MPTP is the interaction of ether carbonyl with a thiol group through the formation of hydrogen bonds between them. In the present work, the effect of buterol on the coupling of mitochondria and survival of DOX-resistant tumor cells was studied in comparison with the thiol reagent N-ethylmaleimide (NEM). It was shown that NEM and buterol sensitize tumor cells and mitochondria to DOX. For the first time the direct effect of DOX on isolated mitochondria was demonstrated, which manifested itself as a rapid decrease in the membrane potential (∆Ψm) in the presence of reduced iron and thiol reagents.

Materials and Methods

All chemicals were purchased from Sigma-Aldrich (United States) and were of the highest purity available. Buterol was synthesized as described (Patent 2292209, Russia).

Cell lines and survival

Human breast cancer cell lines differing in their sensitivity to doxorubicin (DOX) – sensitive (MCF-7) and resistant (MCF-7/DOX-R) were obtained from American Type Culture Collection. The cells were maintained routinely by growth in DMEM medium supplemented with 2 mM glutamine, 10% heat-inactivated FBS, 2.5 mg/ml of sodium bicarbonate, 100 units penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO₂-95% air at 37°C. Exponentially growing cells were used for all experiments. The viability of the cells treated with DOX, buterol, or NEM was determined by the MTT assay [19]. Cells (3 x 10⁴ cells 0.2 ml⁻¹) were seeded in 96-well plates and kept in an incubator at 37°C overnight. After treatment with the corresponding reagent, cells were incubated for an additional 48 hr, typically the reagent solution was added to each well containing suspended cells in 200 μl of medium. After an additional 4 h of incubation at 37°C with MTT (0.05 mg ml⁻¹), the supernatant was...
removed, and 150 μl of DMSO was added to each well. Then plates were read with a "Uniplan" AIFR-01 device (Russia) equipped with a 543 nm filter. Cell viability was defined as the amount of converted dye in stimulated cells versus untreated controls.

Preparation of rat liver mitochondria

Mitochondria were isolated from adult male Wistar rats according to a standard differential centrifugation procedure. Local requirements for the care and use of laboratory animals were carefully followed.Briefly, the liver was homogenized in ice-cold isolation buffer containing 220 mM mannitol, 70 mM sucrose, 1 mM EGTA, 0.3 % BSA and 10 mM HEPES-Tris (pH 7.4). The homogenate was centrifuged at 600 g for 7 min at 4°C, and the supernatant fraction was then centrifuged at 9,000 g for 10 min to sediment mitochondria. The mitochondria were washed twice in the above medium without EGTA and BSA. The final mitochondrial pellet was suspended in the washing medium to yield 60-80 mg protein/ml and kept on ice for analysis. Protein content was measured by the Biuret method with bovine serum albumin as standard.

Mitochondrial respiration was measured polarographically by a Clark electrode with computer registration. Mitochondria were incubated in a medium containing 120 mM KCl, 10mM HEPES, 1.5mM phosphate, pH 7.25 and 4mM substrate of oxidation. Respiração was measured in a thermostated 1-ml cuvette at 26°C under continuous stirring. The mitochondrial membrane potential was monitored by a TPP+-selective electrode with computer registration [18]. The concentration of TPP+ in the cuvette was 1μM, and the concentration of the mitochondrial protein was 1.5 mg/ml.

Statistical analysis

The data represent the means ± standard error of means (S.E.M.) or are the typical traces of five identical experiments with the use of different mitochondrial preparations. The experiments with cells were independently reproduced three to five times, and in each experiment the cell line was tested in triplicate. The statistical significance of difference was estimated by the Student’s t-test.

Results

Because it was proposed that the synthetic progesterone analogue buterol can enhance the antiproliferative effect of doxorubicin via binding of MDR-transporter thiol groups, its action on cancer cells was compared with the effect of the thiol-binding agent N-ethylmaleimide. The effect of NEM and buterol on the viability of tumor cells was studied on hormone-dependent MCF-7 tumor cells. DOX-resistant cells of this line were ten times less sensitive to DOX (Figure1 A). NEM decreased the survival of both cell types in a dose-dependent manner, inducing the percent of survival of MCF-7/R incubated with NEM or buterol alone is statistically different from that of MCF-7/R incubated with NEM or buterol in combination with DOX.

Thus, NEM and buterol significantly increase the sensitivity of MCF-7/R cells to DOX. Their efficiency was estimated from the reversal index, which shows the ratio of IC50 for DOX in the control and in the presence of reversal agents [20]. According to the calculated index, NEM and buterol increased the chemosensitivity of cells 9 and 4.5 times, respectively, indicating their potential MDR reversal activity.

To determine the role of mitochondria in cell death induced by NEM, buterol, and DOX, the effect of these compounds on the coupling of mitochondria was studied. It is well known that NEM influences the mitochondrial respiration by inhibiting complex I of the respiration chain [21,24]. At a concentration of 500 μM and more, NEM completely blocks respiration upon oxidation of NAD-dependent substrates by binding the subunits of complex I and does not affect the oxidation of succinate, a substrate of complex II of the respiration chain. As it has been shown earlier, buterol has no effect on respiration upon oxidation of both substrates [18]. In the next series of experiments, we studied the effect of NEM and buterol on oxidative phosphorylation and the mitochondrial membrane potential during succinate oxidation, i.e., under the conditions that enable one to reveal thiol-dependent mitochondrial processes not associated with the respiration chain. Highly coupled rat liver mitochondria were used as a test system.

(Table 1) presents the data on the effect of NEM and buterol on oxidative phosphorylation, which is determined from the respiration rate in the presence of ADP and the Respiratory Control Ratio (RCR). The two parameters reflect the degree of mitochondrial coupling. Without affecting the uncoupled respiration in the presence of DNP, both compounds decreased the ADP-stimulated respiration (V1), which led to a decrease of the coupling. The RCR decreased by 37% in the presence of 500 μM buterol and by 60% in the presence of 500 μM NEM. This effect was particularly pronounced during the registration of changes in ∆Ψm in response to the addition of ADP (Figures 2A and 2B). In the presence of buterol and NEM, the phosphorylation time increased, and the complete restoration of ∆Ψm did not occur by contrast to control.

DOX at concentrations from 100 μM to 1 mM produced no effect on mitochondria in all these tests. It is shown in (Figure 3A) that the effect of DOX on mitochondria began to show up in the presence of iron. Whereas DOX alone, as well as FeSO4 and FeCl3, did not affect ∆Ψm, DOX in combination with iron induced a slow decrease in ∆Ψm. The effect evolved with time and depended on the degree of iron

<table>
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<th>Additions</th>
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<th>DNP</th>
<th>RCR</th>
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<tr>
<td></td>
<td>V1</td>
<td>V2</td>
<td></td>
</tr>
<tr>
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<td>150 ± 9</td>
<td>40 ± 8</td>
<td>240 ± 15</td>
</tr>
<tr>
<td>buterol</td>
<td>150 ± 11</td>
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<td>240 ± 15</td>
</tr>
<tr>
<td>100 μM</td>
<td>136 ± 9</td>
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<tr>
<td>500 μM</td>
<td>130 ± 7</td>
<td>56 ± 9</td>
<td>240 ± 17</td>
</tr>
<tr>
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<td>151 ± 9</td>
<td>49 ± 9</td>
<td>248 ± 14</td>
</tr>
<tr>
<td>50 μM</td>
<td>117 ± 12</td>
<td>51 ± 11</td>
<td>240 ± 18</td>
</tr>
<tr>
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<td>46 ± 7</td>
<td>240 ± 16</td>
</tr>
<tr>
<td>500 μM</td>
<td>52 ± 9</td>
<td>34 ± 8</td>
<td>220 ± 18</td>
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Respiration rates in the presence of 0.2 mM ADP (V1 and V2) and 50 μM DNP are presented. Substrate of oxidation was 4 mM succinate in the presence of 1 μM rotenone. RCR is the respiratory control ratio. * Statistical significance p < 0.05.

Table 1: Effect of buterol and NEM on the respiration of mitochondria
reduction. If the decrease in $\Delta \Psi_m$ in the presence of Fe$^{3+}$ occurred after 5 min of incubation with DOX, the effect in the presence of Fe$^{2+}$ became evident immediately after DOX addition.

After the addition of the thiol reagents, the effect of DOX in combination with reduced iron sharply increases (Figure 3B). Because NEM alone at higher concentrations, above 250 $\mu$M, decreases $\Delta \Psi_m$ [18], it was used at low concentrations. At a NEM concentration of 50 $\mu$M, which does not influence $\Delta \Psi_m$, the effect of DOX on both the rate and amplitude of the drop of $\Delta \Psi_m$ enhanced. Buterol at a concentration of 200 $\mu$M produced a stronger effect than 50 $\mu$M NEM.

As seen in (Figure 3C), the effect of DOX in combination with reduced iron decreased in the presence of added catalase, which diminishes the concentration of hydrogen peroxide. A comparison of the effects of iron, NEM, and buterol on the rate of DOX-induced
decrease in \( \Delta \Psi_m \) shows that DOX is most effective in the presence of all above indicated reagents and that the blockade of thiol groups abruptly increases its effect on the coupling of mitochondria under iron-induced oxidative stress.

**Discussion**

The results of the study indicated that the efficiency of DOX depends on the presence of free thiol groups in cells and mitochondria. The blocking of SH-groups by NEM and buterol sensitizes DOX-resistant tumor cells to DOX. As it follows from the results, this effect can be accomplished by two mechanisms, which are associated with a decrease in the coupling of mitochondria and can operate in cells. The first consists in a decrease in the rate of oxidative phosphorylation and hence the rate of ATP formation in mitochondria. In cells, the decrease in the ATP level can affect the activity of P-glycoprotein present in MCF-7 tumor cells since the transport of DOX out of the cell requires ATP. In addition, the binding of thiol groups has a direct effect on the activity of P-glycoprotein. As it was shown earlier, the ATPase activity of P-glycoprotein is inactivated by NEM, which modifies cysteine residues within nucleotide-binding domains [6].

The other mechanism is associated with the activation of oxidative stress during the blocking of SH-groups in mitochondria and cells. It is known that mitochondrial SH-containing proteins in the matrix and at the surface of the membrane of mitochondria are involved in the antioxidant defense [21-24]. Their binding or oxidation can stimulate the development of oxidative stress, particularly in the presence of pro-oxidants. DOX is capable of entering the redox cycle via one-electron transfer reactions [25,26]. It has been shown recently that DOX induces short- and long-term effects on mitochondria of various human carcinoma cell lines [27]. DOX (5 μM) remarkably diminished the activity of the respiratory complex I and increased the ROS production in MCF-7 cells after long-term incubation [27]. It was also shown that, in the presence of iron, DOX affects mitochondria with a long lag phase, namely, slowly (within 16 min) decreases the activity of NADH-cytochrome c reductase (complex I of respiratory chain) of bovine heart submitochondrial particles [14]. In the presence of reduced iron, as it follows from our data, DOX depolarizes mitochondria without the lag phase, which may be explained by a rapid stimulation of oxidative stress upon their simultaneous action since reduced iron may react with \( O_2 \) to produce superoxide radical and with hydrogen peroxide to produce highly reactive hydroxy radicals. DOX can directly bind iron and cycle between the iron(II) and iron(III) states in the presence of oxygen, also producing ROS [28]. In cells, the effect of iron is realized through the decomplexation of Fe(II) by DOX from the [4Fe-4S] cluster of cytoplasmic aconitase with the formation of the DOX-Fe(II) complex [29,30].

It was shown on isolated rat heart mitochondria that DOX decreases the net accumulation of calcium, disturbing the calcium-dependent regulation of the mitochondrial permeability transition pore [31]. On the other hand, there was no discernable dissipation of \( \Delta \Psi_m \) and no detectable effect of the drug on the potential-dependent calcium unipor in these experiments. However, mitochondrial depolarization is an early sign of cell death. Mitochondrial changes induced by DOX in cancer cells involve membrane depolarization, which precedes alterations in the mitochondrial respiration, the cellular ATP level, and enzymatic activities [27]. Our data indicate that buterol and NEM enhance the influence of DOX on \( \Delta \Psi_m \). It can be assumed that this effect is accomplished in vivo because the redox state of thiols is responsible for the normal functioning of mitochondria and cells. Moreover, it is highly probable that oxidative stress potentiates the effect of DOX by opening the access to more specific targets, which requires further verification. The common specific targets for NEM and buterol are the SH-groups of adenylate translocase, which are localized on the external side of the inner membrane [18], NEM-sensitive sulfhydryl groups of NADH:ubiquinone oxidoreductase located at the inner membrane–matrix interface [32], and oligomycin-sensitive thiols of ATP-ase, which belong to the intramembranous \( F_0 \) -subunit [33]. Based on our and other data [6], it can be assumed that the sensitization to DOX is also accomplished through the binding of the functional thiol groups of P-glycoprotein. By contrast to NEM, which irreversibly blocks thiol groups, buterol non-covalently binds to thiol-containing compounds to form a hydrogen bond between caronyl in the acid residue of the ester at carbon C3 and the SH-group of cysteine. Owing to this, it is less toxic than NEM and can be considered as a potential sensitizing agent in chemotherapy.

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**References**


