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REVIEW

Classification of Mitochondrial Protonophoric Uncouplers and their Modifications in Biological Environment

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Abstract—In this review, we analyze diversity of mitochondrial uncouplers, a class of compounds, which was in the focus of Vladimir Skulachev's attention throughout his scientific career, starting from the basics of bioenergetics and validation of Mitchell's chemiosmotic theory to the development of concepts of mild uncoupling and its therapeutic role. The review is the first to put forward the idea of classifying uncouplers by the type of a functional group that provides their protonophoric activity, i.e., the ability to transfer protons across the membrane, causing its depolarization and thereby uncoupling the ATP synthesis from the operation of proton pumps in the electron transport chain. In particular, it is shown that anionic and zwitterionic uncouplers can be divided into groups of OH-, NH-, SH-, and CH-acids. Of importance, here we consider metabolic transformations of mitochondrial uncouplers determining tissue-specificity of their action.

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INTRODUCTION

Protonophores comprise a subgroup in a general group of ionophores. Ionophores are low molecular weight compounds of diverse chemical structure, which are capable of forming complexes with ions and transfer them across natural and artificial membranes. In particular, the 12-membered cyclic peptide valinomycin is capable of transporting potassium cations across membranes with high potassium-sodium selectivity [1]. In addition to electrogenic ionophores that transport ions together with their charge, there are non-electrogenic ionophores that exchange cations for protons or anions for hydroxide ions. The best known among them is the polyether antibiotic nigericin [2] that exchanges potassium ions for protons, and the dye of bacterial origin prodigiosin [3] that exchanges chloride ions for hydroxide ions.

In this review we focus on the special type of ionophores – electrogenic transporters of hydrogen ions, which were coined protonophores. It was found out that these compounds are able to uncouple both oxidative and photosynthetic phosphorylation, i.e. to disrupt coupling of proton pump functioning in electron transport chains of mitochondria, chloroplasts, and bacteria with ATP synthesis on the energy-transforming membranes. Under normal conditions, oxidation of respiratory substrates in the inner mitochondrial membrane is coupled with the process of ADP phosphorylation to ATP, which is a universal energy "currency" in cells. Uncouplers disconnect these two processes, so that mitochondrial respiration is not accompanied with ATP synthesis.

According to Mitchell's theory, uncouplers perform their functions via transporting protons across lipid parts of membranes, hence, they are protonophores [4, 5]. The simplest electric analogy of this system is a battery (respiratory chain) and a lantern

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(ATP-synthase). If a shunt resistor is inserted between them, the lantern is switched off. Opening of proton channels formed by the special proton-translocating proteins of the UCP family (Uncoupling Proteins) or addition of special protonophoric uncouplers (compounds that selectively transport hydrogen ions across the membrane) could serve as such shunt resistors. Among those, the uncouplers 2,4-dinitrophenol (DNP) and carbonyl cyanide m-chlorophenylhydrazone (CCCP) are best known. They are weak acids containing aromatic moieties, which help the molecule to effectively cross the membrane even when carrying negative charge despite the high energy barrier for the anion to enter the hydrophobic region of the membrane.

Due to disruptions in the cell bioenergetics caused by protonophores they are considered as important compounds for biochemistry, cell biology, and physiology. In addition to fundamental studies, they are used in agriculture and forestry industry due to their antimicrobial, insecticide, and herbicide (pesticide) properties. In addition to the widely used DNP, pentachlorophenol [6], 2-(1-methylpropyl)-4,6-dinitrophenol (dinoseb) [7, 8], as well as diarylamine fluazinam [9], and other compounds are used in industry. However, not the industrial use of protonophores, but their potential application in pharmacology is important for us. It is known that protonophores exhibit protection against muliple significant pathologies in animal models. They are cardio- [10], neuro- [11, 12], nephro- [13], and radio- [14] protectors, and exert antidiabetic effects [15-17]; and this list can be extended further. Potential of uncouplers as anticancer preparations has been mentioned in many studies [18]. Moreover, low doses of DNP were shown to significantly extend lifespan of rats [19], mice [20], yeast [21], and Drosophila flies [22].

Classic protonophores are organic acids with pK_a close to physiological pH values, which have an extended system of π-electrons assumed to be capable of delocalizing negative charge. This facilitates penetration of the anionic form of a protonophore (P^-) across the membrane in response to the applied voltage. Protonophores differ fundamentally from the penetrating acids or bases, such as salicylic acid or 9-aminoacridine, which are capable of crossing the membrane in the neutral form, but cannot penetrate the membrane in the charged form. As a result, they only can change pH gradient on the membrane due to their concentration gradient, but cannot dissipate membrane potential in mitochondria. Measurement of external concentration of salicylic acid could help to assess pH gradient on mitochondrial and bacterial membranes [23, 24], which is usually around 0.5-1 pH units [25].

In the recent study by Bertholet et al. [26], an electric current through an individual mitoplast attached to a glass micropipette was measured using the patch-clamp technique. The addition of 50 μM DNP resulted in a significant increase in the current through a fragment of the inner mitochondrial membrane, while in the case of plasma membrane of the same surface area no increase in the current was observed at 50 μM DNP. However, higher concentration of DNP (500 μM and 1 mM) increased the current through the plasma membrane. The DNP-induced current through mitoplast was suppressed by the addition of the specific inhibitor of adenine nucleotide translocator 1 (ANT1) carboxyatractyloside (CATR). Similar results were obtained by another method in the earlier studies conducted in Skulachev's laboratory demonstrating that the uncoupling effect of DNP on mitochondria is partially mediated by interaction with ANT1 [27]. In addition to the effect of CATR, Bertholet et al. investigated [26] mitoplasts derived from the mice with knockout of the ANT1 gene. It was shown that the effect of other well-known uncouplers, CCCP and carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), also is partially mediated by the interaction with ANT1 [26, 28]. These results are in agreement with the results of studies conducted in 1960s-1970s reporting ability of uncouplers to bind to proteins [29] and the existence of specific binding sites for uncouplers on the membranes [30, 31].

CHEMICAL STRUCTURES OF PROTONOPHORES, THEIR DIVERSITY AND CLASSIFICATION

Several reviews have been published in recent years that summarize the data of numerous studies on various uncouplers [32-36]. However, no classification of uncouplers has been discussed in these reviews. According to the type of the charged form of the molecules, which participate in proton transfer across membranes, protonophores could be divided into three main classes: anionic, cationic, and zwitterionic (Fig. 1). We suggest this classification in the present review.

In the earlier studies, protonophores were often divided into two types: type 1 included those compounds that transport protons as monomers, and type 2 – transport as dimers [37, 38]. The dimer-based mechanism was postulated for DNP [39], tetrachlorotrifluoromethyl benzimidazole (TTFB) [40], 5,6-dichloro-2-trifluoromethylbenzimidazole (DTFB) [41], and pentachlorophenol [42] based on measuring electric current through planar bilayer lipid membrane (BLM). The current across BLMs induced by these protonophores increased quadratically with the increase in their concentration under certain conditions. On the other hand, the uncoupling activity

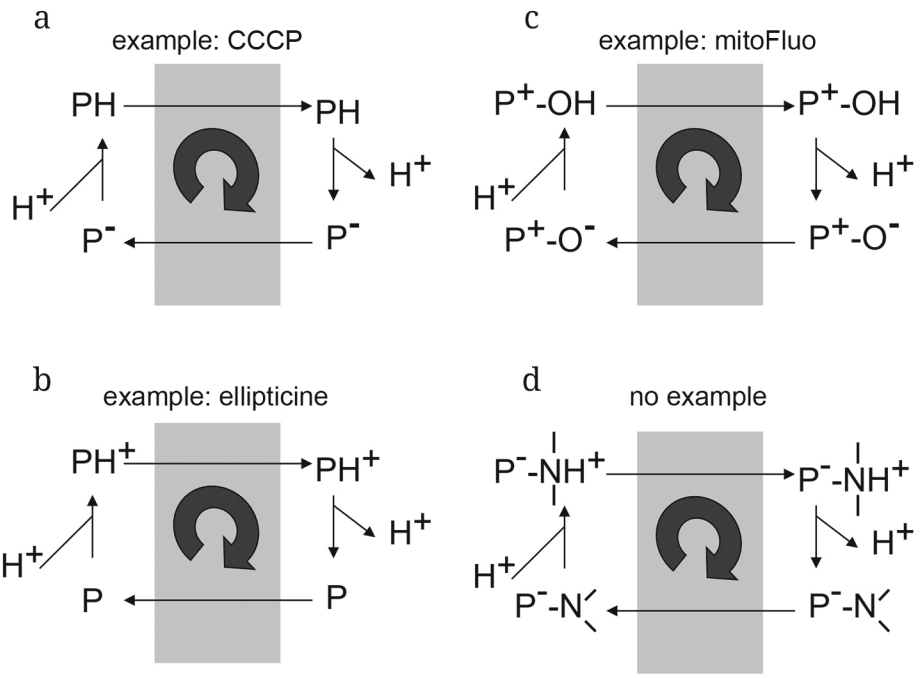

Fig. 1. Operation cycles of anionic protonophore (a), cationic protonophore (b), zwitterionic protonophore in cationic charged form (c), and zwitterionic protonophore in anionic charged form (d).

in isolated mitochondria depended linearly on the concentration of these compounds [43, 44]. Of note, the formation of dimers in the case of pentachlorophenol depended significantly on the solvent and was suppressed upon the addition of water to organic solvent [45]. The formation of dimers of uncouplers was also reduced in the case of BLM formed from the lipids dissolved in chlorodecane but not from the lipids dissolved in the commonly used decane [44]. It could be suggested that unlike the case of planar BLM, the uncoupling activity of these compounds in mitochondria proceeds via the monomeric mechanism due to the presence of a large number of proteins in these membranes, which affect physicochemical properties of the membrane. Hence, the previously suggested classification of protonophores into two types could not correspond to their properties in biological environment.

Anionic protonophores. At present, anionic protonophores comprise the most numerous group of compounds that includes the well-known uncouplers such as DNP and CCCP. Such protonophores could exhibit very high rate of operation, because anions penetrate through membranes much more effectively than cations due to the presence of the dipole potential on lipid membranes [46, 47]. The dipole potential is formed by the oriented dipoles of phospholipid heads and a layer of tightly bound water, with large contribution provided by carbonyls of ester bonds between fatty acids and glycerol [48]. In turn, anionic protonophores could be divided into classes according to the type of a group from which proton is cleaved: OH-acids, NH-acids, SH-acids, and CH-acids.

Based on the results of investigation of uncouplers on BLM, a classic scheme of operation of anionic protonophores has been suggested: an anionic form of a protonophore (P^-) crosses the membrane in response to the applied potential, then it is protonated (transforming into the PH-form), diffuses in a neutral form to the opposite side along the concentration gradient, and, finally, is deprotonated, thus completing the full cycle of protonophore operation (Fig. 1a). Such a carousel-like proton cycling may occur with proton release into water and without it. The latter variant was termed ‘small carousel’ by Markin and Chizmadzhev [49]. In this model, transport of the anionic form of a protonophore P^- is determined by electrogenicity and voltage dependence. Proton selectivity of protonophore functioning is usually evaluated from the potential of the open circuit in the presence of pH gradient, while comparison with the Nernst equation allows one to quantitatively estimate the selectivity. High permeability of the neutral (PH) form of a protonophore, resulting in its equal concentrations at the opposite sides of the membrane $[PH]_1 = [PH]_2$, determines the proton selectivity of a protonophore. Provided that protonation/deprotonation reactions at the membrane are at equilibrium, we have got $[PH] = [P^-]\cdot[H^+]/K_a$ at both sides of the membrane. It follows from this equation that the ratio of $[P^-]_1$ to $[P^-]_2$ is equal to the ratio $[H^+]_2$ to $[H^+]_1$. It means that the pH gradient leads to formation

of the gradient of anionic forms of the protonophore P^- at two sides of the membrane. In this case, invariability of the near-membrane pH, which is provided by high buffer capacity of aqueous solution, is an important issue. The question arises: at what pH value the highest permeability of protonophore will be achieved? Theoretical estimates indicate that this pH value corresponds to the value of pK_a plus half of the logarithm of the ratio of permeabilities of the neutral and anionic forms of the protonophore (see equation 3.8 in the book by Markin and Chizmadzhev [49]) [50]. Correspondingly, in the case of equal permeabilities of these two forms, we would obtain maximum permeability of the protonophore at the pH value equal to pK_a.

Above, we presented a kinetic model of protonophore operation. To have a possibility of predicting the protonophoric activity of new potential drugs, several authors have made attempts to create theoretical models based on the structure of compounds [51-56]. Most of these models suggest that the key factors determining the uncoupling activity are hydrophobicity of the compound and its pK_a. In addition, the importance of the permeability not only of the neutral form, but also of the deprotonated anion has been noted. It has been reported in the review by Kotova and Antonenko [34] that validity of such models is limited due to contribution of the proteins in the inner mitochondrial membrane to the uncoupling activity of protonophores.

The question arises: how closely associated are the protonophore properties on BLM and their uncoupling capacity in mitochondria? Correlation between these parameters has been shown to be rather strong [57-59], but there are striking exceptions for certain compounds [34, 60]. Correlation between protonophoric activity in mitochondria and in artificial membranes is significantly better in the case of using liposomes instead of BLMs [58]. Even better correlation has been observed if the protonophoric activity in mitochondria is assessed from their swelling under certain conditions [61] or from the pH change in the suspension of mitochondria in the presence of valinomycin [38].

Uncouplers based on OH-acids. All synthetic phenols uncouple mitochondria to varying degrees. Various substituted phenols have been analyzed previously in a number of reviews [30, 37, 38], and it has been concluded that their protonophoric activity is determined by their lipophilicity and the pK_a value. In addition to these two parameters, the activity is affected by certain other factors. In particular, 3,5-dibromo-4-hydroxybenzonitrile has almost the same lipophilicity and pK_a, as DNP, but is 6-9-fold more active in mitochondria [30, 62]. How could we explain this? Two main scenarios could be considered. (1) Translocation across the membrane is a complex process associated with adaptation of the molecule to the local environment. (2) Another possible cause could be interaction of substituted phenols with proteins as described in the case of DNP [63].

The uncoupling action of halogen-substituted phenols has been thoroughly investigated in the earlier studies [51, 59, 64]. Pentachlorophenol, which uncouples mitochondria at submicromolar concentrations, has been found to be the most active among them [43]. Its strong protonophoric activity has been demonstrated on BLM [42, 58, 60, 65]. However, pentachlorophenol has been found to inhibit respiratory complex II with high efficiency [66]. Pentachlorophenol is widely used in the forestry industry as a wood preservative, as well as pesticide in agriculture [6]. The compound itself and its esters are also used in peptide synthesis as reagents activating carboxyl group. Extensive use of pentachlorophenol caused significant contamination of the environment with this compound, which is toxic to humans and, in particular, is a carcinogen.

The problem of environmental contamination is even more significant in the case of using another phenol – triclosan (5-chloro-2-(2′,4′-dichlorophenoxy) phenol (Fig. 2). This compound exerts strong protonophoric action on the model lipid membranes, while its uncoupling effect in mitochondria is rather weak [67]. Actually, triclosan induces proton current through BLM more effectively than CCCP, while in mitochondria it should be added at a concentration 100-fold higher to induce the same effect as CCCP. The reason for such discrepancy has not been elucidated yet. The weak uncoupling activity of triclosan could be associated with its inability to interact with mitochondrial proteins that facilitate proton transfer by uncouplers [34]. Triclosan is well known as an antibacterial agent, which has been widely used in personal hygiene products. Currently, its use is legally limited due to harmful effects on water ecosystems via contaminated wastewater.

The best-known uncoupling phenol is DNP (Fig. 2), which for some time was used as a drug for stimulating metabolism in obesity treatment. High interest in this compound arose during and after World War I, when the toxic effect of DNP to the workers, which participated in its production for the war needs, was reported. Studies of the effects of low doses of DNP revealed its ability to reduce the amount of excessive fat both in animals and humans. This led to the use of DNP, as well as dinitrocresol, for obesity treatment. In the period from 1931 to 1934 this over-the-counter medicine was used by ~100,000 individuals [68]. Its effectiveness in terms of weight loss was confirmed in more than 80% of the cases. However, significant side effects were observed.

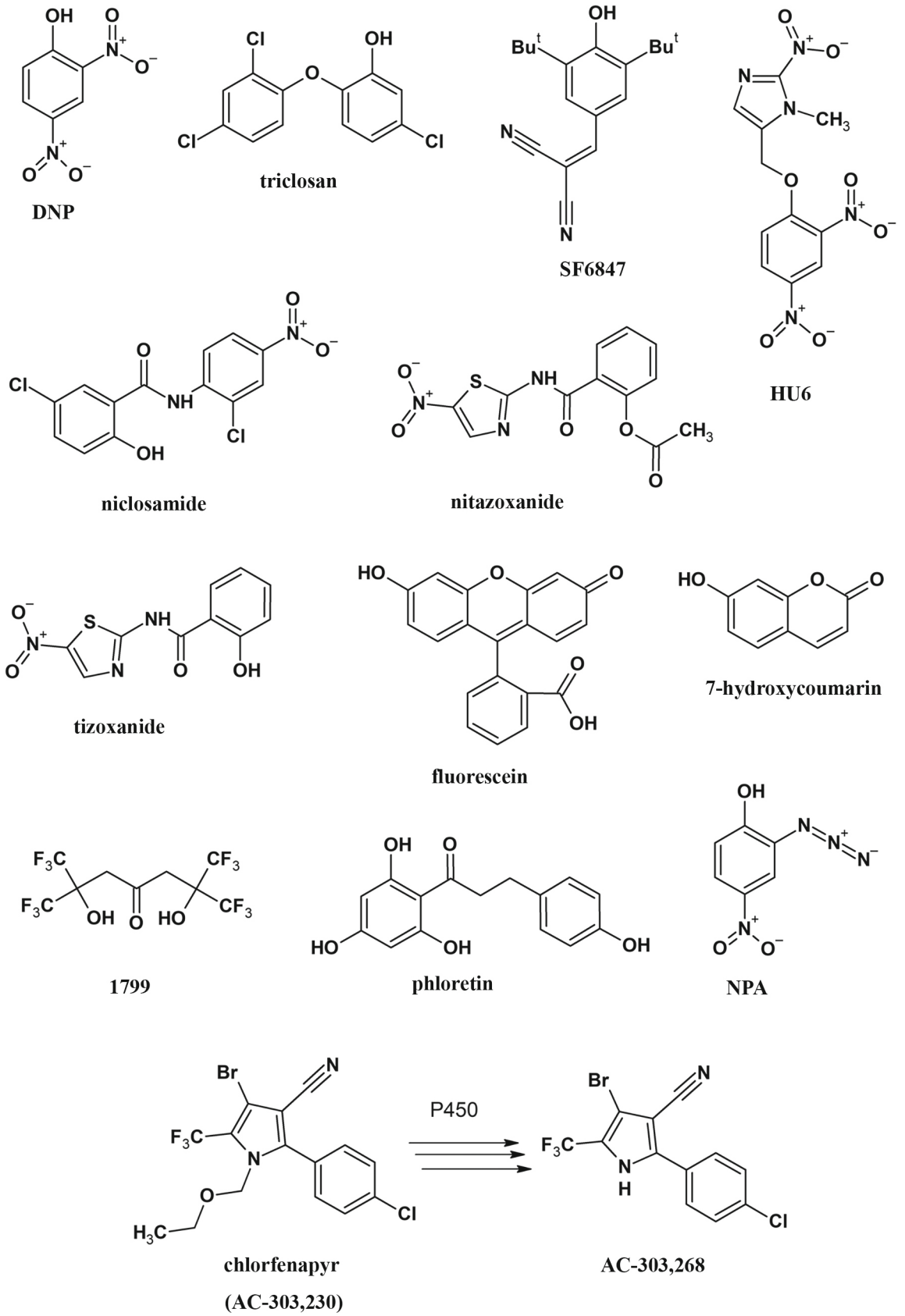

Fig. 2. Chemical structures of DNP, triclosan, SF6847, HU6, niclosamide, nitazoxanide, tizoxanide, fluorescein, 7-hydroxycoumarin, 1799, phloretin, NPA, chlorfenapyr and its metabolite AC-303268.

Some individuals had problems with liver, skin ulcers were formed, and cases of cataract development were reported. These side effects were observed even with some patients taking recommended doses, not due to the use of higher doses. Unfortunately, the cause of enhanced toxicity of DNP for some individuals had not been established. Monitoring the workers at chemical factories producing DNP revealed that high toxicity is exhibited in the individuals with high urine concentration of 2-amino-4-nitrophenol, a product of DNP degradation [68]. Development of cataract was reported in approximately 1% of the patients taking DNP, and this occurred months after completion of the obesity treatment. Search for therapeutics to treat such cataract was unsuccessful, however, surgical intervention helped in the majority of cases. Finally, in 1934 the use of DNP as a drug was prohibited. However, up until now the use of DNP as an effective weight-loss medicine has been reported in some fitness communities [69-71]. Interestingly enough, labels on the packages of DNP in pharmacies in 1930s stated that the excessive effect of DNP could be suppressed by taking baking soda [70]. It was indeed shown in the recent study by Khailova et al. [72] that uncoupling of rat liver mitochondria (RLM) by DNP could be effectively inhibited by millimolar concentrations of bicarbonate. Unfortunately, the mechanism of this effect has not been elucidated. Further, we will consider in detail modern approaches for decreasing side effects of DNP and development of therapeutics on its basis.

After DNP was banned for use as a medicine due to its high toxicity for humans, researchers started to search for its analogues that would not cause side effects and would be less toxic. In particular, it was suggested to use methyl- or ethyl ethers of DNP, which could be hydrolyzed in an organism producing DNP [73]. This idea was developed in later studies [11, 15, 74]. At present, the preparation containing ethyl ether of DNP is being examined in clinical trails as a drug for treatment of Alzheimer's and Parkinson's diseases. The fact is that DNP has manifested itself as a neuroprotector in mouse models [12, 75, 76], as well as cardioprotector [77, 78] and nephroprotector [13]. Another example of DNP ethers is methyl ether of DNP (DNPME, 1-methoxy-2,4-dinitrobenzene), which was investigated in Shulman's laboratory [74] in the USA. The authors demonstrated that DNPME effectively protected mice from development of non-alcoholic fatty liver disease (NAFLD), as well as from insulin tolerance development. The authors mentioned that DNPME hydrolysis and release of DNP occurs predominantly in liver, hence, this preparation is more suitable for treatment of liver disorders than DNP itself. However, particular biochemical pathways mediating DNPME hydrolysis in liver cells were not identified in this study.

Etherification of DNP with (1-methyl-2-nitro-1H-imidazol-5-yl)methanol results in formation of the compound HU6 (5-[(2′,4′-dinitrophenoxy)methyl]-1-methyl-2-nitroimidazole) shown in Fig. 2. HU6 was tested in humans as an anti-obesity agent, as well as in the patients with developed NAFLD [79]. It was concluded that HU6 could become a promising pharmaceutical for treating patients with obesity and NAFLD, as well as with their metabolic complications [79]. According to the results of the study by Kitzman et al. [80], this agent could be also promising for treating heart failure associated with obesity. It was suggested in these studies that HU6 is a precursor of DNP in an organism, which, in turn, mediates its pharmacological effect. However, no details of HU6 metabolism were actually presented.

At present, DNP together with CCCP (Fig. 3) remain the most used uncouplers in the laboratory practice, especially working with isolated mitochondria. It is the opinion of a number of researchers that there is still significant therapeutic potential in using DNP, which could be further increased by improving pharmacokinetics of DNP delivery to target cells. C8-alkyloxy-substituted derivatives of FCCP and DNP were used in the study by Ng et al. [81] for targeted delivery of uncouplers to adipose tissues. The obtained compounds containing ester bond (carbonyl cyanide 4-octyloxyphenylhydrazone and 2,6-dinitro-4-octyloxyphenol) accumulated in adipose tissues and were more effective in acceleration of cellular metabolism than FCCP and DNP; however, the esters were shown to be unstable in the organism of mice, which was considered as a drawback in this study. Several laboratories have synthesized compounds that could accumulate in mitochondria and release DNP upon illumination or addition of hydrogen sulfide. Thus, the light- and H_2S-activated protonophores based on 4-hydroxybenzylidenepropanedinitrile (AG10) [82] and DNP [83-86] were obtained. Scientists hope that mitochondrial targeting of such forms of uncouplers could ensure reduction of their acting concentrations and initiate local uncoupling upon illumination or in the presence of H_2S. It was shown in our recent study that 2-azido-4-nitrophenol, on the contrary, exhibits properties of a protonophore that could be inactivated by light [87], which creates a possibility of local control of mitochondrial uncoupling.

One of the strongest mitochondrial uncouplers is SF6847 ([(3,5-di-tert-butyl-4-hydroxyphenyl)methylidene]propanedinitrile) (Fig. 2). This substituted phenol uncouples mitochondria at nanomolar concentrations [88, 89]. The authors explain the enhanced activity by high stability of the anionic form of SF6847 in the membrane. According to calculations, the anion has a planar structure, which facilitates effective delocalization of the charge involving the

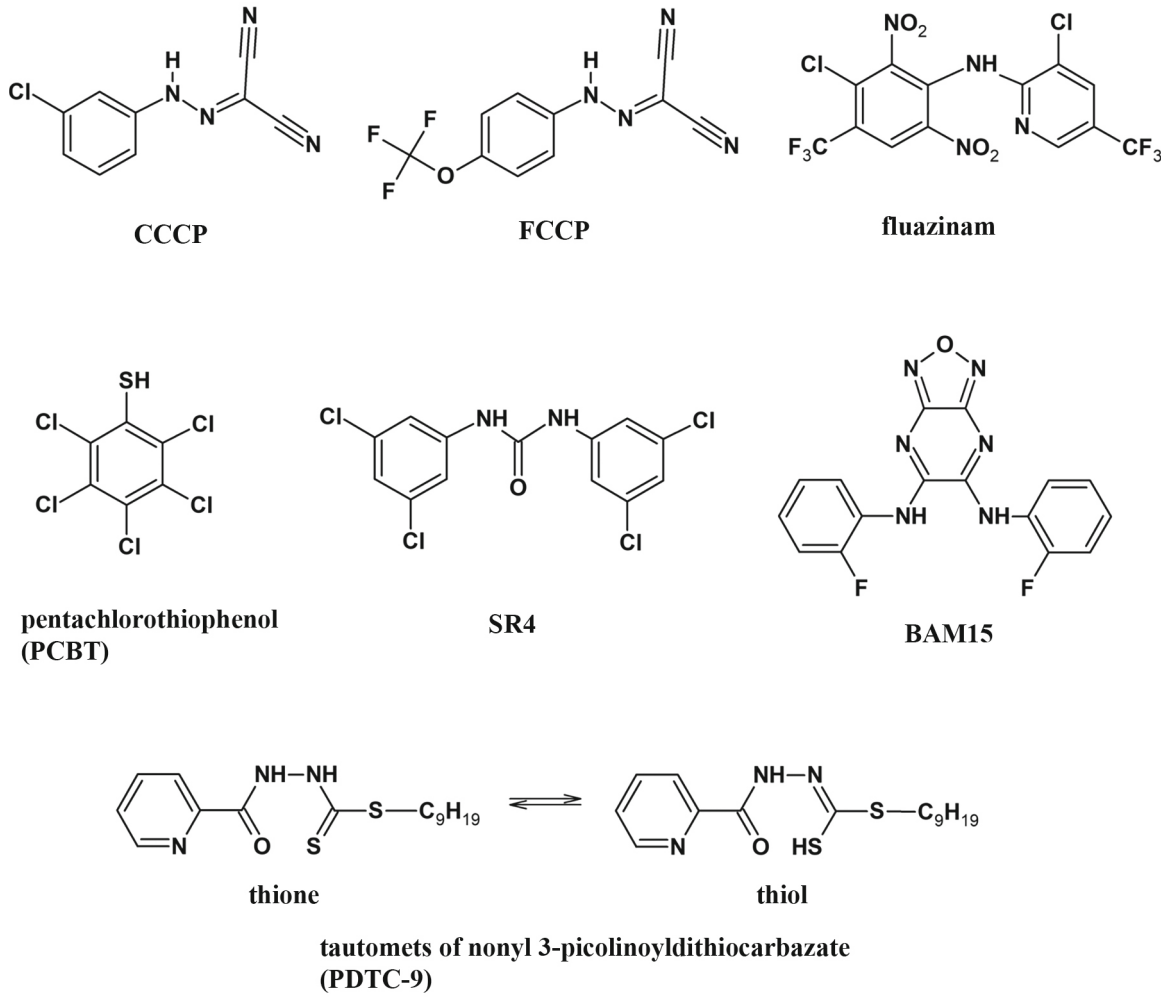

Fig. 3. Chemical structures of CCCP, FCCP, fluazinam, BAM15, pentachlorothiophenol (PCBT), SR4, and nonyl 3-picolinoylcarbazate (PDTC-9).

malononitrile group [90]. Low acting concentration of SF6847 [89] could indicate that the uncoupler itself rather than its complex with a protein operates as a protonophore. However, it has been suggested in the study by Grivennikova et al. [91] that the uncoupling action of SF6847 could be partially mediated by its binding to respiratory Complex I. It has been discovered by Starkov et al. [92, 93] that the uncoupling activity of SF6847 and other strong protonophores is suppressed by 6-ketocholestanol, known as a modifier of the membrane dipole potential [94]. Therefore, the association of the recoupling with the action of 6-ketocholestanol on the lipid part of the membrane cannot be ruled out [93, 95]. SF6847 is also known as an inhibitor of tyrosine kinase [96, 97] and a potential anticancer agent, usually named tyrphostin or malonoben [96, 97].

Another strong uncoupler, niclosamide, belongs to the group of salicylanilides and is the best-known representative of this numerous group of compounds (Fig. 2). Despite its high uncoupling activity, this agent exhibits low cytotoxicity and is used as an anthelmintic drug. Based on the approval of the Food and Drug Administration of USA (FDA), at present, niclosamide is tested for fighting many other diseases including COVID-19 [98]. The fact is that one of the critical stages of virus entry into cytoplasm is acidification of endosomes, which could be suppressed by uncouplers [99, 100]. That is why many uncouplers exhibit antiviral activity. With regards to the mechanism of action, it is known that niclosamide and other salicylanilides induce proton current through BLM [101, 102], however, more detailed investigation of the proton-transporting properties of salicylanilides were conducted using the compound S13 (3-tert-butyl-4′-nitro-2′,5-dichlorosalicylanilide) as an example. In the experiments with BLM it was shown that classical monomeric model of proton transport described above for CCCP is fully applicable for S13 [103]. The only difference consisted in the fact that permeabilities of the protonated and deprotonated forms of S13 were approximately 10-fold higher

than those of CCCP. Due to poor solubility, it is difficult to measure pK_a of S13, but in the presence of liposomes the pK_a value was 7.0-7.3 [103]. S13 is a strong uncoupler of oxidative phosphorylation being second only to SF6847 [38]. Hydroxyl serves as a proton-donor group in salicylanilides; for example, its methylation in niclosamide results in the loss of the uncoupling activity [104]. Of note, salicylanilides contain amino group in the form of CO–NH-linker between two aryl groups, so that the amino group proton could participate in formation of a hydrogen bond with the hydroxyl oxygen atom in the salicylic residue, which could facilitate delocalization of the negative charge with formation of additional 6-membered cycle, thus increasing permeability of the compounds through lipid membranes [105, 106].

Nitazoxanide has been also approved by FDA as an anthelmintic drug. In a human organism it is rapidly deacetylated and transformed into tizoxanide (Fig. 2), an analogue of the above-described niclosamide, and exhibits uncoupling properties both in mitochondria and whole cells [107, 108]. Unlike niclosamide, nitazoxanide has good bioavailability and can easily penetrate into blood circulation in the case of peroral administration. Based on the data that nitazoxanide inhibits formation of atherosclerotic plaques in the $ApoE^{-/-}$ mice with liver steatosis fed according to the so-called 'Western diet' [108], its use as a new antiatherosclerosis agent with high clinical potential was suggested. As shown in another study [109], administration of nitazoxanide through a tube caused therapeutic effect in liver steatosis induced by high-fat-diet in the C57BL/6J mice. This study also confirmed that nitazoxanide is a promising therapeutic agent against liver pathologies.

It is known that many natural phenols uncouple oxidative phosphorylation in mitochondria [110]. Among those phloretin [111] (Fig. 2), as well as quercetin [112] and hyperforin [113] should be mentioned. Many plant-derived metabolites of phenolic nature exert mitochondrial uncoupling [112] and increase electric current through BLM [110]. At micromolar concentrations, phloretin is known as a strong modifier of dipole potential in BLM [114, 115] (Fig. 2). Phloretin molecule has a large dipole moment and is adsorbed at the membrane-water interface in an oriented manner, which leads to suppression of proton current mediated by anionic uncouplers and stimulation of current mediated by cationic protonophores [116]. Interestingly, natural phenols could be brominated, as, for example, (4,5,6-tribromo-2-(2′,4′-dibromophenoxy)phenol also known as P01F08 [117]. This compound exhibits high uncoupling capacity and has been investigated as a potential anticancer agent.

It is well-known that not only substituted phenols could be uncouplers and anionic protonophores of the OH-series. Fluorescein also has a suitable hydroxyl with pK_a (~7) in its more complex aromatic core; however, fluorescein itself does not exhibit uncoupling properties (Fig. 2). This could be explained by the presence of a free carboxyl group in the fluorescein molecule. Nevertheless, butyl ester of fluorescein is also inactive, while the octyl ester of fluorescein exhibits uncoupling properties at submicromolar concentrations [118]. Thus, it could be concluded that fluorescein does not possess enough lipophilicity for protonophoric activity and acquires it upon pending a lipophilic tail. Lipophilic fluorescein derivatives could be of interest as antibacterial agents [119].

Similar phenomenon, namely, the emergence of protonophoric activity based on proton-donating property of a hydroxyl substituent in the aromatic system, caused by the addition of optimal-length alkyl substituents to this system, has been demonstrated in our study of 7-hydroxycoumarin derivatives (Fig. 2). Coumarins (derivatives of 2H-1-benzopyran-2-one) comprise a large class of natural heterocyclic compounds exhibiting a wide range of therapeutic effects [120]. Uncoupling activity of ostruthin [121] and some other 7-hydroxycoumarin (umbelliferone) derivatives [122] with hydroxyl pK_a of 7.5 [123] has been reported previously. It was shown in our recent study that the introduction of hydrophobic substituents to 7-hydroxycoumarin results in creation of effective uncouplers acting at micromolar or even submicromolar concentrations [124, 125]. Detailed investigation of the uncoupling activity of octyl and decyl esters of 7-hydroxycoumarin-3-carboxylic acid on RLM revealed a very interesting feature, namely, spontaneous decay of the activity occurring due to the loss of alkyl substituents [125] as a result of enzymatic hydrolysis of the esters. Surprisingly, the uncoupling remains constant in the case of fluorescein octyl ester [118]. The phenomenon of time-limited uncoupling and metabolism of uncouplers will be discussed in more detail further in the review.

All examples of anionic protonophores of the OH-type described above are compounds with aromatic hydroxyl. However, it is known that uncouplers could be found among the compounds with aliphatic hydroxyl, such as, for example compound 1799 (2,6-dihydroxy-2,6-bis(trifluoromethyl)-1,1,1,7,7,7-hexafluoroheptane-4-one) (Fig. 2), which is a strong uncoupler and protonophore [126]. It is known that the compound 1799 increases permeability of BLM [58, 60]. However, the mechanism of its action is unknown, and can be only assumed. Protonophoric action of 1799 indicates that aromaticity is not needed for effective penetration of anionic form of protonophore across the membrane. The absence of the effect of aromaticity on the membrane permeability has been also noted for tricyclohexylphosphonium derivatives [127].

Another variant of non-aromatic hydroxyls with suitable pK_a are fatty acids. It has been known for a long time that fatty acids uncouple mitochondria at concentrations of tens of micromoles. At the same time, they exhibit very weak ability to increase conductance of planar BLMs: noticeable currents have been observed only for the membranes formed from liposomes [128] according to the method of Montal and Mueller [129]. Moreover, it was shown that the uncoupling effect of fatty acids in mitochondria is partially mediated by the proteins – ADP/ATP-antiporter and UCP [27, 63, 128, 130-137]. Interestingly enough, dicarboxylic fatty acids are also capable of uncoupling mitochondria; more precisely they stimulate respiration, but not dissipate mitochondrial membrane potential [138]. This rather complicated phenomenon was explained by the interaction of such compounds with respiratory complex II in mitochondria. Dicarboxylic fatty acids do not increase current in BLM, but penetrate across the BLM in a neutral form [139]. It should be noted that carboxyl groups in the vicinity of aromatic ring usually do not mediate uncoupling as can be demonstrated with salicylic acid as an example [140]. But in some cases, the compounds with such structure could function as weak uncouplers, as in the case of BT2 (3,6-dichlorobenzo[b]thiophene-2-carboxylic acid), which uncouples mitochondria at hundreds of micromoles and exhibits a cardioprotective effect [141]. Azido-aryl-derivatives of various physiologically active compounds are often used for searching target proteins [142]. Upon illumination with near ultraviolet light, the azido group loses N_2 and is transformed into the highly reactive nitrene, which is capable of reacting with various groups in proteins, in particular, amino groups. 2-Azido-4-nitrophenol (NPA; Fig. 2) was synthesized as the closest analogue of DNP and was used for investigation of interactions of uncouplers with mitochondrial proteins [143]. NPA was shown to be even more active as an uncoupler than DNP (active concentration of NPA is approximately 3-4-fold lower than that of DNP) [87, 144]. Based on the studies of isolated mitochondria, NPA has specific binding sites on unidentified mitochondrial proteins; moreover, this affinity depends significantly on the presence of other known uncouplers such as DNP and CCCP [30, 143]. Among these proteins, a band, corresponding to the molecular mass ~30,000 Da, was found. In the case of azido-derivatives of fatty acids exposed to UV illumination, researchers observed covalent binding to the protein with molecular mass of ~30 kDa, which was identified as ANT1 [145].

It has been shown recently that the antibiotic pyrrolomycin is also, most likely, an anionic protonophore of OH-acid type [146, 147]. The pH-dependence of pyrrolomycin D activity in BLM exhibited an optimum at pH 9 [147]. Unlike synthetic protonophores, pyrrolomycins are produced by the bacteria *Streptomyces vitaminophilus*, *Streptomyces* sp., and *Streptomyces fumanus*. These compounds, having a large number of chlorine atoms in the molecule, uncouple mitochondria and submitochondrial particles (SMPs) at subnanomolar concentrations [147]. These concentrations are extraordinary low, because usually acting concentrations of uncouplers in SMPs are significantly higher than those in mitochondria. The question on the site of deprotonation in the pyrrolomycin molecule remains open, because in addition to phenolic hydroxyl, deprotonation may also occur at the nitrogen atom in the pyrrole ring. However, antibacterial and protonophoric activities of pyrrolomycins I and J, having methylated phenolic hydroxyl, are significantly reduced [146].

Based on the natural antibiotic – derivative of halogenated pyrrole dioxapyrrolomycin [7], the insecticide chlorfenapyr was developed. Its toxicity is too high for crop treatment, but it is used for treatment of home gardens and forests where there are no edible plants. Chlorfenapyr itself does not exert the uncoupling effect on RLM; however, in insect cells N-dealkylation occurs resulting in the formation of free pyrrole, presumably with participation of cytochromes P450 (CYP450) [148]. Structures of chlorfenapyr and the product of its metabolism with uncoupling properties are presented in Fig. 2. In fact, the chlorfenapyr metabolite, although related to pyrrolomycins, performs the mitochondrial uncoupling as NH-acid.

Uncouplers based on NH-acids. This numerous group includes compounds that contain nitrogen both within and outside the aromatic ring. The best known uncoupler CCCP, as well as its close analogue FCCP (Fig. 3), both introduced by Heytler at the beginning of 1960s [126, 149, 150], belong to this group. Despite the fact that these compounds are unstable in the presence of thiol-reagents [150-155], CCCP became very popular, especially in the tests involving mitochondrial uncoupling in cells. Most likely, this is associated with its low acting concentrations (hundreds of nanomoles for isolated mitochondria and several micromoles for intact cells), as well as with its rather low toxicity [14, 156, 157]. Another advantage of CCCP is its weak inhibiting effect on mitochondrial respiratory pumps, which is observed only at concentrations tens-fold higher than the uncoupling concentrations. With regard to the mechanism of the protonophoric action, CCCP is a typical anionic protonophore with $pK_a \approx 6$ and maximum protonophoric activity at pH around 8 [158-160]. The difference between pK_a and pH-optimum of the activity is explained by a large difference in permeability for the anionic and neutral forms of the uncoupler.

Similar to DNP, CCCP was tested in cells and animal physiological models of various pathologies, where it was proved to have properties of neuroprotector [161] and cardioprotector [78]. Similar to the case of DNP, azido-CCCP was synthesized (N_3CCP, carbonyl cyanide 2-nitro-4-azidophenyl hydrazone) and its interaction with mitochondria in the dark and under illumination with UV light was investigated; conjugation with some mitochondrial proteins was observed under illumination [31, 162]. Unfortunately, these proteins have not been yet identified, the label was observed in the band corresponding to the protein with molecular weight of 10,000-15,000 Da. Azido-CCCP was poorly investigated as an uncoupler: there are even no data on comparison of its acting concentrations with those for CCCP in mitochondria and no data at all on its protonophoric activity in BLM.

It was mentioned above that CCCP and its more active analogue FCCP could react with thiols in a chemical system in the absence of mitochondria. However, the issue of the possibility of interaction of CCCP and FCCP with natural thiols in cells, and primarily with glutathione (GSH), remains unsolved. This problem becomes especially important because, according to our data, CCCP and FCCP can interact with N-acetyl cysteine (NAC) in a chemical system [155]. The fact is that NAC as an antioxidant is often used in the experiments with cells (as well as *in vivo*) together with CCCP and FCCP [163-169]. In particular, it was shown that cardioprotective effect of FCCP could be completely eliminated by the addition of NAC [163], which was explained as an effect of FCCP on the level of reactive oxygen species (ROS) in heart cells. However, another possibility exists, that involves simple chemical modification of FCCP upon the addition of NAC to this system, which could block the uncoupling action.

Another synthetic anionic protonophore of the NH-acid series – BAM15 (N^5,N^6-bis(2-fluorophenyl) [2,1,3]oxadiazolo[4,5-b]pyrazine-5,6-diamine) (Fig. 3), introduced by Santos's research group in 2013 [170], could be considered as currently the most investigated from the physiological point of view. BAM15 exhibits strong hepatoprotective properties [171] and is effective in murine obesity models [172]. The chemical structure of BAM15 is more complex than that of CCCP, hence, the issue of its protonation-deprotonation is not so trivial. By using the capillary electrophoresis method, it was shown that BAM15 could be deprotonated in two steps with pK_a 6.44 and 7.99 forming mono- and di- anions, respectively [50]. The proton transfer across BLM mediated by BAM15 could be suppressed by the addition of phloretin, which confirms the anionic nature of this protonophore [50]. It has been stated previously that BAM15 has significant advantage compared to CCCP, because it does not change permeability and electrical activity of the plasma membrane [170], however, this conclusion was challenged in the study by Firsov et al. [50]. In addition to the direct protonophoric action, BAM15 is capable of inducing proton transport across the inner mitochondrial membrane via its interaction with the ANT1 protein [26, 28, 50].

Fluazinam is also a very strong uncoupler of the NH-type, which belongs to the group of diarylamines (Fig. 3). Noteworthy, diarylamines comprise a large group of compounds synthesized with the primary goal to create effective pesticides [7]. The protonophoric action of fluazinam on BLM, demonstrated by Khailova et al. [155], appeared to be close to that of FCCP. The uncoupling concentrations of fluazinam were shown to be subnanomolar, i.e., even less than those of SF6847 [173]. However, fluazinam is rapidly metabolized in RLM with formation of conjugates with glutathione facilitated by glutathione-S-transferase (GST) [155, 173-175].

Similar metabolic transformation was observed in our recent study [176] on another group of NH-type uncouplers – derivatives of N-phenylthiophen-2-amine [177]. Details of the transformation will be discussed further in the section devoted to metabolism of uncouplers. The protonophore, discovered almost 10 year ago – endosidin-9 (ES9, 5-bromo-N-(4-nitrophenyl)thiophene-2-sulfonamide), which is N-arylthiophenesulfonamide – compound related to anilinothiophenes, also belongs to the NH-acid type of protonophores [178].

Uncouplers of the NH-type also include derivatives of the well-known fluorescent dye NBD (7-nitrobenz-2-oxa-1,3-diazole), modified by introduction of amino group at position 4 of the benzene ring, which could be deprotonated with pK_a ~ 10 [179]. A series of 4-alkylamino-7-nitrobenz-2-oxa-1,3-diazoles was synthesized, for which it was shown that starting with octyl the uncoupling and protonophoric properties have been observed [180]. The maximum uncoupling effect on mitochondria was observed precisely for the N-octyl derivative with acting concentration being tens of micromoles. The N-dodecyl-derivative was shown to be a significantly stronger protonophore than the octyl analogue in experiments on BLM [180]. Such difference between the activities in mitochondria and BLM could be associated with the ability of 7-nitro-4-octylaminobenz-2-oxa-1,3-diazole to interact with ANT1, thereby promoting additional proton flow with participation of this protein [180, 181].

All the above-mentioned uncouplers of the NH-acid type are synthetic compounds. However, it was shown recently that the natural toxin aetokthonotoxin, which is produced by the cyanobacteria *Aetokthonos hydrillicola* that causes poisoning in fishes and birds, is a strong uncoupler of oxidative

phosphorylation, and this uncoupling is likely the main mechanism of the toxicity [182]. This toxin contains an unusual bi-indole group and has five bromine atoms as substituents. Methylation of the nitrogen atom in the indole ring results in the loss of the uncoupling activity in animal cells at micromolar concentrations of this toxin. It was shown that at the same concentrations aetokthonotoxin induces electric current through BLM at neutral pH.

Compounds based on diphenylurea such as SR4 (N,N′-bis(3,5-dichlorophenyl)urea) (Fig. 3) and analogues could be also assigned with a certain caution to protonophores of the NH-acid type [183]. Some authors, on the contrary, associate protonophoric properties of SR4 with its ability to bind hydroxide ions as well as other inorganic anions [184]. N,N′-diphenyl urea is a natural compound – a plant hormone, which controls, in particular, the formation of flowers. SR4 was shown to dissipate membrane potential and stimulate respiration at submicromolar concentrations [183]. Furthermore, derivatives of diphenylurea increased proton permeability of liposome membranes [185], which indicates their ability to act as classical protonophores. These compounds were investigated during the search for effective anticancer agents [186]. For example, Sorafenib, which is a derivative of diphenylurea with a picolinamide fragment, is used as an anticancer agent, and its main mechanism is inhibition of kinases [187]. On the other hand, it was shown that Sorafenib uncouples mitochondria, and this property could be significant for the anticancer effect [188, 189]. Interestingly enough, similar compounds also could transport chloride ions and other anions across model and natural membranes [184].

Uncouplers based on CH-acids. C–H bond has, as a rule, a negligibly weak capacity for dissociation, especially in an aqueous environment. However, in the case of carboranes it was possible to observe dissociation in different protic and aprotic solvents [190]. Moreover, decachloro-*o*-carborane has pK_a ~ 7, and its uncoupling action was demonstrated in the study by Liberman et al. [191] already in 1970. The uncoupling activity of ortho-carborane, unlike meta- and para-carborane, in line with higher acidity of ortho-isomers as compared to others [190], was confirmed in the study by Rokitskaya et al. [192]. The uncoupling effect on mitochondria correlated with the protonophoric activity in BLM and liposomes [192]. To the best of our knowledge, these compounds have not been tested in physiological experiments with animals. However, there are many studies on application of carboranes as pharmacophores; a large number of compounds – conjugates with carboranes were synthesized [193]. Part of them exhibited the uncoupling activity and was tested in tissue cultures [194].

Uncouplers based on SH-acids. Uncoupling action of pentachlorothiophenol (PCBT; Fig. 3) in mitochondria stimulating their respiration in the state 4 was investigated in the early study by Wilson et al. [43]. In particular, it was shown that PCBT uncouples mitochondria at submicromolar concentrations, while it practically does not induce current through BLM [43]. In the later (1983) study by Smejtek et al. [195] it was clarified that in the study by Wilson et al. [43] in 1971, photosensitivity and poor water solubility of PCBT was not taken into account, and in fact permeability induced by PCBT in BLM was approximately 10-fold higher, than the one at the same concentration of pentachlorophenol [195]. The pH-dependence of BLM permeability in induced by PCBT had maximum at pH ~ 6; and pK_a of PCBT was estimated as 4.3 [43], while pK_a of pentachlorophenol was ~4.7. All these data imply that uncouplers based on SH-acids have high activity and their application is limited only by their low chemical stability in biological environment.

This conclusion has been supported by the results of another study on the uncoupling activity of alkyl acyldithiocarbazates, and, in particular, of nonyl 3-picolinoyldithiocarbazate (PDTC-9) [196], which uncouples mitochondria and induces proton permeability of liposomes at micromolar concentrations. In aqueous solutions PDTC-9 exists as two tautomers, with one of them being thione, and another – thiol (Fig. 3). Methylation of the nitrogen atom N2 in thione or the sulfur atom in thiol results in complete loss of the uncoupling activity. Considering that one of the tautomers is a NH-acid, and another – SH-acid, PDTC-9 could be assigned both to NH- and SH-protonophores. According to the data reported by Terada et al. [196], PDTC-9 exhibits weak reactivity with such natural thiols as cysteine and GSH. Several structural analogues of PDTC-9 were tested, and it was shown, in particular, that their activity increases with an increase in the length of alkyl substituent reaching maximum in the case of nonyl; further increase in the alkyl substituent length leads to a decrease in the uncoupling activity [196].

Cationic protonophores. This group of uncouplers is not numerous in comparison with the group of anionic protonophores. These compounds exist in protonated cationic and deprotonated neutral forms. It is essential that the protonated form in this group of compounds is a charged molecule and not a zwitterion. Schematic representation of functioning of these protonophores is shown in Fig. 1b. The cationic charged form penetrates across the membranes significantly less effective than the anionic one due to the membrane dipole potential, hence, cationic protonophores usually are effective at higher concentrations than the anionic ones. Another feature

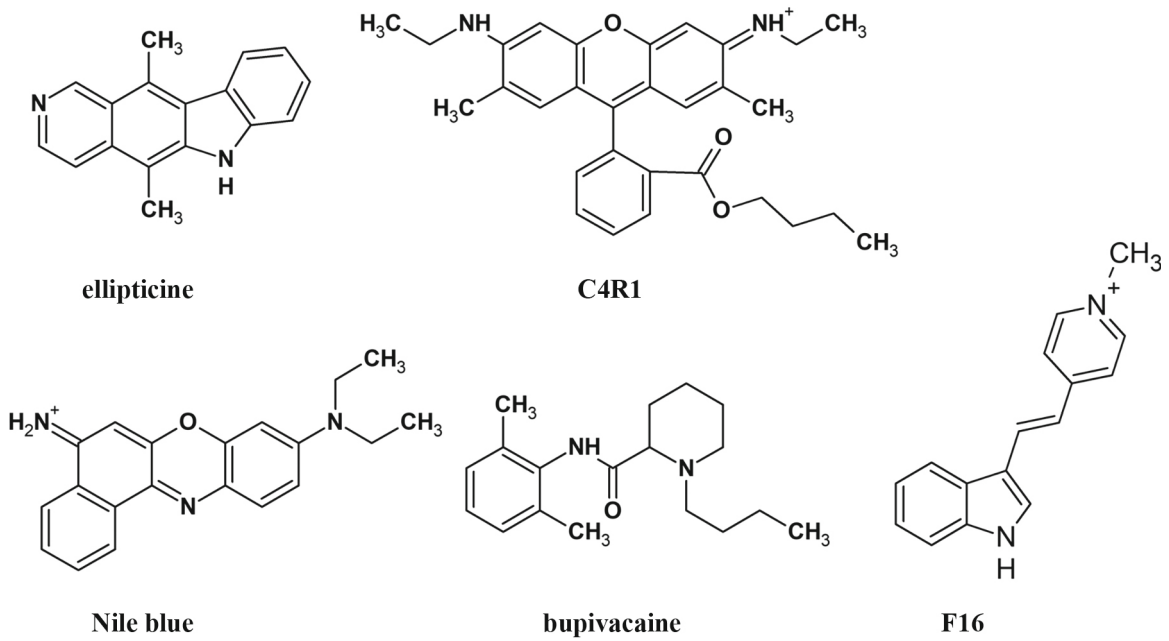

Fig. 4. Chemical structures of ellipticine, C4R1, Nile Blue, bupivacaine, and F16.

of cationic protonophores is their ability to accumulate in the mitochondrial matrix, because mitochondrial membrane potential is negative inside. Ellipticine is the best-known uncoupler of this type [197] (Fig. 4). These compounds are practically not used in the laboratory practice as uncouplers, because they produce effect at very high concentrations and the mechanism of their action is poorly understood. However, ellipticine and related compounds exhibit anticancer activity, and in this relation were investigated in great detail [198]. The rhodamine derivative C4R1 [199, 200] and the Nile Blue dye [201] could also be assigned to cationic uncouplers.

Ellipticine is an anticancer alkaloid isolated from the plants *Ochrosia elliptica*. It was shown that it uncouples mitochondria at concentrations of tens of micromoles with maximum at ~100 μM [197]. Ellipticine contains a pyridine ring (Fig. 4), in which methylation of nitrogen atom results in almost complete loss of the uncoupling activity. This observation indicates that protonation-deprotonation of the nitrogen atom is responsible for the protonophoric action of ellipticine. The measured value of pK_a of this nitrogen atom is close to 7.0 [202]. It was shown that in cancer cells ellipticine is accumulated in nuclei and mitochondria, furthermore, fluorescence of ellipticine in mitochondria depends on the pH gradient on the membrane [203].

As reported in 1984, rhodamine 6G, rhodamine 19 ethyl ester, uncouples mitochondria at micromolar concentrations [204]. It was shown in the following studies that the butyl ester (C4R1; Fig. 4) exhibits the highest uncoupling activity in the series of rhodamine derivatives with alkyl substituents of varying length [199]. Surprisingly, attempts to detect the process of deprotonation in rhodamine 6G failed. It seems that there was an error in the study by Duvvuri et al. [205]: in fact, there is no pH dependence of the rhodamine 6G spectrum. It was shown with the help of capillary electrophoresis that rhodamine 6G does not exhibit pK_a in the physiological range of pH [206], while this method was successful in reliable determination of pK_a for many other uncouplers. It was also shown that the derivatives of rhodamine 19 increase current across BLM and behave as protonophores [207]. This current is stimulated by phloretin, i.e., it increases with a decrease in membrane dipole potential [116]. At the same time, the ATPase enzyme is apparently involved in the uncoupling effect of C4R1 on mitochondria [200]. Weak toxicity of C4R1 was demonstrated in the experiments with mice; in addition, this rhodamine stimulated weight loss in rats maintained on a high fat diet [208]. Alkyl-rhodamines also exhibited antibacterial properties [209, 210].

Other dyes, such as Nile Blue (Fig. 4), pyronin Y, and acridine orange also show properties of cationic uncouplers [201]. However, Nile Blue is an inhibitor of ATP synthase, and it has been suggested that, similar to C4R1, it operates as an uncoupler in mitochondria with participation of this protein. This dye was not investigated on BLM, however, its structurally close derivative increased proton current across a lipid membrane [211].

Bupivacaine (Fig. 4) is a local anesthetic with piperidine heterocycle, which can be protonated at

neutral pH. The use of bupivacaine was approved by FDA. It was shown that, similarly to some other anesthetics [212], it decreases mitochondrial membrane potential and stimulates their respiration at millimolar concentrations, i.e. it functions as an uncoupler [213, 214]. Evaluation of pK_a of protonation of the piperidine ring in bupivacaine gave the value of 8.2 [215]. Investigation of bupivacaine in liposomes showed its protonophoric properties, however, the authors suggest that the mechanism of the uncoupling effect of this anesthetic involves the formation of selective proton channels by its aggregates [216], which is in agreement with the data on the ability of bupivacaine to form defects in the lipid membrane, obtained by Terada et al. [214] with planar BLMs.

Screening a wide range of synthetic compounds for anticancer effects revealed the cationic compound F16 ((E)-4-(3-indolylvinyl)-N-methylpyridinium iodide) with high antitumor activity [217] (Fig. 4). The authors suggested that this anticancer effect is associated with accumulation of this cation in mitochondria of tumor cells, causing induction of a mitochondrial pore, release of cytochrome *c*, and finally the apoptosis. It was shown both in the first study by Fantin et al. [217], and in the later study [218] that F16 stimulates respiration of mitochondria and behaves as an uncoupler. The authors of the later study by Wang et al. [218] concluded that the uncoupling activity of F16 is precisely responsible for its anticancer effects. Isomers of F16 were synthesized (o-F16, (E)-2-(3-indolylvinyl)-N-methylpyridinium iodide and m-F16, (E)-3-(3-indolylvinyl)-N-methylpyridinium iodide), which differ only by orientation of indole and pyridine rings of the molecule [219]. It was found that o-F16 exhibits properties of a strong uncoupler, while m-F16 is practically inactive [219]. The authors associate this property with lower acidity of the meta-isomer. It should be mentioned that the mechanism of deprotonation of F-16 and o-F16 is poorly understood. Dissociation of proton from these compounds could result in the formation of both neutral molecules without charged groups, and zwitterions, as was postulated in the study by Xu et al. [219]. The completely neutral deprotonated forms of F16 and o-F16 were previously isolated and characterized with the help of IR- and mass-spectroscopy [220] (scheme in Fig. 4). We believe that formation of the less hydrophobic zwitterion is more probable in the case of deprotonation of m-F16. This fact could also explain the reduced activity of m-F16. Of note, if indeed the deprotonated form of F16 is a zwitterion, this uncoupler should be assigned to the next group in our classification, namely the group of zwitterionic uncouplers. To the best of our knowledge, the protonophoric action of F16 on BLM has not been investigated. In recent years, works on synthesis of the conjugates of F16 with other anticancer agents were published. In particular, the conjugate of F16 with betulinic acid exhibited significantly higher cytotoxicity towards tumor cells than individual betulinic acid or F16 [221]. In later studies, the list of anticancer preparations based on F16 was significantly expanded [222-224].

Zwitterionic protonophores. Relatively recently zwitterionic protonophores have been discovered, i.e., compounds with deprotonated form carrying simultaneously positive and negative charges comprising a zwitterion [225, 226]. Mechanism of their functioning is presented in Fig. 1c. It is essential that zwitterionic and cationic forms of such protonophores should exhibit high permeability across the membrane. One could imagine the process, when the zwitterionic form of protonophores could be combined with anionic form as a pair for proton transport (Fig. 1d), however, such compounds have not been observed experimentally. The majority of the confirmed zwitterionic protonophores are conjugates of triphenylphosphonium with anionic protonophores (Fig. 5). It was shown that these protonophores initiate proton current through BLM, which is stimulated by decreasing dipole potential upon the addition of phloretin or including certain lipids in the composition of BLMs [116], i.e. in this regard they behave as cationic protonophores. Furthermore, these compounds are accumulated in the cell mitochondria, which allows considering them as mitochondria-targeted protonophores [225]. It must be noted that similar to anionic protonophores, zwitterionic protonophores could be classified according to the type of proton-donor group: OH-, NH-, and CH-acids.

Conjugates of anionic uncouplers with triphenylphosphonium cations. The first attempt to synthesize a mitochondria-targeted protonophore was made in 2006 in the research group of Murphy and Smith [227] by conjugating triphenylphosphonium with DNP. However, the obtained compound termed mitoDNP (3-(4′-hydroxy-3′,5′-dinitrophenyl)propyltriphenylphosphonium methanesulfonate) (Fig. 5) did not exhibit uncoupling properties in mitochondria, which made questionable the initial assumption about the existence of zwitterionic protonophores. However, in 2014 a conjugate of triphenylphosphonium and fluorescein was synthesized, which was named mitoFluo (10-[(2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoyl)oxy] decyltriphenylphosphonium bromide) (Fig. 5); this compound appeared to be more advantageous [225]. It was shown that mitoFluo uncouples mitochondria at submicromolar concentrations, and is accumulated in mitochondria, which is easily detected due to the bright fluorescence of this compound. The weak uncoupling effect of mitoDNP could be explained by: (1) the weaker uncoupling effect of DNP

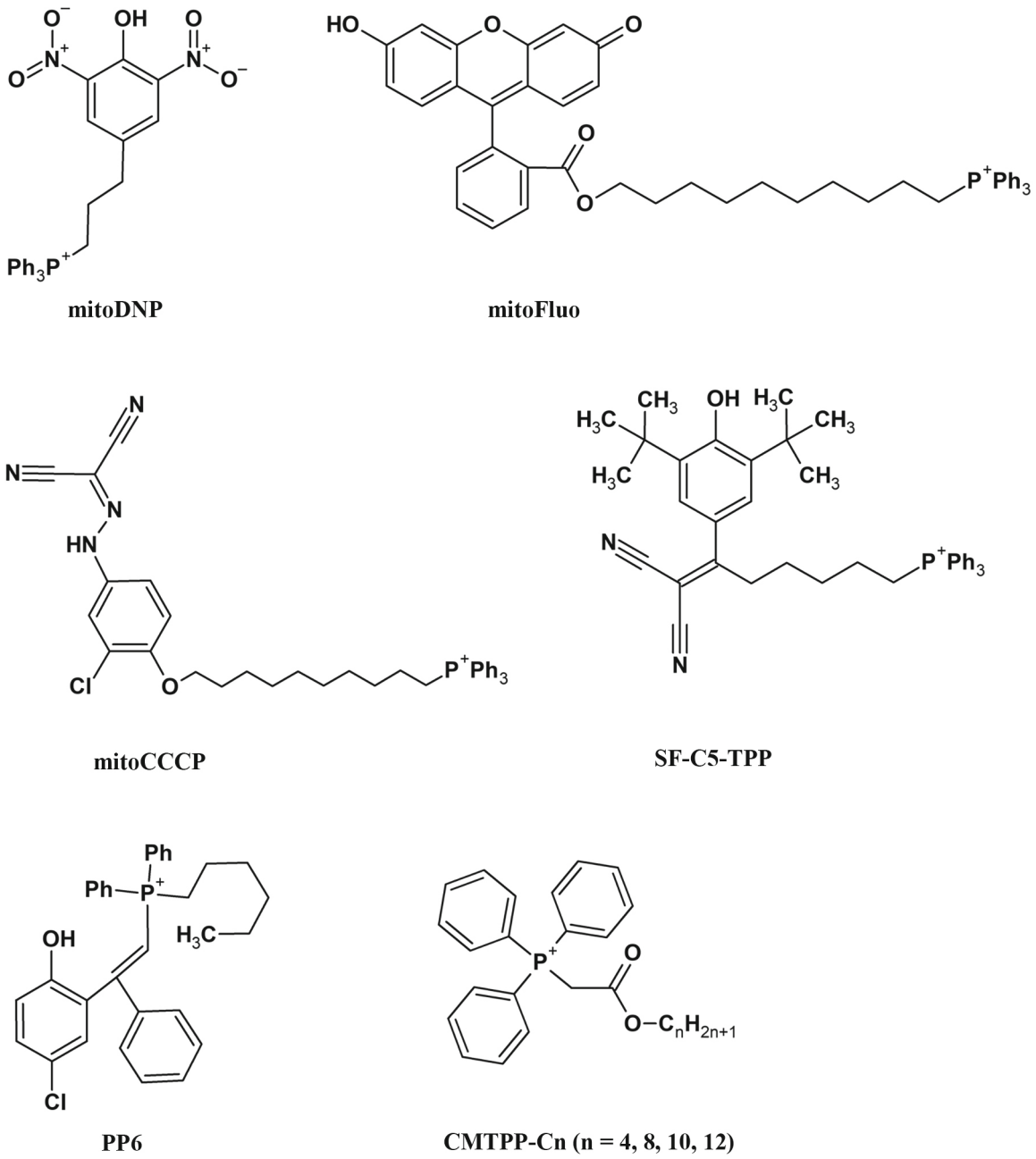

Fig. 5. Chemical structures of mitoDNP, mitoFluo, mitoCCCP, SF-C5-TPP, PP6, CMTPP-Cn (precursors of phosphonium ylides).

in comparison with alkyl-fluorescein and (2) by the too short trimethylene linker in mitoDNP. The mitoFluo compound contained the decyl linker, and the uncoupling effect was significantly reduced in the case of a similar compound with the butyl linker [228]. MitoFluo induced electric current through BLM, which was selective for protons [225]. It was shown that *in vivo* mitoFluo exerts neuro- and nephro-protective effects in the models of brain trauma and rat kidney ischemia-reperfusion, respectively [228]. MitoFluo, as well as mitoNBD – the conjugate of decyltriphenylphosphonium with NBD (7-nitrobenz-2-oxa-1,3-diazole) also exhibited strong antibacterial properties especially towards Gram-positive bacteria [119, 229].

The success with mitoFluo stimulated research in this direction: conjugates of triphenylphosphonium with CCCP (mitoCCCP, 10-([4′-(dicyanomethylene) hydrazinyl-2′-chlorophenyl]oxy)decyltriphenylphosphonium bromide) [230] and with the strongest uncoupler SF6847 (mitoSF, covalent conjugates of [(3,5-di-tert-butyl-4-hydroxyphenyl)methylidene]propanedinitrile with alkyltriphenylphosphonium) were synthesized [231]. It should be noted that attachment

of triphenylphosphonium to an anionic protonophore does not change its proton-donor group, hence, mitoDNP, mitoFluo, and mitoSF could be considered as OH-acids, similar to corresponding anionic protonophores, while mitoNBD and mitoCCCP – as NH-acids. MitoCCCP exhibited very weak uncoupling effect at submicromolar concentrations, while the conjugate with SF6847 uncoupled mitochondria at submicromolar concentrations. Among the derivatives of SF6847, the conjugate with the pentyl linker (SF-C5-TPP, (6-[3′,5′-di-tert-butyl-4′-hydroxyphenyl]-7,7-dicyanohept-6-en-1-yl)triphenylphosphonium bromide) exhibited the highest activity, while the uncoupling activity was significantly reduced in the case of butyl- and decyl- linkers. It was also shown that the compound with butyl linker is accumulated in mitochondria in response to their energization. In isolated RLM, SF-C5-TPP exhibited significantly lower uncoupling activity as compared to SF6847 itself (approximately by 2 orders of magnitude), however, it effectively decreased membrane potential in mitochondria in cell culture, which could be associated with the ability of SF-C5-TPP to accumulate in cell mitochondria. Furthermore, it induced proton current across BLM, which was stimulated by the addition of phloretin. This indicates that transmembrane diffusion of the protonated cation is the rate-limiting step in the protonophoric cycle of SF-C5-TPP. Of note, mitoCCCP was found to be a strong uncoupler in the system of inverted submitochondrial particles, as well as in subbacterial particles, while this compound was a weak uncoupler in isolated mitochondria [230].

In another series of studies, derivatives of [(E)-2-(5-chloro-2-hydroxyphenyl)-2-phenylethenyl]phosphonium with substituents of varying length at the phosphorus atom, which could be considered as conjugates of phosphonium with para-chlorophenol (PP6; Fig. 5) were investigated [226, 232, 233]. The most active compounds in this series exhibited the uncoupling effect in mitochondria at micromolar concentrations, suppressed growth of *Bacillus subtilis* bacteria, and also induced proton current in BLMs. Compounds with higher hydrophobicity were able to create nonspecific defects in lipid membranes and even cause hemolysis of erythrocytes.

Precursors of phosphonium ylides. Phosphonium ylides are often used in the Wittig reaction. They comprise zwitterions with the positive charge at the phosphorus atom and the negative charge at the closest carbon atom [234]. It was shown in our recent study [235] that the cations of (alkyloxycarbonylmethyl) triphenylphosphonium (Fig. 5) could release protons of the methylene group and, in this way, to participate in the proton transfer across lipid membranes. It was found out that the precursor of the phosphonium ylide CMTPP-C8 ((octyloxycarbonylmethyl)triphenylphosphonium bromide) stabilized by the ester bond, is able to uncouple mitochondria at micromolar concentrations and induce proton current across BLM. Hence, the precursors of stabilized ylides, as well as the carboranes described above, are protonophoric uncoulplers based on CH-acids. CMTPP-C8 and its analogues exhibited moderate cytotoxicity in cell culture and caused a decrease in the mitochondrial membrane potential in cells at concentrations that did not cause a significant decrease in cell survival. CMTPP-C12 dissipated pH gradient in thylakoid membranes of chloroplasts and exhibited antimicrobial activity. It was also shown that methylation of phenyl residues significantly increased the protonophoric activity of these compounds, so the uncoupling concentrations in mitochondria decreased to the level of tens of nanomoles [236].

Uncouplers not characterized in model systems. At present not all described uncouplers can be classified. The data available in the literature demonstrate uncoupling activity of certain compounds, which were investigated only in biological systems (cell cultures and mice), while their physicochemical properties and mechanisms of uncoupling were not explored. In particular, the compound CZ5 (ethyl (2E)-5-(4-chlorophenyl)-2-[(3,5-dibromo-4-hydroxyphenyl)methylidene]-7-methyl-3-oxo-2,3-dihydro-5H-[1,3]thiazolo[3,2-a]pyrimidine-6-carboxylate), described in the study by Fu et al. [237], most likely operates as an anionic uncoupler, because it is a derivative of 2,6-dibromophenol. The same could be said about OPC-163493, a triazole-containing compound (4-(5-methyl-2-(4-(trifluoromethyl)phenyl)-1,3-thiazol-4-yl)-1H-1,2,3-triazole-5-carbonitrile), which most likely could be deprotonated at alkaline pH [17]. However, classification of these and some other protonophores described in the literature requires further investigation.

Cation-dependent protonophores. It has been noted above in this review that the transporter of potassium ions nigericin (Fig. 6a) is a non-electrogenic ionophore and cannot uncouple mitochondria at low concentrations; its main function is the induction of potassium/H^+-exchange. However, it was shown that at high concentrations nigericin is capable of mitochondrial uncoupling, i.e. accelerating mitochondrial respiration [238]. Moreover, such well-known uncoupler of oxidative phosphorylation as usnic acid (UA) (Fig. 6a) [239, 240] is also an ionophore transporting divalent cations [241]. Below the mechanism of their protonophoric function will be discussed in detail.

It was shown previously that nigericin at concentrations of tens of micromoles is able to stimulate mitochondrial respiration and reduce the efficiency of oxidative phosphorylation [238]. Acceleration of

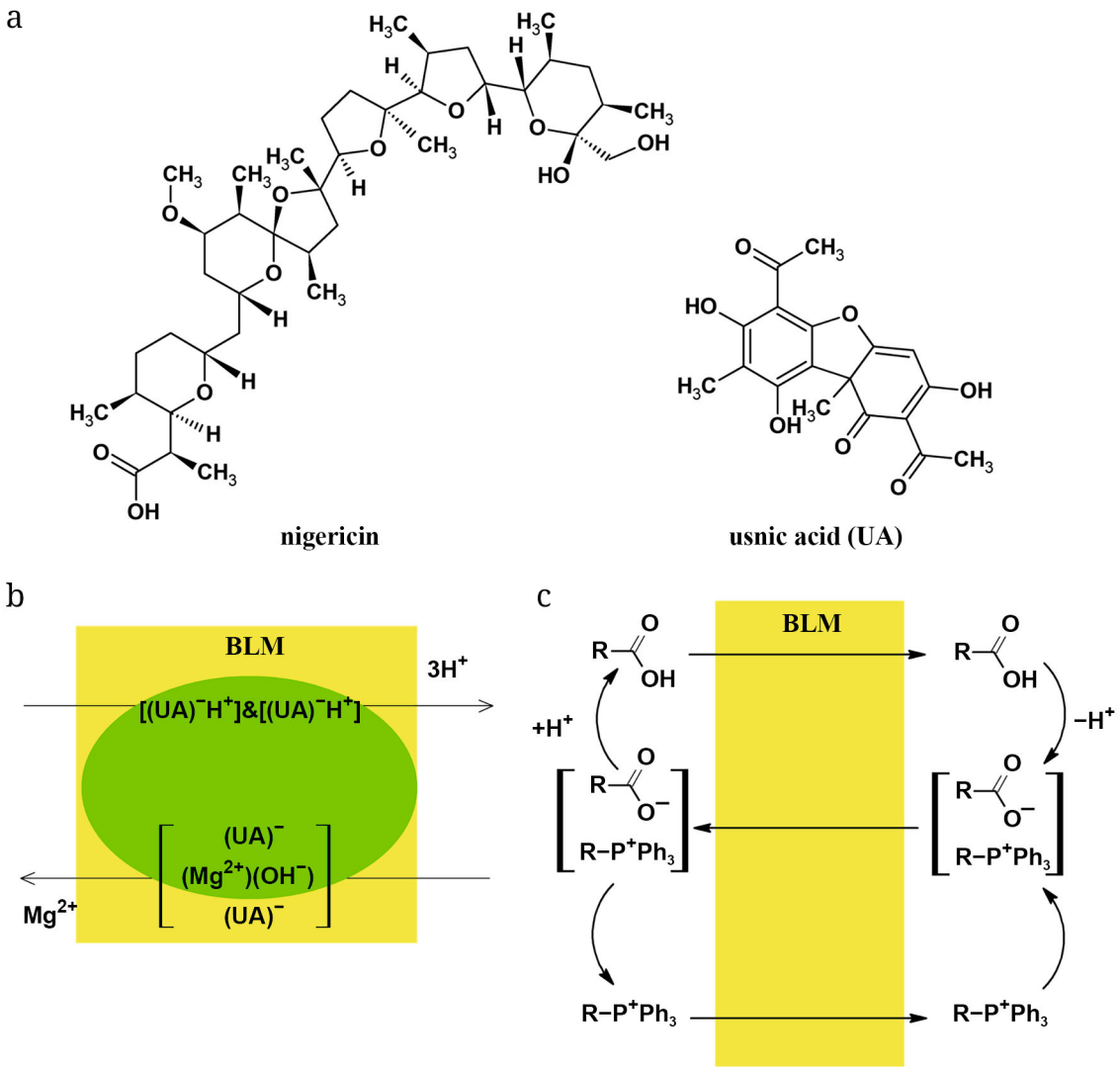

Fig. 6. Chemical structure of nigericin and usnic acid (UA) (a). Scheme of functioning of cation-dependent protonophores (b). Scheme of functioning of complexes of fatty acids with lipophilic cations (c). BLM, bilayer lipid membrane.

respiration was clearly pronounced in the case of using succinate as a substrate of respiratory complex II, while in the case of using substrates of respiratory complex I (glutamate and malate) nigericin inhibited respiration of mitochondria [242]. Both stimulation of respiration and its inhibition were observed only in the presence of potassium ions in the medium, while no effect was observed in presence of other cations. This is in accordance with the cation selectivity of nigericin as an ionophore. Inhibition of respiration in the case of using glutamate and malate could be possibly associated with inhibition of transporters of these compounds in the mitochondrial membrane.

Nigericin at micromolar concentrations increased permeability of BLM proportionally to the square of concentration [243]. The authors hypothesized that this pattern of membrane permeability could be explained by formation of nigericin dimers in complex with potassium ions and proton, in particular, proton is transported across BLM in the form of a neutral complex with nigericin, while potassium cation is transported in the charged form by the dimer of protonated nigericin molecules.

Selectivity of ion transport mediated by nigericin was examined in the study by Markin and Sokolov [244] based on potential of the open circuit in the presence of ion concentration gradient on the membrane. This parameter usually varies in the range from zero to the value calculated from the Nernst equation, i.e. approximately 59 mV per 10-fold concentration gradient of monovalent ions. It was unexpectedly observed by Markin and Sokolov that in the case of nigericin a potential of opposite sign is generated in the presence of the pH gradient, while in the presence of the potassium ion gradient

a 'super-Nernst' potential is generated, i.e., potential higher than 59 mV. The authors developed a theoretical model, which described adequately the obtained data [244]. According to their suggestion, the process does not involve establishing of interfacial equilibrium, but equilibrium between the penetrating components. In the case of complex co-transport of several ions, as in the case of nigericin, its cessation could occur without equalization of electrochemical potentials of these ions, hence, the potential could differ from the Nernst value. The obtained experimental data are consistent with the model, implying that transport stoichiometry of the ions is $2K^+/1H^+$.

Usnic acid (Fig. 6a) is a secondary metabolite of certain lichens, used as a supplement in the weight-loss diets. It has been known for a long time that UA uncouples mitochondria at micromolar concentrations [239, 245]. According to our data [241], the presence of calcium or magnesium ions in the medium is necessary for induction of electric current across BLM by UA; in the presence of the chelating agent EDTA the current becomes zero. The BLM current is proportional to the square of usnic acid concentration, which indicates the formation of UA dimers in the course of the ion transport across BLM. Investigation of selectivity of the UA-mediated ion transport revealed that potentials in the case of pH gradient were approximately 2-fold higher than the Nernst potentials [246]. Use of the model suggested by Markin and Sokolov for description of the UA-mediated ion transport led to the conclusion that the main process of this transport is electrogenic exchange of three protons for calcium or magnesium ion (see scheme in Fig. 6b).

The pentadecapeptide gramicidin A produced by *Bacillus brevis* has the following amino acid sequence: HCO-L-Val^1-Gly^2-L-Ala^3-D-Leu^4-L-Ala^5-D-Val^6-L-Val^7-D-Val^8-L-Trp^9-D-Leu^{10}-L-Trp^{11}-D-Leu^{12}-L-Trp^{13}-D-Leu^{14}-L-Trp^{15}-$NHCH_2CH_2OH$; it is known as a channel-former selective for monovalent cations [247-249]. It is often used for dissipation of membrane potential in various bioenergetic systems such as mitochondria [250], bacteria [251], submitochondrial particles [252], and chloroplasts [253]. As was shown, gramicidin A also can transport protons; and proton transport is 2 orders of magnitude more effective than transport of potassium ions [254]. However, in biological systems gramicidin A primarily functions as a potassium and sodium ionophore, because amount of protons in cells is approximately 7 orders of magnitude lower than that of potassium ions. From this point of view, it formally could be assigned to the group of cation-dependent protonophores. However, in reality, the mechanism is fundamentally different involving the formation of an ionic channel and transport of protons along the hydrophilic wall of the channel inside the membrane. Wide use of gramicidin A as an uncoupler is limited due to its high cytotoxicity associated with equalization of potassium and sodium ion gradients on the plasma membrane of cells [255, 256], as well as by its low permeability across cellular and subcellular membranes [253, 257]. Some derivatives of gramicidin A exhibit significantly lower cytotoxicity, but, at the same time, maintain high protonophoric activity [255, 258]. It was shown that one of such analogues with replacement of valine at the first position with glutamic acid exhibits neuroprotective and nephroprotective properties, similar to other uncouplers [259].

Complexes of fatty acids with hydrophobic cations (and other transporters) as protonophores. Certain amounts of fatty acids are always present in biological membranes, which themselves cannot be considered as protonophores due to low efficiency of penetration of fatty acid anionic form across lipid membranes. However, there are compounds that form complexes with anions of fatty acids and to certain extent facilitate their translocation across the membrane. Among those relatively popular compounds there are conjugates of decyltriphenylphosphonium with plastoquinone or ubiquinone, which were named SkQ1 (plastoquinoyldecyltriphenylphosphonium bromide) and mitoQ (ubiquinonyldecyltriphenylphosphonium methanesulfonate), respectively. It was shown that at micromolar concentrations SkQ1 (or dodecyltriphenylphosphonium C_{12}TPP) uncouples mitochondria due to interaction with endogenous fatty acids [260]. In the review by Childress et al. [32] mitoQ and C_{12}TPP are described as usual protonophores, which is not precisely correct. Below the mechanism of action of these and similar compounds exhibiting the uncoupling effect due to interaction with fatty acids will be considered in more detail.

Complexes of fatty acids with lipophilic cations. It was shown previously that fatty acids are capable of forming complexes with lipophilic cations in lipid membranes [260, 261]. These complexes facilitate diffusion of fatty acid anions across the membranes, which results in protonophoric activity due to high membrane permeability of the neutral form of fatty acid. Scheme of this process is presented in Fig. 6c. Derivatives of alkyltriphenylphosphonium, rhodamine 19, rhodamine B, and berberine could serve as lipophilic cations [260-263], as well as some local anesthetics of a cationic nature [264]. In general, it has been known for a long time that hydrophobic cations form complexes with hydrophobic anions in lipid membranes [46, 264, 265]. Interestingly enough, anions of classical anionic uncouplers can play a role of such hydrophobic anions, and the addition of lipophilic cations results in significant acceleration of proton transfer by CCCP and DNP [266-268].

Derivatives of urea and thiourea. It was shown using liposomes loaded with the pH indicator pyranine that lipophilic cations in combination with thiourea-containing compounds could transport hydrogen ions [269]. Previously, it was reported that such compounds are transporters of inorganic anions, including chloride ions [184]. Complexes of fatty acid carboxyls with a thiourea group are assembled via formation of several hydrogen bonds between them. The uncoupling effect of one of the thiourea derivatives, NT-1505 (N-allyl-N′,N′-dibenzyl-S-ethylthioisourea hydroiodide) was demonstrated in the study by Antonenko et al. [270] both in isolated mitochondria and in the neuron culture. It was also shown that NT-1505 increases proton current across BLM in the presence of palmitate. According to the studies by York et al. [186, 271], the ability of transporting fatty acid anions is characteristic not only for the derivatives of thiourea, but also for other urea derivatives such as, for example, N,N′-bis(3,5-dichlorophenyl)urea also known as compound SR4. Protonophoric properties of SR4 have been described above in the section devoted to NH-acids, i.e. these compounds could serve as protonophores even without fatty acids.

Uncoupling via formation of defects in the membrane. The process of uncoupling in mitochondria implies preservation of membrane integrity and formation of selective pathway for hydrogen ions in the presence of the added proton transporter. However, the main signs of uncoupling, namely stimulation of respiration and depolarization of the mitochondrial membrane, could be also caused by compounds inducing formation of non-selective pores in lipid membranes [272-276], or even through direct membrane disruption [277-279]. In some cases, the true uncoupling and formation of non-selective leakage is difficult to distinguish. For example, in the case of anesthetic bupivacaine described above it is not clear whether this compound is a cationic protonophore, or it induces defects in the membrane. Below this quite contradictory issue will be discussed in more detail.

Detergent action and uncoupling. Classical action of detergents occurs at concentrations above the critical micelle concentration (CMC) and results in complete solubilization of membrane components (proteins and lipids). However, the uncoupling effect of classical detergents could be observed at concentrations significantly lower than CMC [277-279]. This is in line with the fact that detergents induce formation of non-specific channels in BLMs at concentrations, which are several orders of magnitude lower than the concentration of micelle formation [280]. Under these conditions, BLMs maintain their integrity and are not disrupted when regular potentials are applied. Changes in spontaneous curvature of monolayers in the bilayer due to non-lamellar structure of the majority of detergents could play a significant role in formation of such transient defects by lowering the energy of formation of hydrophilic cavities and transient channels in the membrane, which defines its ionic permeability [281]. As examples, the action of minocycline [282] and SkQ1 [283] could be considered, which cause leakage in both mitochondrial and artificial membranes. Terada and colleagues [284] described the induction of ionic transport through the mitochondrial membrane and BLM by the dicationic cyanine dye triS-C4(5) (2,2′-[5-(3-butyl-4-methyl-1,3-thiazol-2-ylidene)penta-1,3-diene-1,3-diyl]bis[3-butyl-4-methyl-1,3-thiazolium] diiodide), which depended on the presence of phosphate in the medium. The authors suggested that it is exactly the complex of triS-C4(5) with phosphate that is capable of inducing transient defects in the lipid membrane, which manifested themselves in mitochondria as uncoupling of oxidative phosphorylation.

Interaction of lauryl sulfate with UCP1 and ANT1 proteins. It was noted earlier in this review that anionic uncouplers could interact with ANT1 and activate proton leakage through this protein. It was shown that this protein could also interact with sodium lauryl (dodecyl) sulfate (SDS) causing uncoupling in mitochondria, which is inhibited by the specific inhibitor of ANT1 carboxyatractyloside [27]. Furthermore, it was shown that SDS stimulates proton leakage via the UCP1 protein [133]. In this experiment the used concentrations of SDS (micromoles) were significantly lower than CMC (~8 mM).

Nonprotonophoric uncoupling. Quite a few agents are known that provide the pattern of uncoupling (stimulation of respiration, cessation of ATP synthesis, and drop of membrane potential) without relation to the described above proton uncoupling. A classic example is an effect of arsenate [285, 286] Arsenate is a substrate of ATP synthase; ADP-arsenate, formed as a result of this reaction, is unstable in water and decomposes rapidly to initial arsenate and ADP. This causes stimulation of respiration in mitochondria in the presence of ADP, which, however, is not accompanied by ATP synthesis. Phenomenologically, in the presence of arsenate, mitochondria are transformed into the continuous ‘third’ state. The Ca^{2+}/H^{+}-exchanger A23187 (calcimycin) could be another example, which causes the uncoupling because the electrogenic calcium uniporter is present in the mitochondrial membrane [287]. The list of such examples could be continued, but they are not the subject of this review. It should be also mentioned that freezing-thawing cycles transform mitochondria into the completely uncoupled state due to the formation of multiple defects in its inner membrane [288].

METABOLIC TRANSFORMATIONS OF PROTONOPHORES IN CELLS

It is obvious that protonophores, similar to the majority of xenobiotics, are subjected to metabolic transformations in organisms. Chemical modifications of xenobiotics, including drugs, environmental contaminants, or food supplements, which are compounds foreign for cells, predominantly occur via enzymatic processes classified as phase I and phase II reactions. These metabolic pathways are directed towards increasing hydrophilicity of xenobiotics, which facilitates their excretion from an organism by phase III transporters. Phase I reactions include functionalization of a compound via oxidation, reduction, or hydrolysis, which makes the molecule more reactive in the subsequent conjugation during phase II. The key player in oxidation is the family of cytochrome P450 enzymes, which introduce polar groups such as hydroxyl. For example, oxidation of benzene derivatives to phenol mediated by CYP450 increases their solubility. The reactions of reduction are less common; they involve transformation of nitro groups into amines. Hydrolytic enzymes, such as esterases, cleave ester or amide bonds, as occurs with aspirin, which is transformed into salicylic acid [289]. It is remarkable that in phase I super-reactive intermediate products, such as epoxides, sometimes could be formed, which could exert toxic effects, if they are not detoxified effectively.

Phase II reactions include conjugation of water-soluble fragments with initial compounds or with compounds modified during phase I. Glucuronization catalyzed by uridine diphosphate (UDP)-glucuronosyltransferase (UGT) attaches glucuronic acid from UDP-glucuronic acid to substrates [290] such as morphine forming highly soluble, excretable glucuronides [291]. Sulfotransferases (SULT) transfer sulfate groups from 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to phenols and alcohols [292], as in the case of sulfation of paracetamol. Glutathione-S-transferases (GST) link glutathione with electrophilic centers [293], thus neutralizing metabolites formed, for example, in the case of acetaminophen overdose. N-acetyltransferases (NAT) catalyze the transfer of an acetyl group from acetyl-CoA to aromatic amines [294], while methyltransferases, such as catechol-O-methyltransferase (COMT), catalyze the transfer of a methyl group from S-adenosylmethionine (SAM) to substrates such as catecholamines [295]. Conjugation with amino acids, although less common, links xenobiotics with glycine or taurine; one of the examples of such processes is excretion of benzoic acid with formation of hippuric acid.

Concerning metabolic transformations of protonophoric uncouplers, the following reactions could be of interest: phase I reactions involving hydrolysis of ester bonds catalyzed, in particular, by esterases; phase II reactions involving glutathionylation. Among the enzymes with esterase activity, in this review we will consider only enzymes that are primarily involved in detoxification and metabolism of xenobiotics via hydrolysis of ester bonds. Carboxylesterases (CES), members of the superfamily of serine hydrolases, are representatives of this group. CES1 and CES2, main isoforms in humans, hydrolyze substances containing esters (such as clopidogrel, irinotecan) and toxic compounds from the environment. They belong to the α/β-hydrolase-fold family with the catalytic triad (Ser-His-Glu/Asp) and conserved oxyanion hole (for stabilization of negative charge at the deprotonated oxygen in the intermediate state), which provides broad substrate specificity. CES enzymes are localized in endoplasmic reticulum and plasma, where they process lipophilic esters into hydrophilic metabolites for excretion. There are also data on CES activity associated with mitochondria [296, 297].

Glutathionylation involved in xenobiotic metabolism is associated primarily with conjugation of GSH with electrophilic xenobiotics, which represents a critical mechanism of detoxification mediated by glutatione-S-transferases. This process, a part of the mercapturate pathway, neutralizes harmful compounds by increasing their solubility in water, facilitating excretion through urine or bile [298]. The reaction involves a nucleophilic attack of the thiole group in GSH on electrophilic centers of xenobiotics forming glutathione-S-conjugates. These conjugates are further processed by enzymes such as γ-glutamyl transferase and dipeptidase for the production of cysteine S-conjugates, which are further acetylated with formation of mercapturic acids (conjugates of N-acetylcysteine) for elimination [298]. Glutathione-S-transferases comprise a superfamily of enzymes playing a central role in metabolism of phase II via conjugation of the GSH tripeptide (γ-glutamyl-cysteinyl-glycine) with electrophilic xenobiotics and endogenous toxins. The conjugation increases solubility of these compounds facilitating their excretion via phase III transporters, such as proteins associated with multidrug resistance.

Ester derivatives of 7-hydroxycoumarin and esterases. Structure and uncoupling properties of 7-hydroxycoumarin ester derivatives have been described above. In particular, rapid hydrolysis of the compounds of this series in RLM and the observed tissue specificity of the inactivation was mentioned before. 7-Hydroxycoumarin is a natural pH-dependent fluorophore, which is derived from medicinal plants of the Umbelliferae family (hence, the second name – umbelliferone) and some others. We have synthesized two series of esters: with umbelliferone-3-carboxylic acid and umbelliferone-4-acetic acid [124, 125].

Compounds of both series exhibited pronounced uncoupling activity in mitochondria. Surprisingly, this activity disappeared within minutes, unlike the case of classical uncouplers DNP and CCCP. Taking into account that protonophoric activity of the esters in model membranes (BLMs and liposomes) did not change with time, it was suggested that the uncoupling activity in mitochondria disappeared due to enzymatic hydrolysis of the esters. Indeed, thin layer chromatography (TLC) showed that the umbelliferone-containing esters, incubated in the presence of isolated RLM, were almost completely converted into initial acids within minutes, while no hydrolysis was observed in the case of fluorescein octyl ester [118]. We hypothesized that the mitochondrial aldehyde dehydrogenase ALDH2 could be a good candidate for the role of an enzyme catalyzing hydrolysis of the umbelliferone-containing esters, because this enzyme exhibits high esterase activity [299]. Moreover, this activity is sensitive to the same inhibitors as the main activity of this enzyme – oxidation of acetaldehyde. The known inhibitor of aldehyde dehydrogenases disulfiram suppressed the drop in the uncoupling activity of the umbelliferone esters [124]. An alternative candidate of such enzymatic activity is the carboxyl esterase CES1, which plays a significant role in metabolism of xenobiotics. Its activity is also inhibited by disulfiram [300, 301]. Certain isoforms of carboxyl esterases are located predominantly in liver, while only low amounts of these enzymes are detected in heart and kidney, which could explain the tissue specificity of the mitochondrial uncoupling by the umbelliferone derivatives, namely: the spontaneous disappearance of the uncoupling activity of the umbelliferone esters found in RLM was not observed in rat heart and kidney mitochondria [125]. Hence, the umbelliferone esters could be considered as tissue-specific uncouplers.

Diarylamines and glutathione-S-transferases. Above we have already discussed diarylamines, a group of compounds with some representatives exhibiting properties of anionic uncouplers due to an ability of their NH group to lose a proton. The best-known compound among them is fluazinam (Fig. 3), which uncouples mitochondria at nanomolar concentrations [155, 174, 175]. This compound is a broad-spectrum fungicide widely used in agriculture. In the pioneer study published in 1991 it was shown that the time course of oxygen consumption by isolated RLM after the addition of fluazinam is biphasic, namely: stimulation of mitochondrial respiration by fluazinam is followed byreversal of the respiration rate to the initial level on the minute time scale [174]. Efficiency of the reversal depended significantly on the level of glutathione (GSH) in mitochondria, and in the presence of GSH-depleting agents the reversal practically disappeared. The author suggested that the restoration of the respiration rate is associated with conjugation of fluazinam with GSH, which is catalyzed by mitochondrial glutathione-S-transferase. The analog of fluazinam B-3 (N-[2,4-dinitro-6-trifluoromethyl-3-chlorophenyl]-5-(trifluoromethyl)pyridine-2-amine), discovered in the study by Brandt et al. [175], also exhibited very high uncoupling activity, but, unlike fluazinam itself, was not metabolized by RLM due to the absence of electrophilic substituent in the third position of the benzene ring. According to the data reported by Clarke et al. [9], fluazinam was found to be the most reactive substrate of glutathione-S-transferase among the very wide range of agrochemical compounds. In our study [173], the addition of GSH to RLM enhanced the fluazinam inactivation. The formation of the conjugates of fluazinam and GSH was detected experimentally using methods of TLC and chromato-mass spectrometry (LC-MS): catalytic replacement of chlorine and one of the nitro groups with glutathione occurred in the process. It is worth noting that the phase of fluazinam inactivation is absent in the case of rat heart mitochondria, i.e. fluazinam exhibits the properties of a tissue-specific uncoupler [173].

N-phenylthiophenamines and glutathione-S-transferases. N-phenylthiophenamines comprise a class of organic compounds having a thiophene ring (5-membered aromatic heterocycle containing sulfur atom) attached through an amino group to a phenyl ring. Interestingly enough, in 1970 the paper was published by Buechel and Schaefer [302] describing effects of a large series of anilinothiophene derivatives on isolated RLM. The study mainly concentrated on three groups of compounds: 2-anilino-3,5-dinitrothiophenes, 2-anilino-5-halo-3,4-dinitrothiophenes and derivatives of 2-hydroxy-3,5-dinitrothiophene. Buechel and Schaefer [302] revealed the relationship between the structure and activity, demonstrating the mandatory requirement of NH group for the uncoupling activity: the absence of this group resulted in the complete loss of activity. Electron acceptor substituents in the phenyl ring, such as nitro ($-NO_2$), trifluoromethyl ($-CF_3$), and chlorine, enhanced the activity likely due to lowering of pK_a. It is worth mentioning that some of these compounds exhibited properties similar to those described later for fluazinam [174, 177], namely, the ability to stimulate mitochondrial respiration, which disappeared within minutes. However, the authors explained this phenomenon not by chemical modification of anilinothiophenes, but by their interaction with non-identified components of the mitochondrial respiratory chain. Noteworthy, the study by Buechel and Schaefer [302] (1970) was conducted in the era, when the modern ideas of bioenergetics just started to emerge. The biphasic uncoupling action of

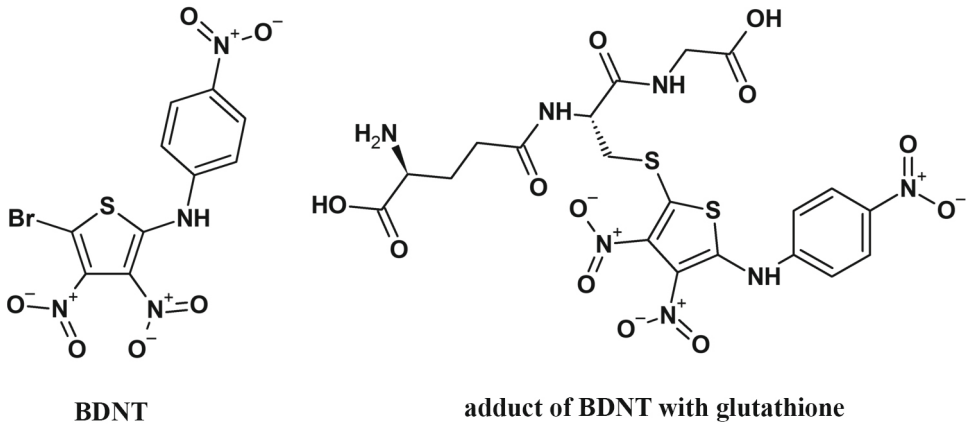

Fig. 7. Chemical structures of BDNT and its adduct with glutathione.

5-bromo-3,4-dinitro-N-(4-nitrophenyl)thiophen-2-amine (BDNT) in RLM was investigated in great detail in our recent study [176]. The restoration of the membrane potential level after the BDNT-induced decrease became more pronounced upon the addition of GSH to incubation medium and practically disappeared upon complete depletion of intramitochondrial GSH pool. In the case of rat heart mitochondria (RHM), the restoration of the decreased membrane potential did not occur. By using capillary electrophoresis and LC-MS, the formation of BDNT conjugates with glutathione (Fig. 7) was found upon incubation with RLM, and these conjugates were absent in the case of incubation with RHM. In was concluded that BDNT is a substrate of the glutathione-S-transferase (GST) enzyme, which catalyzed the formation of the BDNT conjugate with GSH: similar to the case of fluazinam, the nitro group of thiophene is replaced with glutathione. Higher expression of GST or predominance of another isoform in liver as compared to heart could be responsible for the tissue-specific action of BDNT. It should be mentioned that BDNT caused depolarization of mitochondria in the culture of fibroblasts, but not in liver cells (HepG2). BDNT induced proton-selective current through planar BLM. The current intensity decreased upon the addition of phloretin, which indicated the anionic nature of the protonophore. The pK_a value of BDNT was found to be 7.38.

CONCLUSIONS

By classifying mitochondrial uncouplers, we were able for the first time to systematize numerous examples of uncouplers present in the literature. This allowed us to review the chemical diversity of uncouplers, as well as to identify gaps in the table of chemical structures. In particular, among the known zwitterionic uncouplers there are many examples with a proton cycle performed by the pair zwitterion-cation, while there are yet no examples with a proton cycle performed by the pair zwitterion-anion in the literature (Fig. 1). Such a compound could consist of triphenylborate and a fragment containing amino group capable of protonating-deprotonating under physiological pH. It should be examined in future, how effective this molecule (or the similar one) is in transporting hydrogen ions across lipid membranes. Such ‘anionic’ zwitterionic protonophores could be much more active than ‘cationic’ zwitterionic protonophores, because anions are transported significantly more effectively across lipid membranes as compared to cations of the similar structure due to the presence of membrane dipole potential.

A goal in the search for the most active uncouplers is usually their selective action in the uncoupling of oxidative phosphorylation without affecting other processes vital for the cell functioning. Penetration of these compounds across the plasma membrane and their predominant accumulation in mitochondria are essential issues. In this regard, cationic uncouplers as well as zwitterionic uncouplers that accumulate in mitochondria due to the inner membrane potential of approximately −180 mV should have a certain advantage. However, this advantage is offset by the weak protonophoric activity of cationic uncouplers; likely, this is the reason why most of the commonly used uncouplers belong to the class of anionic protonophores.

On the other hand, as has been mentioned previously [33], a therapeutic effect of uncouplers could be significantly improved by using the compounds that have advantages in certain tissues. In particular, the use of DNP ethers for treating non-alcoholic steatohepatitis (NASH) was demonstrated to be successful [15]. Ethyl and methyl ethers of DNP are easily absorbed from intestine to blood and are delivered to liver, where they are rapidly transformed

into DNP by hydrolases. Hence, in this case the uncoupling agents emerge locally, which should reduce side effects of DNP. This means that application of tissue-specific uncouplers is of great interest in medicine. Ideally, such uncouplers could actively work in one tissue type, while exhibiting low activity (ideally no activity at all) in other tissues. In the series of studies on the uncoupling activity of fluazinam and anilinothiophenes, it was demonstrated that these compounds are subjected to rapid glutathionylation in liver, while in heart and kidney their activity is preserved [173, 176]. In another series of studies, the tissue-specific activity was demonstrated for the derivatives of 7-hydroxycoumarin [125]. Hence, tissue-specific inactivation together with formation of an active uncoupler in the specific tissue could be also used for achieving partial or complete tissue specificity of the corresponding uncouplers.

Another approach in the search for tissue-specific uncouplers is the use of ability of certain compounds to mediate proton transfer across membranes via interaction with the proteins of the inner mitochondrial membrane, primarily with the protein transporters of the SLC25 family. In particular, it was shown that DNP uncouples mitochondria partially via interaction with the adenine nucleotide translocator ANT1 [26, 27, 63]. Considering that the patterns of ANT1 expression differ significantly in different tissues, the ability of DNP to uncouple mitochondria could vary significantly: it is shown that the uncoupling activity of DNP is significantly higher in heart mitochondria than in liver mitochondria [63]. Hence, even such widely known and used uncoupler as DNP could be considered as a tissue-specific uncoupler. In this regard, fatty acids could be also considered as uncouplers specific for brown adipose tissue due to participation of the UCP1 protein in their uncoupling activity. Further studies should reveal uncouplers with higher tissue specificity than DNP, moreover, the protein partner of such an uncoupler should not be necessary ANT1. In particular, it was shown that the uncoupling effect of the cationic uncoupler C4R1 involves the proton ATPase of mitochondria [200].

Vladimir Petrovich Skulachev has strongly boosted studies of uncouplers by suggesting the concept of 'mild uncoupling' [286, 303]. This concept is based on the steep dependence of the ROS formation rate on mitochondrial membrane potential. Considering that organism aging is often associated with oxidative stress and accumulation of oxidation products, uncouplers could cause an increase in life span. Indeed, it was shown that low doses of DNP reliably increase life span of rats [19], mice [20], yeast [21], and Drosophila flies [22]. Investigation of life span of animals is a rather complex experimental task; however, fortunately, uncouplers exhibit therapeutic properties, which are easier to investigate, with many of them mentioned above. One could hope that all the facts taken together would make this review useful for readers and inspire researchers to further investigate new uncouplers and elucidate mechanisms of their action.

Abbreviations

ANT1	adenine nucleotide translocator 1 (ATP/ADP-antiporter)
BDNT	5-bromo-3,4-dinitro-N-(4-nitrophenyl) thiophen-2-amine
C4R1	butyl ester of Rhodamine 19
CCCP	carbonyl cyanide 3 chlorophenylhydrazone
CES	carboxylesterases
DNP	2,4-dinitrophenol
DNPME	1-methoxy-2,4-dinitrobenzene
o-F16	F16, and m-F16, (E)-2-, (E)-3-, and (E)-4-(3-indolylvinyl)-N-methylpyridinium iodide, respectively
FCCP	carbonyl cyanide p-trifluoromethoxyphenylhydrazone
GSH	glutathione
HU6	5-[(2′,4′-dinitrophenoxy)methyl]-1-methyl-2-nitroimidazole
BLM	bilayer lipid membrane
mitoCCCP	10-([4′-(dicyanomethylene)hydrazinyl-2′-chlorophenyl]oxy)decyltriphenylphosphonium bromide
mitoDNP	3-(4′-hydroxy-3′,5′-dinitrophenyl)propyltriphenylphosphonium methanesulfonate
mitoFluo	10-[(2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoyl)oxy]decyltriphenylphosphonium bromide
NAC	N-acetyl cysteine
PCBT	pentachlorothiophenol
PDTC-9	nonyl 3-picolinoyldithiocarbazate
S13	3-tert-butyl-4′-nitro-2′,5-dichlorosalicylanilide
SF6847	[(3,5-di-tert-butyl-4-hydroxyphenyl) methylidene]propanedinitrile
SF-C5-TPP	(6-[3′,5′-di-tert-butyl-4′-hydroxyphenyl]-7,7-dicyanohept-6-en-1-yl)triphenylphosphonium bromide
SkQ1	10-(6′-plastoquinonyl)decyltriphenylphosphonium
SR4	N,N′-bis(3,5-dichlorophenyl)urea
UA	usnic acid
UCP	uncoupling proteins

Contributions

Y. N. Antonenko – concept and supervision of the work; Y. N. Antonenko, E. A. Kotova, V. S. Krasnov, and R. S. Kirsanov – writing and editing of the paper.

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This work does not contain any studies involving human and animal subjects.

Conflict of interest
The authors of this work declare that they have no conflicts of interest.

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