Significance of the mitochondrial permeability transition in the regulation of cell death

Ph.D. Thesis

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Abbreviations

ANT	adenine nucleotide translocase
ATP	adenosine triphosphate
Bcl-2	B-cell lymphoma
BH	Bcl-2 homology
CsA	cyclosporine A
CypD	cyclophilin D
$\Delta\psi$	mitochondrial membrane potential
H_2O_2	hydrogen peroxide
JNK	c-Jun N-terminal kinase
MMP	mitochondrial membrane permeabilization
MPT	mitochondrial permeability transition
OM	outer membrane
PARP	poly(ADP-ribose) polymerase
RNS	reactive nitrogen species
ROS	reactive oxygen species
siRNA	small interfering RNA
SOD	superoxide dismutase
VDAC	voltage-dependent anionic channel

Introduction

It is well-known that dysregulation of the balance between cell death and survival leads to development of various diseases such as cancer, autoimmune diseases and neurodegenerative disorders. Influence on cell death processes provides opportunity for treatment of these diseases. Both the inhibition as well as the activation of cell death could be efficient for the *clinician* depending on the nature of the disease.

Apoptosis is a highly programmed cell death which can be activated by various factors. Mitochondria play a key role in the apoptotic process; their damage, which involves permeabilization of the mitochondrial membrane, activates a series of events that leads to cell death.

Based on the recent developments in mitochondrial research, increased pharmacological and pharmaceutical efforts have lead to the emergence of „mitochondrial medicine" as a new field of biomedical research. Targeting of biologically active molecules to mitochondria in living cells will open avenues for manipulating mitochondrial functions, which may result in the selective protection, repair, or eradication of cells.

We previously synthesized a panel of different structures carrying a cyclic nitroxide group and demonstrated their SOD (superoxide dismutase) mimetic characteristics; in addition, some of these structures were shown to have cytoprotective properties. In the present work, we studied the effect of new apolar mitochondria targeted triphenylphosphonium derivates (SOD mimetics) and PARP inhibitors in mitochondria related cell death in different experimental circumstances in order to investigate their possible therapeutic applications.

The role of mitochondria in cell death

Mitochondria play a critical role in maintaining the bioenergetic status of cells and, in addition, have a second crucial function: the control of cell death processes. These cell death processes have long been considered to be an important target for drug discovery.. Several studies have shown that apoptosis induction is strongly associated with the anticancer activity of many chemical agents. Moreover, chemoresistance of cancer cells often results from defects in apoptosis signaling. Therefore, alternative mitochondrial cell death mechanisms, such as mitochondrial permeability transition (MPT)-mediated necrotic cell death or autophagy could play important roles in anti-cancer therapy.

The mitochondrial permeability transition (M)

The MPT pore, a non-specific channel originally thought to span both mitochondrial membranes, mediates the increases in mitochondrial permeability associated with cell death. The pore itself is permeable to solutes up to 1.5 kDa. This causes equilibration of H^+ across the inner membrane, which dissipates $\Delta\Psi_m$ and inhibits ATP production. A concomitant influx of water causes swelling of the mitochondria, which stretches the membranes to the point where the outer membrane fails. The mitochondrial pore is redox, Ca^{2+}, voltage, adenine nucleotide, and pH sensitive.

The composition of PT pores remains controversial. Based upon biochemical and pharmacological studies, the pore was proposed to consist of the voltage-dependent anion channel (VDAC) in the outer membrane, the adenine nucleotide translocase (ANT) in the inner membrane and cyclophilin D (CypD) in the matrix. However, recent genetic knockout studies challenge the validity of this model by showing that the MPT still occurs in mitochondria that are deficient in ANT, VDAC and even CypD, although some properties of the MPT are altered.

The Bcl-2 family

Bcl-2 is the prototype member of a family of proteins containing at least one Bcl-2 homology (BH) region. For classification purposes, the family may be divided into antiapoptotic multidomain proteins (prototypes: Bcl-2, Bcl-XL), which contain four BH domains (BH1234); proapoptotic multidomain proteins (prototypes: Bax, Bak), which contain three BH domains (BH123); and proapoptotic BH3-only proteins (prototypes: Bid, Bad). The main site of action of Bcl-2-like proteins is probably the mitochondrial membrane. As a rule, BH1234 proteins mainly reside in OM, where they protect mitochondria against MMP, presumably by binding to and neutralizing other proapoptotic proteins from the Bcl-2 family, which on the contrary induce MMP. Some data indicate that Bcl-2 and Bcl-XL can interact with sessile mitochondrial proteins including ANT and VDAC. In vitro, the overexpression of Bcl-2 in cells or the addition of Bcl-2 to isolated mitochondria reduces the PT probability.

PARP inhibitors

The nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP-1) is activated in response to DNA damage. Single- and/or double-strand DNA breaks induce the production of branched chain ADP-ribose polymers that are covalently attached to numerous nuclear proteins like histones or the PARP itself and this process represents an early event in DNA repair.

Overactivation of PARP can lead to depletion of cellular NAD^+ and ATP which results in cell dysfunction and cause necrotic cell death. In the absence of DNA damage, PARP is not necessary for survival. Therefore PARP-1 inhibition is a promising mechanistic target for drug development in the context of various forms of inflammation, ischemia, and cancer therapy.

Although it is well-documented that inhibition of PARP-1 has cytoprotective effects against oxidative stress, there is growing evidence suggesting that inhibition of PARP-1 sensitizes cells to DNA-damaging agents.

Although several classes of PARP inhibitors move toward clinical development, new compounds are still needed. In our previous studies we found that modification of cardiovascular drugs, such as mexiletine, amiodarone, or trimetazidine, with pyrroline nitroxide precursors provided the parent compounds with additional antioxidant and radical scavenging activity. For example, alkylation of trimetazidine secondary amine with a 2,2,5,5-tetramethyl-2,5-dihydropyrrolin-3-ylmethyl group provided protection from ischemia-reperfusion induced contractile dysfunction. This approach well suits the new stream of drug research, e.g., incorporation of two drug pharmacophores in a single molecule with the intention to exert dual (cardiovascular and antioxidant) action. We considered the combination of nitroxides and their sterically hindered amine precursors with PARP inhibitors, realizing that most of the deleterious processes resulting from PARP activation are initiated by harmful reactive oxygen species (ROS) and reactive nitrogen species (RNS). These types of compounds would inhibit not only poly-ADP-ribosylation but simultaneously would suppress or decrease the harmful effect of initiator ROS and RNS as well.

Mitochondria targeted triphenylphosphonium derivative

The presence of mitochondrial membrane potential ($\Delta\Psi_m$) alterations is an important characteristic of cancer cells. Many groups have shown that the mitochondrial transmembrane potential in carcinoma cell lines is significantly higher than in normal cell lines. If the plasma and mitochondrial transmembrane potentials are both negative, molecules with cationic properties can be driven electrophoretically through these membranes, resulting in their accumulation inside the energized mitochondria of tumor cells. Triphenylphosphonium derivatives of vitamin E, ubiquinone, N-tert-butyl-α-phenylnitrone, and, more recently, nitroxides, were the first antioxidant molecules shown to predominantly localize in mitochondria. These initial attempts demonstrated that mitochondria-targeted antioxidants may have a highly cytoprotective effect, particularly against apoptosis.

Lipophilic organic cations, such as rhodamine-123 and ^{3}H-tetraphenylphosphonium (^{3}H-TPP), were used for to measure mitochondrial potentials in tumor cells. Although mitochondria-directed triphenylphosphonium derivatives have limited tumor specificity, cytostatic agents targeted to the mitochondria may have therapeutic significance. In fact, paramagnetically-modified triphenylphosphonium salts have been widely used for biophysical studies of membranes for decades; and their therapeutic potential as mitochondria-targeting, small, non-vitamin-like antioxidants is becoming increasingly appreciated.

Aims of the study

We synthesized a number of PARP-1 inhibitor compounds (benzimidazole derivatives), that have SOD mimetic activity. The aim of the present study was the following:

- To screen their inhibitory effect on the PARP activation and cell death induced by H_2O_2
- To analyse their antioxidant effect/ hydroxyl radical scavenging ability
- To elucidate their oxidative metabolism on a rat model
- To investigate the relationship between PARP inhibitory and antioxidant effect

It has been shown, that a number of mitochondria targeted antioxidant molecules by coupling with a triphenilphosphonium-group were more effective even at a lower concentration then their unsubstituted counterparts. We investigated new triphenylphosphonium derivates of mitochondria-directed SOD mimetic nitroxides, however we observed that changing the hydrophobicity of these compounds, by attaching a bulky apolar side-chain, reversed their cytoprotective effects, resulting in cell death even at micromolar concentration. Therefore, the aim of the present study was the following:

- To verify the accumulation of HO-3814 in the mitochondria
- To describe the effect of hydrophobic derivatization on cell death by measuring cell viability of three tumor cell lines
- To compare the effect of the apolar SOD mimetic (HO-3814) with that of well-known anti-cancer drugs on cell viability in tumor cell lines
- To determine the type of cell death induced by HO-3814

- To investigate the molecular mechanism of the cytotoxic effect of this compound
- To describe the effect of HO-3814 on the mitochondrial permeability transition and on the stability of the mitochondrial membrane potential ($\Delta\Psi_m$)
- To show the effect of Cyclophilin D suppression by siRNA technique on cell death and mitochondrial depolarisation induced by HO-3814 using a PANC-1 cell model
- To show the effect of Bcl-2 overexpression on HO-3814 induced mitochondrial depolarisation and cell death

Conclusions

The inhibitory effect of benzimidazole derivatives on the PARP activation and cell death induced by H_2O_2

Among the tested compounds **3h** and **4h** were found to be the best-performing PARP inhibitors. We found low correlation between the cell death inhibitory and PARP inhibitory effect.

Their antioxidant effect/ hydroxyl radical scavenging ability

We established that compound **4h** appears to be the best antioxidant and PARP inhibitor regarding the PARP enzyme inhibition, cell death inhibition, and hydroxyl radical-scavenging results.

Their oxidative metabolism on a rat model

We found that sterically hindered secondary amine moiety of PARP inhibitor **4h** is oxidized to nitroxide **3h**, while nitroxide **3h** and its hydroxylamine are in equilibrium in the rat model.

The relationship between PARP inhibitory and antioxidant effect

We observed that the results of PARP inhibition and antioxidant studies did not correlate. The cell death inhibition is based not only on the PARP enzyme inhibition but probably on the ROS scavenging activity also.

In summary these results indicate the advantages of combining an antioxidant nitroxide or nitroxide precursor with a PARP inhibitor molecule to decrease or eliminate the deleterious processes initiated by reactive oxygen and reactive nitrogen species (ROS and RNS). In this

respect these compounds may have therapeutic potential in the future, however further biological studies are needed.

Intracellular localization of HO-3814

We verified, that this compound was accumulated in the mitochondria using mass spectrometric detection after cellular subfractionation.

Effect of hydrophobic derivatization on cell death in three tumor cell lines

We presented evidences at the first time that increasing lipophilicity of mitochondria targeted SOD mimetics inverted their cytoprotective properties inducing cell death in three different tumor cell lines used, indicating that the cytotoxic effect of HO-3814 could be the result of a general, rather than a cell-specific mechanism.

Comparison of the effect of HO-3814 and well-known anti-cancer drugs on cell viabilities

We found that PANC-1 cells were much more sensitive to HO-3814 than cisplatin. Because gemcitabine is the first line drug in pancreatic tumor therapy, we tested the sensitivity of PANC-1 cells toward gemcitabine. We found that gemcitabine even at the highest concentration used, caused only a slight decrease in the viability of PANC-1 cells under our experimental conditions, indicating that HO-3814 was much more effective in inducing cell death in PANC-1 cells than either of the anti-cancer drugs used. These results suggest that apolar mitochondria-directed SOD mimetics may have therapeutic potential in the management of pancreatic tumors.

Determination of the type of cell death induced by HO-3814

We established that HO-3814-induced cell death was predominantly necrotic.

The molecular mechanism of the cytotoxic effect of HO-3814

We analysed the effect of the mitochondrial permeability transition inhibitor, cyclosporine A, as well as inhibitors of ERK, p38, JNK and Akt kinase signalling pathways, a PARP inhibitor, a caspase inhibitor, and the antioxidants quercetine and N-acetyl-cysteine, on the cell viability of PANC-1 cells treated with HO-3814. We found that none of them had any effect on HO-3814 induced cell death indicating that most likely, none of these intracellular pathways were involved.

Effect of HO-3814 on isolated mitochondria

HO-3814 was found to provoke mitochondrial swelling and loss of the mitochondrial membrane potential destabilizing the mitochondrial membrane system that was not inhibited by cyclosporine, suggesting that CypD was likely not involved in the mitochondrial permeability transition.

Effect of Cyclophilin D suppression by siRNA technique on cell death and mitochondrial depolarisation induced by HO-3814

We showed that HO-3814- induced cell death and mitochondrial depolarisation was not affected by cyclophylin D-suppression confirming our results for isolated mitochondria.

Effect of Bcl-2 overexpression on HO-3814 induced mitochondrial depolarization and cell death

When PANC-1 cells were overexpressing Bcl-2, a mitochondrial membrane system stabilizing protein, we found that the overexpression diminished the effects of HO-3814. These data suggest that HO-3814 induces cell death by destabilization of the mitochondrial inner and outer membrane systems, resulting in a collapse of membrane potential, leading to necrotic cell death.

In summary we provided evidence that changing hydrophobicity of mitochondria directed SOD mimetics reversed their cytoprotective effect inducing permeabilization of the mitochondrial membrane systems and necrotic cell death. Traditionally, necrosis of cancer cells was associated with poor prognosis since the resulting chronic inflammation was found to encourage tumor growth. However, malfunctioning of apoptotic mechanisms in many types of tumor cells increased the value of necrosis as a clinical focus recently. In this respect, hydrophobic mitochondria directed SOD mimetics may have therapeutic potential in the future.

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