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

Ph.D. Thesis

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2011

1. Contents

2. Abbreviations

ADK	adenylate kinase
AIF	apoptosis inducing factor
Akt/PKB	protein kinase B
ANT	adenine nucleotide translocase
ATP	adenosine triphosphate
Bcl-2	B-cell lymphoma
BH	Bcl-2 homology
BRCA1/2	breast cancer associated gene-1 and -2
CsA	cyclosporine A
CypD	cyclophilin D
cyt-c	cytochrome *c*
$\Delta\psi$	mitochondrial membrane potential
DMEM	Dulbecco's modified Eagle's medium
ECL	enhanced chemiluminescence
EPR	electron paramagnetic resonance
Etoposide	4' - demethyl - epipodophyllotoxin 9 - [4,6-O-(*R*)-ethylidene-beta-Dglucopyranoside], 4' - (dihydrogen phosphate)
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FKHR	forkhead homolog rhabdomyosarcoma
G-418	(2R,3S,4R,5R,6S)-5-amino-6-[(1R,2S,3S,4R,6S)-4,6-diamino-3-[(2R,3R,4R,5R)-3,5-dihydroxy-5-methyl-4-methylaminooxan-2-yl]oxy-2 hydroxycyclohexyl]oxy-2-(1-hydroxyethyl)oxane-3,4-diol
GSK-3	glycogen synthase kinase-3
H_2O_2	hydrogen peroxide
HPLC-MS	quantitative high-pressure liquid chromatography-mass spectrometry
IC_{50}	half maximal (50%) inhibitory concentration
IM	inner membrane
IMM	inner mitochondrial membrane
IMS	intermembrane space
ip	intraperitoneal
JC-1	5,5,6,6-tetracloro-1,1,3,3 -tetraethylbenzimidazolyl-carbocyanine iodide

JNK	c-Jun N-terminal kinase
LD_{50}	lethal dose 50%
MAPK	mitogen-activated protein kinase
MEM	minimum Eagle's medium
MMP	mitochondrial membrane permeabilization
MPT	mitochondrial permeability transition
mPT	mitochondrial permeability transition
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFκB	nuclear factor κB
•OH	hydroxyl radical
OM	outer membrane
OMM	outer mitochondrial membrane
PARP	poly(ADP-ribose) polymerase
PET	positron emission tomography
PI	propidium iodide
PiC	mitochondrial phosphate carrier
PTPC	permeability transition pore complex
Rh123	rhodamine123
RNS	reactive nitrogen species
ROS	reactive oxygen species
siRNA	small interfering RNA
SOD	superoxide dismutase
Taxol	(2α,4α,5β,7β,10β,13α)-4,10-bis (acetyloxy)-13-{[(2*R*,3*S*)-3-(benzoylamino)-2-hydroxy-3-phenylpropanoyl]oxy} -1,7-dihydroxy-9-oxo-5,20-epoxytax-11-en-2-yl benzoate
tBid	truncated Bid
VDAC	voltage-dependent anionic channel

3. 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 (Bognar et al., 2006). 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.

3.1. 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 (Szewczyk and Wojtczak, 2002), a view supported by their central role in apoptosis as the “point of no return”, following the release of proapoptotic factors from the mitochondrial intermembrane pool (Garber, 2005). Several studies have shown that apoptosis induction is strongly

associated with the anticancer activity of many chemical agents (Hail, 2005). Moreover, chemoresistance of cancer cells often results from defects in apoptosis signaling (Fischer and Schulze-Osthoff, 2005; Reed and Pellecchia, 2005; Sjöström and Bergh, 2001). Furthermore, constitutively activated survival pathways, in particular the mitogen-activated protein kinases (MAPK), protein kinase B/Akt and nuclear factor NFκB, all may work in concert to prevent effective therapy (Eberle et al. 2007). Therefore, alternative mitochondrial cell death mechanisms, such as mitochondrial permeability transition (mPT)-mediated necrotic cell death or autophagy (Lemasters et al., 1998), could play important roles in anti-cancer therapy (Proskuryakov and Gabai, 2010; Szigeti et al, 2006).

3.1.1. The mitochondrial permeability transition (MPT)

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 (Di et al., 2007; Halestrap et al., 2004; Kroemer et al., 2007).

The composition of PT pores remains controversial (Lemasters et al., 2009) 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 (Baines, 2009; Murphy and Steenbergen, 2008) (Figure 1). Pharmacological inhibitors of ANT and CypD, e.g., bongkrekic acid and cyclosporine-A, respectively, can inhibit MPT (Baines et al., 2005; de Macedo et al., 1993; Haworth and Hunter, 2000). 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 (Kokoszka et al., 2004; Krauskopf et al., 2006). For example in ANT deficient mitochondria, the ANT ligand, atractyloside, no longer promotes PT pore opening, whereas in CypD deficient mitochondria, MPT induction requires greater Ca^{2+} and is not blocked by CsA (Lemasters et al., 2009).

It has long been known that inorganic phosphate sensitizes the MPT pore to induction by Ca^{2+} and oxidants. Presumably, therefore the pore must possess a Pi-binding site. While there are several Pi binding proteins in the mitochondrion, the most obvious candidate is the mitochondrial PiC, a ubiquitously expressed ~30 kDa inner mitochondrial membrane protein (Palmieri, 2004). The PiC's physiological role is to mediate the re-uptake of Pi back into the mitochondrial matrix, which it co-transports with H^+ (Palmieri, 2004). However, the mitochondrial PiC is now beginning to be scrutinized as a potential regulator of MPT and cell death. Indeed, the PiC is attractive as a MPT pore component for several reasons. Firstly, the aforementioned role of Pi is the induction of MPT. Secondly, ANT, which we know is a regulator of the MPT pore, tightly associates with the PiC in the so-called "synthasome" (Ko et al., 2003). Thirdly, reconstitution of the PiC in liposomes can yield a non-specific pore under certain conditions (Schroers et al., 1997). Finally, the PiC is expressed in all tissues. There have been two recent studies that suggest that the PiC could be part of the MPT pore. Using a genetic screen for death-inducing proteins, Alcala et al. reported that overexpression of the PiC dominantly induced mitochondrial dysfunction and apoptosis in non-cardiac cells (Alcala et al., 2008). Halestrap's group has taken a different approach. Utilizing GST-CypD as bait coupled with proteomic analyzes, they identified the PiC as a bona fide CypD-interacting protein (Leung et al., 2008). They then went on to demonstrate that MPT-inducing agents enhanced the PiC-CypD interaction, whereas MPT-blocking compounds reduced it. Most importantly, agents that were able to inhibit mitochondrial Pi transport activity also blocked MPT in isolated mitochondria. Together, these data suggest that the PiC may indeed be a component of the MPT pore (Figure 1). Moreover, the study found that ANT was part of the PiC-CypD complex (Leung et al., 2008), and Halestrap has suggested that alterations in ANTs conformation could influence the PiC (Leung et al., 2008). Such a notion would be consistent with the idea that ANT acts as a regulatory protein rather than one of the actual pore-forming units, and would help reconcile the biochemical and genetic data regarding ANT. Obviously the question still remains as to whether the PiC is in fact the pore-forming channel of the MPT pore or whether it represents yet another regulatory protein such as CypD or ANT. Genetic analyses using RNAi or gene targeting technologies to deplete the PiC are clearly needed before a role for PiC in MPT can be conclusively established.

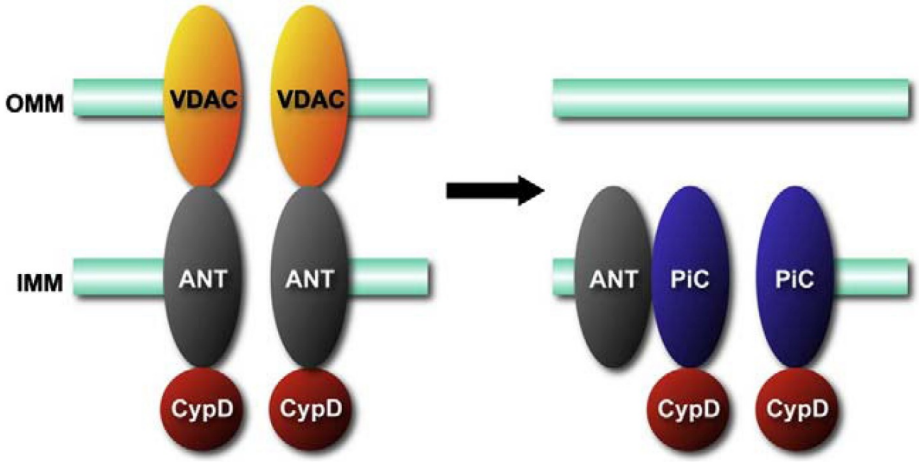


Figure 1: Molecular models for the mitochondrial permeability transition (MPT) pore. Left, the -original model for the MPT pore, consisting of the voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane (OMM), the adenine nucleotide translocase (ANT) in the inner mitochondrial membrane (IMM), and cyclophilin-D (CypD) in the matrix. Right, revised models in light of recent findings in gene-targeted mice. VDAC is no longer part of the model and it appears that an outer membrane component may not even be necessary for this process. ANT now appears to be more of a regulatory protein, and only CypD remains as an established component. In contrast, the mitochondrial phosphate carrier (PiC) has been added to model as a potential candidate for the pore-forming unit of the MPT pore.

3.1.1.1. Signs of outer mitochondrial permeabilization

Outer mitochondrial membrane (OMM) permeabilization is generally detected by determining the subcellular localization of proteins that are normally retained within the intermembrane space (IMS) by the protein-impermeable outer membrane (OM). Immunoblot detection of such proteins including Cyt *c*, AIF, or adenylate kinase (ADK), in an extramitochondrial compartment (cytosol, nuclei) purified according to subcellular fractionation procedures is interpreted as a reliable sign of OM permeabilization (Kroemer et al., 2007).

3.1.1.2. Signs of inner mitochondrial permeabilization

Inner membrane (IM) permeabilization implies the formation of pores or channels that cause the dissipation of the $\Delta\psi_m$ built across IM. Following IM permeabilization, an increase in mitochondrial matrix volume occurs as a consequence of the massive entry of solutes and water (Hunter et al., 1976). This is the result of the colloid osmotic pressure of the matrix, which is tightly packed with metabolically relevant enzyme complexes. This swelling gives rise to a distension and disorganization of the cristae as well as to a reduction of the electron density of the matrix. Thus electron microscopy constitutes an additional means to investigate the contribution of mitochondria to cell death (Kroemer et al., 2007).

3.1.1.3. Involvement of the MPT in necrosis and apoptosis

CypD is a mitochondrial matrix protein that can interact with inner membrane proteins including ANT, and that participates in the permeability transition causing dissipation of the inner mitochondrial transmembrane potential. The strongest stimulus of permeability transition is cytosolic Ca^{2+}, but additional conditions, including ROS, depletion of ATP and the production of lipid stress signals (e.g. ceramide, ganglioside GD3), favor permeability transition (Kroemer et al., 2006). Knockout of the gene encoding CypD induces resistance to necrotic cell death induced by ROS or Ca^{2+} overload in, for example, hepatocytes and fibroblasts (Baines et al., 2005; Nakagawa et al., 2005; Schinzel et al., 2005). In addition, CypD-deficient mice are resistant to cardiac injury by ischemia – reperfusion (Baines et al.,

2005; Nakagawa et al., 2005) and focal cerebral ischemia (Schinzel et al., 2005). CypD is the mitochondrial target of cyclosporinA, which has also been shown to reduce cell loss induced by necrotic stimuli in, for example, TNFα-treated hepatocytes (Pastorino et al., 1996). By contrast, it seems that apoptotic mitochondrial membrane permeabilization mediated by Bax or Bak is not regulated by CypD (Baines et al., 2005; Nakagawa et al., 2005; Schinzel et al., 2005), suggesting that there are two mechanisms of MMP – one that is strongly connected to necrosis (and is CypD dependent) and another that is linked to apoptosis (and is mediated by pro-apoptotic members of the Bcl-2 family such as Bax and/orBak). In some situations, however, cyclosporin A can inhibit apoptotic death and antiapoptotic proteins of the Bcl-2 family can inhibit permeability transition, suggesting that there is considerable crosstalk between the two pathways (Kroemer et al., 2006). Although the link between necrosis and CypD is impressive, CypD might not be involved in all instances of necrosis and might be involved in some cases of apoptosis (Tsujimoto et al., 2006; Yasuda et al., 2006) (Figure 2).

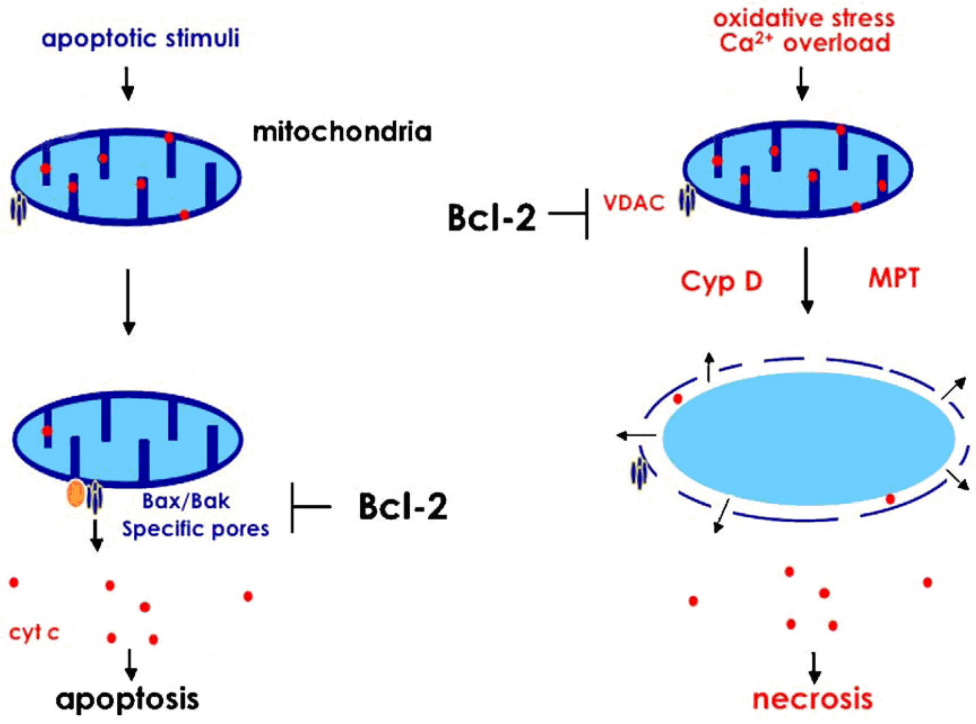


Figure 2: Involvement of the mitochondria in apoptosis and necrosis. During apoptosis, an increase in the permeability of the outer mitochondrial membrane is crucial and is regulated by multidomain pro-apoptotic members of the Bcl-2 family (Bax and Bak), resulting in the release of several apoptogenic factors (e.g. cytochrome c) into the cytoplasm. In contrast, the CypD-dependent MPT (increased permeability of both the outer and inner mitochondrial membranes) is involved in necrosis induced by Ca^{2+} overload and oxidative stress. Both kinds of mitochondrial membrane permeability changes are inhibited by anti-apoptotic members of the Bcl-2 family (Bcl-2 and Bcl-x_L).

3.1.2.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) (Letai et al., 2002). Some members of all the subgroups share an additional COOH-terminal transmembrane domain, which mediates their insertion into the OM and other intracellular membranes (e.g., the ER membrane). The main site of action of Bcl-2-like proteins is probably the mitochondrial membrane (Kroemer and Reed, 2000). 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. In healthy cells, the BH123 protein Bak is associated with the OM, whereas the other BH123 protein Bax resides in the cytosol, under normal circumstances. The expression of at least one of the two BH123 proteins (Bax or Bak) is required for MMP, in a series of different models of apoptosis induction (Wei et al., 2001). Accordingly, fibroblasts from mice that lack both Bax and Bak (but not cells from animals deficient solely for Bax or Bak) are highly resistant against MMP induction and against the activation of cell death by the intrinsic pathway (Wei et al., 2001). As just mentioned, in physiological conditions Bax is a cytosolic protein. However, upon apoptosis induction, Bax inserts into the OM (Wolter et al., 1997), where it is thought to form supramolecular openings, alone or in association with other proapoptotic members such as Bak or truncated Bid (tBid) (Kuwana et al., 2002). Such openings might result from the formation of homooligomeric Bax-containing pores or from the destabilization of the lipid bilayer, resulting in transient discontinuities within OM. Relocalization of Bax is required for its proapoptotic function. Although the precise mechanisms of MMP are debated (Zamzami and Kroemer, 2003), MMP can result from a conformational change of Bax or Bak (with exposure of their NH2 terminus), their full insertion into mitochondrial membranes as homooligomerized multimers, and formation of giant protein-permeable pores (Kuwana et al., 2002). BH3-only proteins can exert their proapoptotic action by two different mechanisms. Some BH3-only proteins (the “facilitators,” prototype: Bad) preferentially interact with BH1234 proteins, dissociating them from other BH3-only or from BH123 proteins, which in turn promote MMP. Others (the “activators,” prototype: tBid) directly activate BH123 proteins to initiate MMP, either by stimulating the

translocation of Bax to mitochondrial membranes or by local effects on Bak (Letai et al., 2002). Some data indicate that Bcl-2 and Bcl-XL can interact with sessile mitochondrial proteins including ANT (Marzo et al., 1998) and VDAC (Shimizu et al., 2000). In vitro, the overexpression of Bcl-2 in cells or the addition of Bcl-2 to isolated mitochondria reduces the PT probability (Marzo et al., 1998; Shimizu et al., 1998). Moreover, recombinant Bcl-2 can inhibit the formation of pores by purified ANT or VDAC reconstituted into artificial membranes (Brenner et al., 2000; Shimizu et al., 1998), while enhancing the ADP/ATP antiporter activity of ANT (Brenner et al., 2000). Bcl-2-like proteins have two functions on mitochondria, namely, the inhibition of pore formation by Bax and Bak as well as the inhibition of pores formed by proteins from the PTPC, such as VDAC and ANT. Therefore two MMP mechanisms may coexist: a Bax-mediated OM permeabilization that occurs independently of any early and direct effect on the IM (Finucane et al., 1999) and a PTPC-mediated permeabilization, which on the contrary involves IM. It appears plausible that either of the two mechanisms may prevail, depending on the cell type and the apoptosis inducer (Kroemer et al., 2007).

3.2. PARP inhibitors

The nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP-1) is activated in response to DNA damage (Virag and Szabo, 2002). 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 (Althaus et al., 1999; D'Amours et al., 1999; de Murcia and Menissier de Murcia, 1994). Overactivation of PARP can lead to depletion of cellular NAD^+ and ATP which results in cell dysfunction and cause necrotic cell death (Berger, 1985; Ha and Snyder, 1999; Jagtap and Szabo, 2005; Oleinick and Evans, 1985). A cellular suicide mechanism of necrosis and apoptosis by PARP activation has been implicated in the pathogenesis of brain injury and neurodegenerative disorders. This observation is confirmed also by the fact that PARP-1 knockout mice were found to be fertile and normally developing but resistant to cerebral ischemia. These findings suggest that in the absence of DNA damage, PARP is not necessary for survival (Eliasson et al., 1997). Therefore PARP-1 inhibition is a promising mechanistic target for drug development in the context of various forms of

inflammation, ischemia, and cancer therapy (Eliasson et al., 1997; Endres et al., 1997; Liaudet et al., 2002; Zingarelli et al., 1998).

The protective effect of PARP-1 inhibitors in a number of experimental models of human diseases, caused by oxidative or nitrosative stress and consequent PARP-1 activation, suggests that these compounds can offer therapeutic advances either to prevent or to slow down disease progression (Plascke et al., 2000; Szabados et al., 2000; Toth et al., 2006).

Although it is well-documented that inhibition of PARP-1 has cytoprotective effects against oxidative stress (Halmosi et al., 2001), there is growing evidence suggesting that inhibition of PARP-1 sensitizes cells to DNA-damaging agents (Oliveira et al., 2005). This later effect of PARP-1 inhibition is attributed to the DNA-damage sensing function of PARP-1, namely that it responds to single- and/or double-strand DNA breaks, and facilitates DNA repair and cell survival. It was shown that cells deficient in breast cancer associated gene-1 and -2 (BRCA1/2) are extremely sensitive to PARP inhibition because of defective double-strand DNA break repair (de Soto et al., 2006). Based on these data, PARP inhibition is considered a useful therapeutic strategy for the treatment of a wider range of tumors bearing a variety of deficiencies in the homologous recombination DNA repair pathway (Bowman et al., 2001). However, we found that inhibition of PARP leads to Akt phosphorylation in oxidative stress (Tapodi et al., 2005). The application of -PARP inhibitors in tumor therapy may activate the phosphatidylinositol-3 kinase (PI-3K)-Akt pathway, which initiates processes like the inactivation of glycogen synthase kinase-3 (GSK-3), caspase-9, Bad or forkhead homolog rhabdomyosarcoma (FKHR) transcription factors (Birkenkamp and Coffer, 2003) leading to cytostatic resistance. We have shown that the PARP-inhibition-induced Akt activation ((Szanto et al., 2009a) leads to Bad phosphorylation that considerably involved in taxol resistance of T24 human urine bladder transitional cancer cell line (Szanto et al., 2009b).

The majority of PARP inhibitors bind to the nicotinamide binding site; e.g., they are competitive inhibitors of NAD^+. It is well-known that nicotinamide forms three hydrogen bonds in the catalytic region of PARP-1, one to the hydroxyl group of Ser904 and two to the backbone amides of Gly863. In addition, π-π interactions with Tyr 907 of the aromatic moiety are also important (Figure 3) (Costantino et al., 2001; Ruf et al., 1998). The proper interaction requires an anti conformation of the amide carbonyl group which is in equilibrium with the syn conformation.

The first PARP-1 inhibitors were nicotinamide and 3-aminobenzamide as benchmark inhibitors (Banasik et al., 1992), but because of their low potency, low aqueous solubility, and

low specificity, new PARP inhibitors were needed. The low potency was attributed to the flexibility of the carboxamide group, and therefore, synthesis of conformationally restricted PARP inhibitors, e.g., lactams or heterocyclic compounds, resulted in more potent compounds by 2-4 orders of magnitude (Horvath, and Szabo, 2007; Jagtap and Szabo, 2005; Peukert et al., 2005; Virag and Szabo, 2002; Woon and Threadqill, 2005). The carboxamide group rotation can also be restricted by intramolecular hydrogen bond as in the case of benzoxazole-4-carboxamides and benzimidazole-4-carboxamides(White et al., 2000).

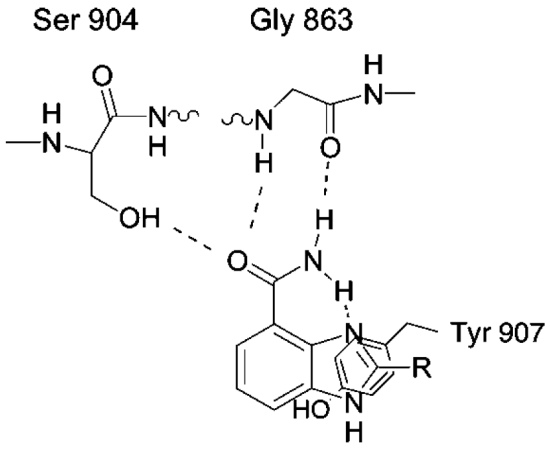


Figure 3: Interactions of 4-carboxamidobenzimidazoles with PARP-1 enzyme active site

Although several classes of PARP inhibitors move toward clinical development, new compounds are still needed (Graziani and Szabo, 2005). In our previous studies we found that modification of cardiovascular drugs, such as mexiletine (Li et al., 2000), amiodarone (Bognar et al., 2006; Kalai et al., 2005), or trimetazidine (Kalai et al., 2006; Kutala et al., 2006), 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 (Morphy et al., 2004), 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.

Cyclic nitroxides can be regarded as synthetic, multifunctional antioxidant molecules, owing their unique features to either their reduced forms, hydroxylamines, or their oxidized forms oxoammonium cations (Lam et al., 2008). It has been shown a decade ago that nitroxides form superoxide dismutase mimetic oxoammonium cations (Krishna et al., 1996), followed by reduction of the latter species with superoxide (eqs 1 and 2 of Figure 4). Hydroxylamines can act as proton and electron donor molecules, reducing any radical species (eq 3 of Figure 4). Nitroxides are also capable of inhibiting •OH formation by oxidizing Fe^{2+} to Fe^{3+} and hence preventing its participation in Fenton reactions (eq 4 of Figure 4) (Glebska et al., 2001). The fully reduced form of the nitroxide, a sterically hindered amine, is easily oxidized to nitroxide by various ROS (eq 5 of Figure 4) (Twomey et al., 1997). This nitroxide is in an equilibrium with the hydroxylamine depending upon the oxidative or reductive nature of its environment (eq 6 of Figure 4) (Bobko et al., 2007).

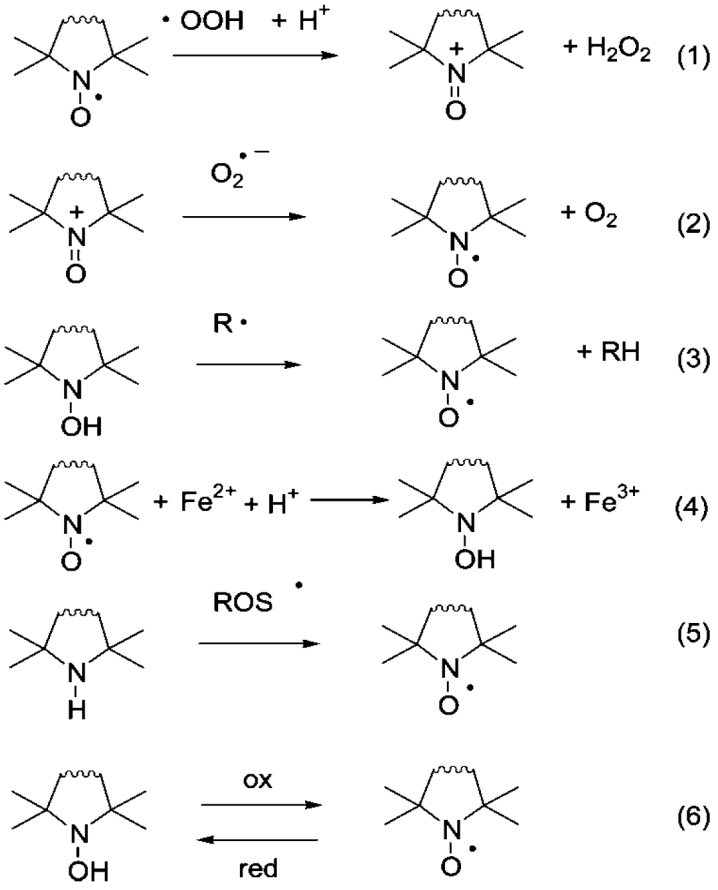


Figure 4: Possible radical scavenging mechanisms and transformations of nitroxides and prenitroxides.

3.3. Mitochondria targeted triphenylphosphonium derivatives

The presence of mitochondrial membrane potential ($\Delta\Psi_m$) alterations is an important characteristic of cancer cells (Kim et al., 2008). Many groups have shown that the mitochondrial transmembrane potential in carcinoma cell lines is significantly higher than in normal cell lines (Wang et al., 2007). 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. Murphy (Murphy, 1997) was the first to employ this property of mitochondrial membrane potential for "electrophoretic" delivery of antioxidants conjugated with hydrophobic cations into organelles. 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 (Kelso et al., 2001; Murphy et al., 2003; Smith et al., 1999). These initial attempts demonstrated that mitochondria-targeted antioxidants may have a highly cytoprotective effect, particularly against apoptosis (Armstrong, 2008; Belikova et al., 2009).

Lipophilic organic cations, such as rhodamine-123 and ^{3}H-tetraphenylphosphonium (^{3}H-TPP), were used for to measure mitochondrial potentials in tumor cells (Hockings and Rogers, 1996; Huang, 2002; Kroemer et al., 1998). In addition, several research groups have used mitochondria-directed, radiolabeled triphenylphosphonium cations, such as 4-(^{18}F-benzyl)triphenylphosphonium, as radiotracers for both myocardial perfusion imaging and tumor imaging by positron emission tomography (PET) (Cheng et al., 2005; Krause et al., 1994; Madar et al., 2006; Min et al., 2004). Some of these groups have also shown that mitochondria-directed ^{3}H-TPP is preferentially taken up by tumor cells over non-mitochondrially targeted 99mTc-sestamibi (Krause et al., 1994; Min et al., 2004). These data show that although mitochondria-directed triphenylphosphonium derivatives have limited tumor specificity, cytostatic agents targeted to the mitochondria may have therapeutic significance (Lena et al., 2009). In fact, paramagnetically-modified triphenylphosphonium salts have been widely used for biophysical studies of membranes for decades (Cafiso and Hubbell 1981); and their therapeutic potential as mitochondria-targeting, small, non-vitamin-like antioxidants is becoming increasingly appreciated (Deassolin et al., 2002; Dhanasekaran et al., 2005).

4. 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

5. Materials and methods

5.1. Materials

A protease inhibitor mixture and all chemicals for cell culture use were purchased from Sigma Aldrich Co. (Budapest, Hungary). Fluorescent dyes JC-1, fluorescein-conjugated annexin V, and propidium iodide were from Molecular Probes (Carlsbad, California). The PD98059, SB203580, JNK inhibitor II, LY294002, Akt Inhibitor IV, quercetine and Caspase3 inhibitor III were from Calbiochem (Darmstadt, Germany). Mitochondria targeted mito-CP (Dhanasekaran et al., 2005), HO-3814 (Balog M. et al., 2007) and HO-435 (Belkin S. et al. 1987), as well as the poly(ADP-ribose) polymerase (PARP) inhibitors, 4h (HO-3089), 3h and 15 were synthesized as previously described. Details of the synthesis of HO-4223 (2-[3-triphenylphosphoniumprop-1-enyl]-2,5,5-trimethylpyrrolidin-1-yloxyl Radical Bromide salt) will be published elsewhere. Cisplatin and gemcitabine (GEMZAR) were obtained from Ebewe Pharma (Unterach, Austria) and Eli Lilly France (Fegersheim, France). The following primer antibodies were used: anti-Bcl-2 (Cell Signalling Technology, Beverly, MA, USA), anti-Cyclophilin D (Calbiochem, Darmstadt, Germany) and anti-β actin (Sigma Aldrich Co, Budapest, Hungary). The anti-mouse IgG and anti-rabbit IgG were from Sigma-Aldrich Co (Budapest, Hungary). All reagents were of the highest purity commercially available.

5.2. Animals

Wistar rats were purchased from Charles River Hungary Breeding Ltd. (Budapest, Hungary). The animals were kept under standard conditions; tap water and rat chow were provided *ad libitum*. The investigation conformed to the *Guide for the Care and Use of Laboratory Animals*, published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and was approved by the Animal Research Review Committee of the University of Pecs.

5.3. Oxidative metabolism studies with EPR

Male, 290-350 g Wistar rats (Charles River, Budapest, Hungary) were used in our study. All animals were housed in wire bottom individual metabolic cages in order to collect urine. Rats were randomly divided into two groups. One group was treated with 2 mg, ip (intraperitoneal), of **4h** (HO-3089) (group 1, *n*) 3) one time. A single dose of **3h** (HO-3088), 2 mg, ip, was administered to the other group (group 2, *n*) 3). Our agents were completely dissolved in 1.0 mL of 0.9% NaCl. Urine was collected on three occasions. The first collection was performed before the treatment. The second fraction was collected right after the injection to 4 h. The third fraction of urine was collected from 4 to 22 h after the treatment. The PbO_2 treatment of urine was exploited as follows: to 1 mL of urine, 50 mg of PbO2 was added and vortexed for 1 min, and then the sample was allowed stay for 10 min and 50 μL from the supernatant was studied by EPR (electron paramagnetic resonance). EPR analyses were performed on Magnettech MS200 spectrometer (X-band) in 50 μL glass capillaries. Modulation was 1400 mG, sweep was 59.5 G, sweep time was 60 s, gain was 500, microwave attenuation was 10 dB, and urine was measured directly after collection.

5.4. Isolation of mitochondria

Rats were sacrificed by decapitation and mitochondria were isolated from the liver by differential centrifugation, as described in a standard protocol (Schneider and Hageboom, 1950). All isolated mitochondria were purified by Percoll gradient centrifugation (Sims, 1990), and mitochondrial protein concentrations were determined using the biuret method with bovine serum albumin as a standard.

5.5. Mitochondrial permeability transition

mPT was monitored by following associated large amplitude swellings via the decrease in the reflectance of 540 nm light (Varbiro et al., 2001) measured at room temperature using a Perkin–Elmer fluorimeter (London, UK). Briefly, mitochondria at a concentration of 1 mg protein/mL, were preincubated in assay buffer (70 mM sucrose, 214 mM mannitol, 20 mM *N*-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid, 5 mM

glutamate, 0.5 mM malate, 0.5 mM phosphate) containing the studied substances for 60 s. Mitochondrial permeability transition was induced by the addition of 150 μM Ca^{2+}, HO-3814, or HO-4223, at the indicated concentrations.

5.6. Mitochondrial membrane potential

Mitochondrial membrane potential was monitored by measuring rhodamine123 (Rh123) fluorescence upon release from mitochondria after induction of permeability transition at room temperature. Fluorescence was measured using a Perkin–Elmer fluorimeter at an excitation wavelength of 495 and an emission wavelength of 535 nm. Briefly, mitochondria at a concentration of 1 mg protein/mL were preincubated in assay buffer (70 mM sucrose, 214 mM mannitol, 20 mM N-2-hydroxyethyl piperazine-N′-2-ethanesulfonic acid, 5 mM glutamate, 0.5 mM malate, and 0.5 mM phosphate) containing 1 μM Rh123 and the studied substances for 60 s. Alterations in mitochondrial membrane potential were induced by the addition of 150 μM Ca^{2+}, HO-3814, or HO-4223, at the indicated concentrations.

5.7 Assay to test inhibitory effects of benzimidazole derivatives on PARP enzyme in vitro

Poly(ADP-ribose) polymerase was isolated from rat liver based on a known method (Shah et al., 1995). The potential inhibitory effect of benzimidazole derivatives was tested using this assay system. The PARP activity was determined in 130 μL of reaction mixture containing 100 mM Tris-HCI buffer, (pH 8.0), 10 mM $MgCl_2$, 10% glycerol, 1.5 mM DTT, 1 mM (adenine- 2,8-^{3}H) NAD^+(4.500 cpm/nmol), 10 μg of activated DNA, and 10 μg of histones. The incubation time was l5 min, and the reaction was stopped by addition of trichloroacetic acid (8%). After addition of 0.5 mg of albumine, precipitation was allowed to proceed for at least 20 min on ice, and the insoluble material was collected on glass filters, washed with 5% perchloric acid. The protein-bound radioactivity was determined using a LS-200 Beckman scintillation counter.

5.8. Cell cultures

PANC-1 (human pancreatic ductal tumor cells, poorly differentiated), HeLa (human cervix carcinoma), T24/83 (human bladder carcinoma) and WRL-68 (human hepatic cell line) cells were obtained from the European Collection of Cell Cultures. Cell lines were grown in a humidified 5% CO_2 atmosphere at 37 °C. PANC-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), WRL-68 and HeLa cells in minimum Eagle's medium (MEM) and T24/83 cells in McCoy's medium. All media contained antibiotic solution (100 U/mL penicillin and 100 μg/mL streptomycin) and 10% fetal bovine serum (FBS, Gibco). Cells were passaged at 3 day intervals.

5.9. Suppression of cyclophillin D expression by siRNA

PANC-1 cells were transiently transfected with siRNA designed by the manufacturer (Santa Cruz Biotechnology) to silence cyclophilin D, in Opti-MEM I Reduced Serum Medium (Invitrogen) using Lipofectamine 2000. For effective silencing, the transfection step was repeated twice, with a 48-h interval between transfections, and experiments on the cells were performed 40 h after the third transfection. Silencing efficiency was tested by Western blot, using a polyclonal anti-cyclophilin D primary antibody.

5.10. Cell viability assay

Cells were seeded on 96-well plates at a starting density of 2×10^4 cells/well and cultured overnight. The next day, various compounds at the indicated concentrations were added to the medium. 24 h later, 0.5% of the water-soluble mitochondrial dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT^+), was added. Incubation was continued for 3 more hours, the medium was removed, and the water-insoluble blue formasan dye, formed stochiometrically from MTT^+, was solubilized using acidic isopropanol. Optical densities were determined using an ELISA 96-well plate reader (Anthos Labtech 2010; Vienna, Austria) at a wavelength of 550 nm.

5.11. Stable transfection

Full-length Bcl-2 cDNA was PCR amplified and subcloned into a pcDNA3.1 vector. The vector-containing bcl-2 or empty pcDNA3.1 vector was transfected into PANC-1 cells with Lipofectamin 2000 according to the manufacturer's protocol (Invitrogen, Carlsbad, California). Cells were then grown in selective medium (10% fetal calf serum, Dulbecco´s modified Eagle´s medium containing 500 μg/mL G-418). Cell clones were subsequently screened by Western blot analysis using anti-Bcl-2 polyclonal antibody to check for increased Bcl-2 protein expression relative to empty pcDNA3.1 transfected cells. Bcl-2-overexpressing clones were selected for further experiments.

5.12. Quantitative high-pressure liquid chromatography-mass spectrometry (HPLC-MS) assay

Cells were harvested, and nuclear, mitochondrial and cytosolic fractions were isolated by differential centrifugation (Szigeti et al., 2006). Subcellular fractions were extracted with acetonitrile, centrifuged at 10000g for 5 min, and the resulting supernatants were assayed using HPLC-MS. HO-3814 calibration standards were dissolved in acetonitrile. Sample solutions were introduced into the ion source at a flow rate of 200 μL per hour via a PEEK capillary (Upchurch Scientific, Inc., Oak Harbor, WA, USA). A Thermo AQA guadrupole mass spectrometer, equipped with an electrosprayionization source (Thermoquest, San José, USA) was used for mass detection. The ionization source was operated in positive mode. Nitrogen was used as the drying gas at 300 °C, at a flow rate of 12 L/min, and the pressure of the nebulizer was set at 20 psi. The scanning mass-to-charge range was 50 to 1000 m/z.

5.13. Western blot analysis

Cells were harvested in cold lysis buffer (0.5 mM sodium metavanadate, 1 mM EDTA, and a protease inhibitor cocktail in phosphate-buffered saline, pH: 7.4). Proteins were precipitated using trichloroacetic acid, washed three times with -20 °C acetone, and subjected to SDS-PAGE. Proteins (30 μg/lane) were separated on 12% SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% low-fat milk for 1 h at room

temperature, then exposed to primer antibodies at 4 °C overnight, at a dilution of 1:1000 in 5% BSA, 1X TBS, and 0.1% Tween20 solution. Appropriate horseradish peroxidase-conjugated secondary antibodies were used for 2 h at room temperature at a 1:5000 dilution. Peroxidase labeling was visualized by enhanced chemiluminescence (ECL) using the ECL Western blot detection system (Amersham Biosciences). Developed films were scanned, and pixel volumes of the bands were quantitated using NIH Image J software.

5.14. Fluorescent microscopy

PANC-1 cells were seeded on poly-L-lysine-coated (2.5-5 μg/cm^2) glass coverslips and cultured at least overnight before experiments. After subjecting cells to the appropriate treatment (as indicated in the figure legends), coverslips were rinsed twice in phosphate-buffered saline, and placed upside down on the top of a small chamber formed by a microscope slide and a press-to-seal silicone isolator. The chamber was filled with phosphate-buffered saline containing 4.5 g/L glucose and 20 mM HEPES pH 7.4. Cells were imaged using a Zeiss Axiovert 25 fluorescent microscope equipped with a ProgRes C12 Plus CCD camera, using a 63 x objective and epifluorescent illumination. Cells were loaded with appropriate dyes for 15 min. The same microscopic field was imaged first with 546nm bandpass excitation and 590nm emission (green filter, red fluorescence), then with 450-490 nm bandpass excitation and 520 nm emission (blue filter, green fluorescence). The resulting images were merged.

5.15. Hydroxyl radical scavenging of benzimidazole derivates (Antiox)

Hydroxyl radical formation was detected using the oxidant-sensitive nonfluorescent probe benzoic acid which is hydroxylated to 2-, 3-, or 4-hydroxybenzoic acid (Miles et al., 1996). Hydroxylation of benzoic acid results in the appearance of intense fluorescence, which makes possible the fluorescence spectroscopic monitoring of the hydroxylation reactions (excitation λ = 305 nm; emission λ = 407 nm). The reaction was studied in 2.5 mL reaction volumes containing 20 mM potassium phosphate buffer (pH 6.8), 0.1 mM benzoic acid, 0.1 mM H_2O_2, and 20 μM Fe^{2+}-EDTA. Data of Table 1 show the concentration of benzimidazoles (in μM) at which the rate of hydroxyl radical induced hydroxylation is inhibited by 50%. The

parent ring (4-carboxamidobenzimidazole) fluorescence λex/λem = 267/357 nm (data not shown) has no influence on the determination of hydroxybenzoic acids. Under Fenton reaction conditions in the presence of compounds **3h** and **4h** no species were formed with fluorescence at 407 nm (data not shown).

5.16. Statistical analysis

Each experiment was repeated at least three times. Values in the figures, tables and text are expressed as mean ± SEM of n observations. Statistical analysis was performed by analysis of variance followed by Student's t-test. Statistical significance was set at $p<0,05$.

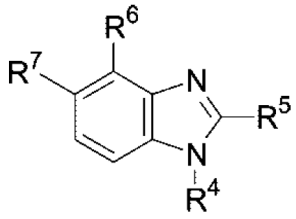
6. Results

6.1. New poly(ADP-ribose) polymerase-1 inhibitors with antioxidant activity

6.1.1. Biological study of compounds 3h, 4h and 15

We showed, that the tested 37 compounds exhibited IC_{50} for PARP inhibition in range between 14 nM and 10 μM in the whole cell system assay and IC_{50} values in range between 9 nM and 60 μM. In the *in vitro* antioxidant assay all of the examined compounds exhibited IC_{50} in range between 0.5 nM and 0.35 μM (data not shown). During the structure-activity relationship monitoring we changed the size of the nitroxide ring (five- or six-membered), the saturation of the ring, the substituents on the pyrroline ring, the spacer between the nitroxide ring and the benzimidazole ring or the oxidation status of the ring nitrogen. The effect of substitution changes in the benzimidazole ring also had been tested. Compounds **3h** and **4h** were found to be the best-performing PARP inhibitors (Table 1). Alkylations of benzimidazole NH or carboxamide nitrogen were detrimental to PARP inhibitory potency, as we experienced for compound **15**. As we presumed, removal of carboxamide and changing its position from 4 to 5 on the benzimidazole ring resulted in a loss of PARP inhibitory activity. Cell death inhibition usually provides information on the cellular potency of the compound investigated. Surprisingly, there was a low correlation between cell death inhibition results and PARP inhibition results. The cell death inhibitory IC_{50} values are below 100 nM, the most effective one being 3h with a sixmembered- ring. The alkylation of carboxamide nitrogen with an isopropyl group did not decrease the cell death inhibitory activity. From these results we conclude that the cell death inhibition, tested as protection of WRL-68 human liver cells from H_2O_2 induced cell death, does not exclusively reflect on PARP inhibitory activity but includes other protective mechanisms of tested compounds as well. This observation inspired us to study hydroxyl radical scavenging activity of the new PARP inhibitors in a Fenton reaction. In this assay the watersoluble hydroxylamines were used and may reduce hydroxyl radicals to water while being oxidized to stable nitroxide free radicals. The formed nitroxides can also oxidize Fe^{2+} to Fe^{3+} and hence prevent its participation in the reaction (Figure 4, eqs 3 and 4). Sterically hindered amines can be oxidized to nitroxides (Figure 4, eq 5). These multiple ways of preventing •OH (hydroxyl radical) formation in the Fenton reaction justify the very low IC_{50} values measured for studied compounds, which were mostly below 10 nM. On the basis of nitrogen oxidative status, obvious structure-activity relationships cannot be

drawn yet; compound 4h appears to be the best antioxidant and PARP inhibitor regarding the PARP enzyme inhibition, cell death inhibition, and hydroxyl radical-scavenging results. The toxicity of 4h was further assessed on eight mice. The LD_{50} for 4h was 740 mg/ kg, that being higher than 500 mg/kg, the acceptable therapy index.



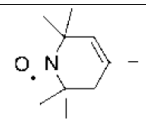
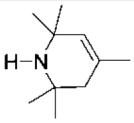
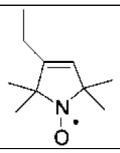
Cpd	R^4	R^5	R^6	R^7	PARP Inh. IC_{50} µM	Inh. Cell death IC_{50} µM	Antiox IC_{50} µM
3h	H		$CONH_2$	H	0.026	15.57±0.220	0.0096
4h	H		$CONH_2$	H	0.014	0.098±0.009	0.0016
15		H	$CONH_2$	H	10	1.985±0.071	0.0018

Table 1. Data for compounds 3h, 4h and 15

6.1.2. Metabolism study of compounds 3h and 4h

During the metabolism studies HCl salts of both compounds (**3h**, **4h**) were administered to rats intraperitoneally (ip) in 8 mg/kg doses, and urine samples were collected before administration, for 4 h after administration, and after 4 h till 18 h. An isotropic triplet in the EPR spectra demonstrates the presence of nitroxide radicals and thus the oxidative metabolism of sterically hindered amine **4h**; i.e., it is metabolized to **3h** in vivo. The concentration of **3h** in the urine sample collected for 4 h is 0.7 nM. However, the EPR absorption increased almost 3-fold upon adding PbO_2 to urine samples, indicating the presence of not only nitroxide but also hydroxylamines oxidizable to nitroxides. The final concentration of radical species was 1.7 nM (Figure 5). The ip administered hydroxylamine HCl salt of **3h** to rats and collection of their urine showed that hydroxylamine mostly (74%) oxidized to nitroxide after 4 h (the concentration was 87 nM, based on EPR measurements), and only 26% remained in the hydroxylamine form. Further EPR active substances were formed upon addition of PbO_2 (Figure 6), and the final concentration of EPR active substance was 116 nM. On the basis of these observations, we conclude 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. Hydroxylamines are rapidly ((0.2-1.2) × 104 M^{-1}) oxidized by ROS ($O_2^{\bullet-}$ or peroxynitnitrite or their decomposition products), although autoxidation during sample collection cannot be excluded either. More detailed studies demonstrated the oxidation of hydroxylamines to nitroxide with rates influenced by ring size and substituents of the nitroxide (Nishimura-Suzuki and Swartz, 1998). Saito et al. demonstrated that both hydroxylamine and nitroxide forms are present, although hydroxylamine is more dominant from a freshly prepared kidney (Saito et al., 2004). Urine collected between 4 and 22 h after administration of **3h** or **4h** practically did not show triplet EPR signal (data not shown), presumably because the loss of the PARP-inhibitor metabolites.

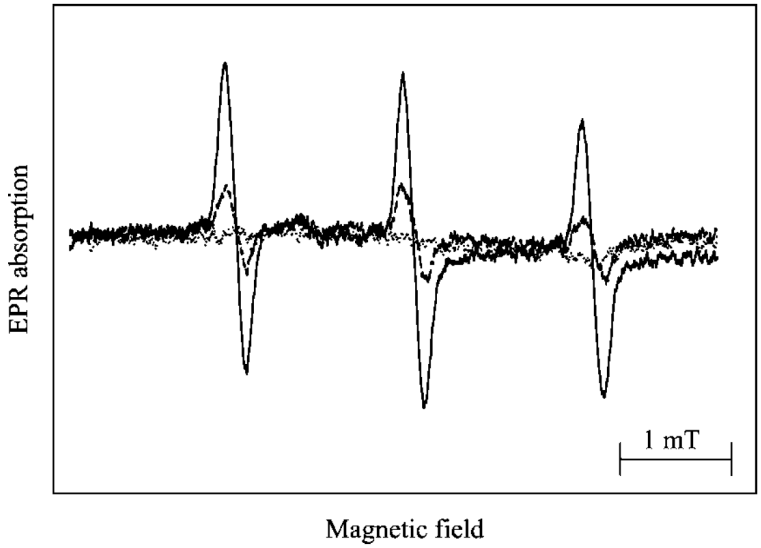


Figure 5: The metabolism of 4h. EPR spectra of collected urine before administration of 4h (· · ·), EPR spectra of collected urine (between 0-4 h) after administration of 4h (- - -), EPR spectra of collected urine (between 0-4 h) after administration of 4h (-) and oxidation further with PbO_2.

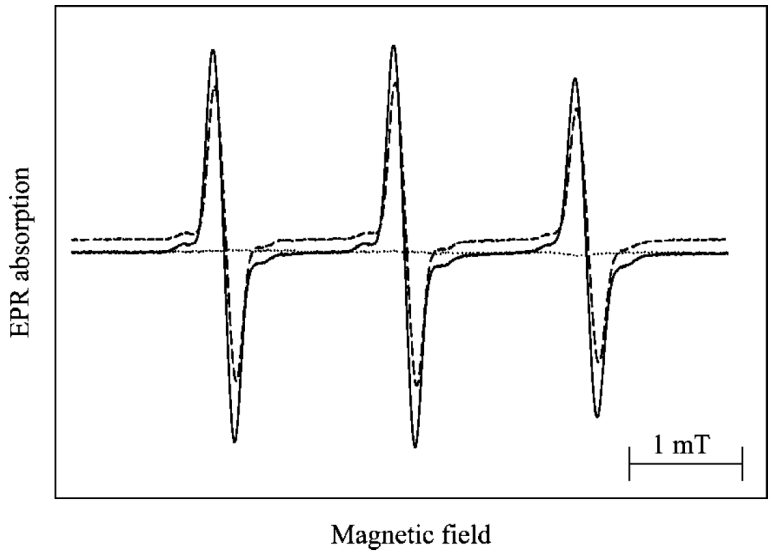


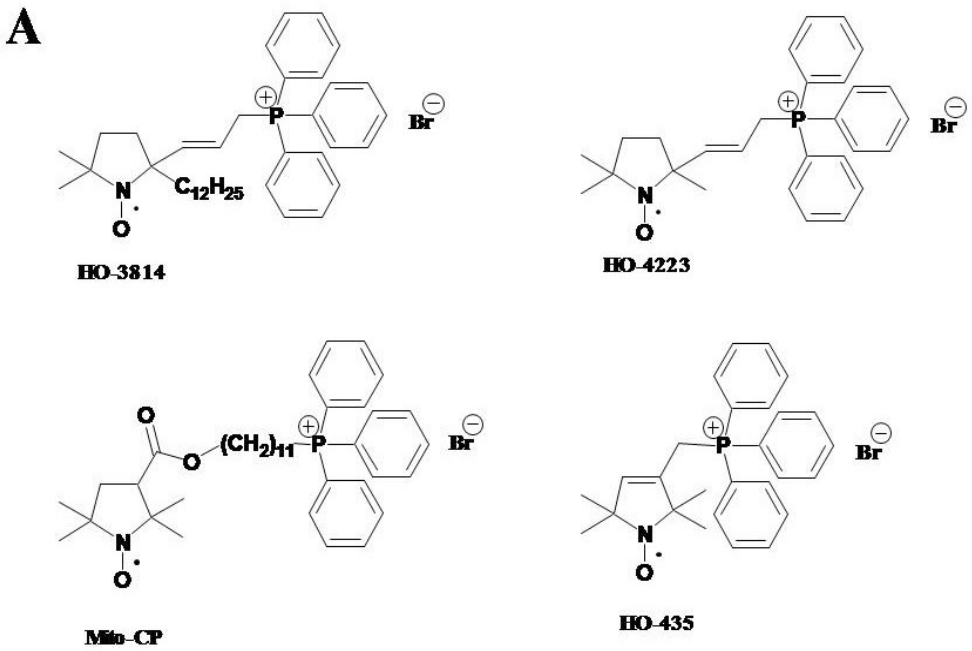
Figure 6: The metabolism of 3h. EPR spectra of collected urine before administration of 3h (· · ·), EPR spectra of collected urine (between 0-4 h) after administration of 3h (- - -), EPR spectra of collected urine (between 0-4 h) after administration of 3h (-) and oxidation further with PbO_2.

6.2. Induction of mitochondria de-stabilization and necrotic cell death by apolar mitochondria-directed SOD mimetics

6.2.1. Effect of mitochondria-directed compounds on the viability of PANC-1 cells

The chemical structures of mitochondria-directed SOD-mimetics (HO-4223 and HO-435) and their hydrophobic derivatives (HO-3814 and Mito-CP) are presented in Figure 7A. To test for mitochondrial localization, we treated PANC-1 cells with equimolar concentrations of these compounds (5 µM) for 24 h, and determined the concentrations in nuclear, mitochondrial, and cytosolic fractions using quantitative HPLC-MS. As reported previously for Mito-CP (Dhanasekaran et al., 2005), more than 98 % of the applied HO-3814 (Figure 7B), as well as the other compounds (data not shown), were found to be localized to the mitochondria.

To compare the effect of mitochondria-directed compounds on PANC-1 cells, an MTT^+ viability assay was performed after exposing the cells to 5 µM of the compounds for 24 h. We observed that both hydrophobically modified compounds, Mito-CP (Dhanasekaran, 2005) and HO-3814, decreased the viability of PANC-1 cells significantly, whereas the non-hydrophobic derivatives (HO-4223 and HO-435) had no effect (Figure 7C).



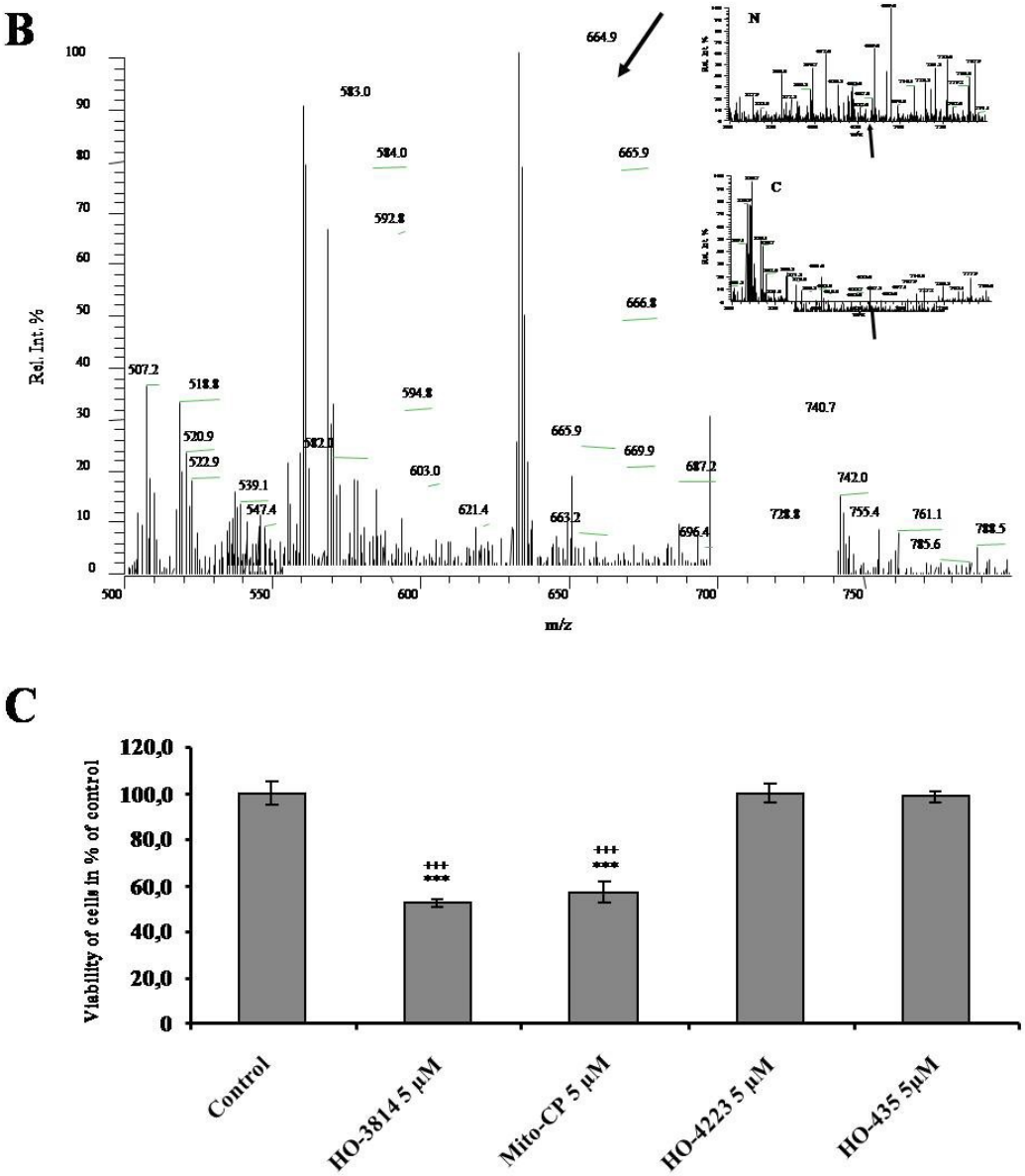
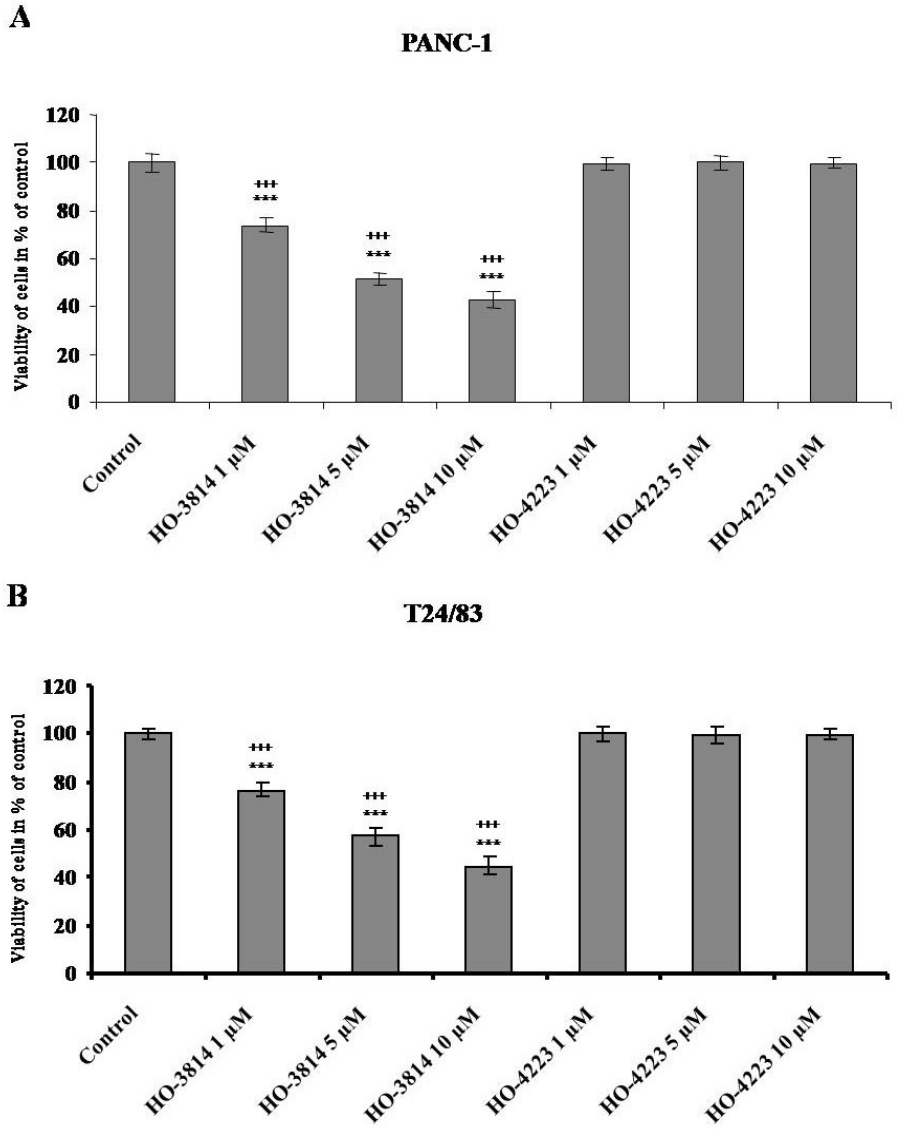


Figure 7: The chemical structures, intracellular localizations and cytotoxicities of the tested compounds.

PANC-1 cells were treated with 5 µM concentrations of the different mitochondria-directed SOD mimetics (**A**) for 24 h. Then, the cellular distribution of the compounds was determined using a quantitative HPLC-MS assay on an aliquot of the cells after subcellular fractionation. Representative mass spectra of mitochondrial (**B**) cytosolic (C; inset) and nuclear (N; inset) fractions of HO-3814 treated cells are presented. Arrows indicate the position of the HO-3814 molecular ion (theoretical value 664.74). Experiments were repeated three times. Viabilities for the rest of the cells were determined by monitoring the formation of water insoluble blue formasan dye from the yellow mitochondrial dye MTT^+ and are presented in panel (**C**) as the % of the untreated (control) cells. Data shown represent the mean ± S.E.M. of eight independent experiments. ***$P<0.001$ compared to control cells; $^{+++}P<0.001$ compared to HO-4223 and HO-435 treated cells (compounds without apolar side chains).

6.2.2. Effect of hydrophobic derivatization on the viabilities of cancer cell lines

To determine if the cytototoxic effect of hydrophobic, mitochondria-directed SOD-mimetics presented in section 6.2.1. is restricted to the PANC-1 cell line, we tested HO-3814 and HO-4223 on two additional cancer cell lines. PANC-1, T24/83 and HeLa cell lines were treated with different concentrations (1, 5, 10 μM) of the studied mitochondria-directed SOD-mimetic compounds for 24 hours. Cell viabilities were evaluated using the MTT^{+} assay, and expressed as the percentage of untreated cells. The results demonstrate that HO-3814 treatment induced substantial cell death in a concentration-dependent manner. Viabilities in all three cancer cell lines were significantly decreased even by the lowest concentration of HO-3814 (Figure 8). In contrast to its hydrophobic derivative, HO-4223 treatment did not have any effect on the viability of the different cell lines tested (Figure 8).



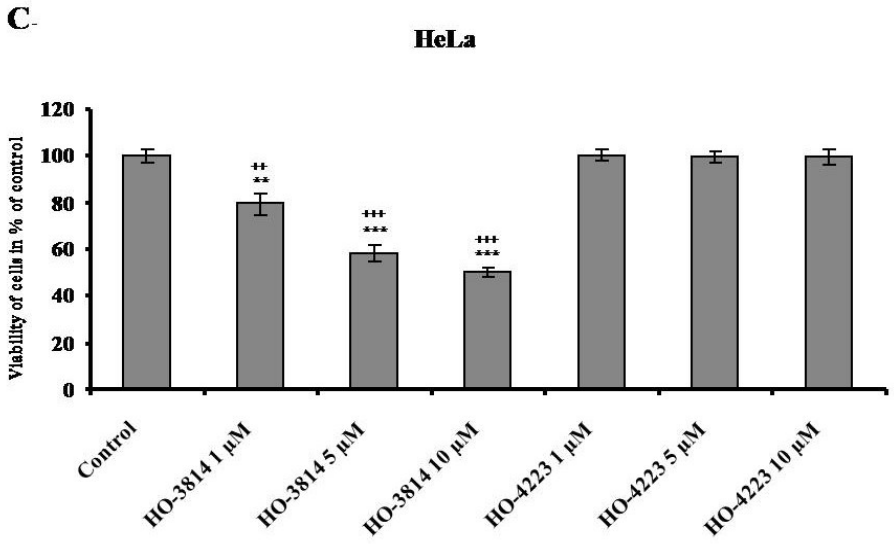


Figure 8: Effect of HO-3814- and HO-4223 treatment on the survival of cancer cell lines. PANC-1 pancreatic ductal tumor cells (**A**), T24/83 bladder carcinoma cells (**B**) and HeLa cervix carcinoma cells (**C**) were treated with different concentrations of HO-3814 and HO-4223 (indicated beneath the columns) for 24 h. Cell viabilities were evaluated using the MTT assay and are expressed as % of untreated (control) cells; data are the mean ± S.E.M. of eight independent experiments. ***P<0.001 compared to control cells; $^{+++}$P<0.001 compared to HO-4223 treated cells (a compound without apolar side chain).

6.2.3. Comparison of the effect of HO-3814 and anti-cancer drugs on cell viabilities

We compared the effect of HO-3814 on cell viability (Figure 9) with that of cisplatin, a widely used anti-tumor agent. Cell lines were treated with 1-10 µM HO-3814 or 5-30 µM cisplatin, and their viabilities were determined. The cancer cell lines showed different sensitivity toward HO-3814 compared to cisplatin. Specifically, PANC-1 cells were much more sensitive to HO-3814 than cisplatin (Figure 9A). However, in the T24/83 cell line, HO-3814 induced cell death was comparable to that induced by cisplatin at the concentrations tested (Figure 9B). Because HO-3814 was used at much lower concentrations than cisplatin, this result still suggests a higher sensitivity to HO-3814 in T24/83 cells. In contrast, HeLa cells were more resistant to HO-3814 than cisplatin at any concentration tested (Figure 9C).
Because PANC-1 cells are derived from a human pancreatic ductal tumor, and gemcitabine (rather than cisplatin) is the first line drug in pancreatic tumor therapy, we tested the sensitivity of PANC-1 cells toward gemcitabine using the same experimental setup as used to compare HO-3814 and cisplatin. We found that even 1 mM of gemcitabine caused only a

slight decrease in the viability of PANC-1 cells under our experimental conditions (Figure 10), indicating that HO-3814 was much more effective in inducing cell death in PANC-1 cells than either of the anti-cancer drugs used.

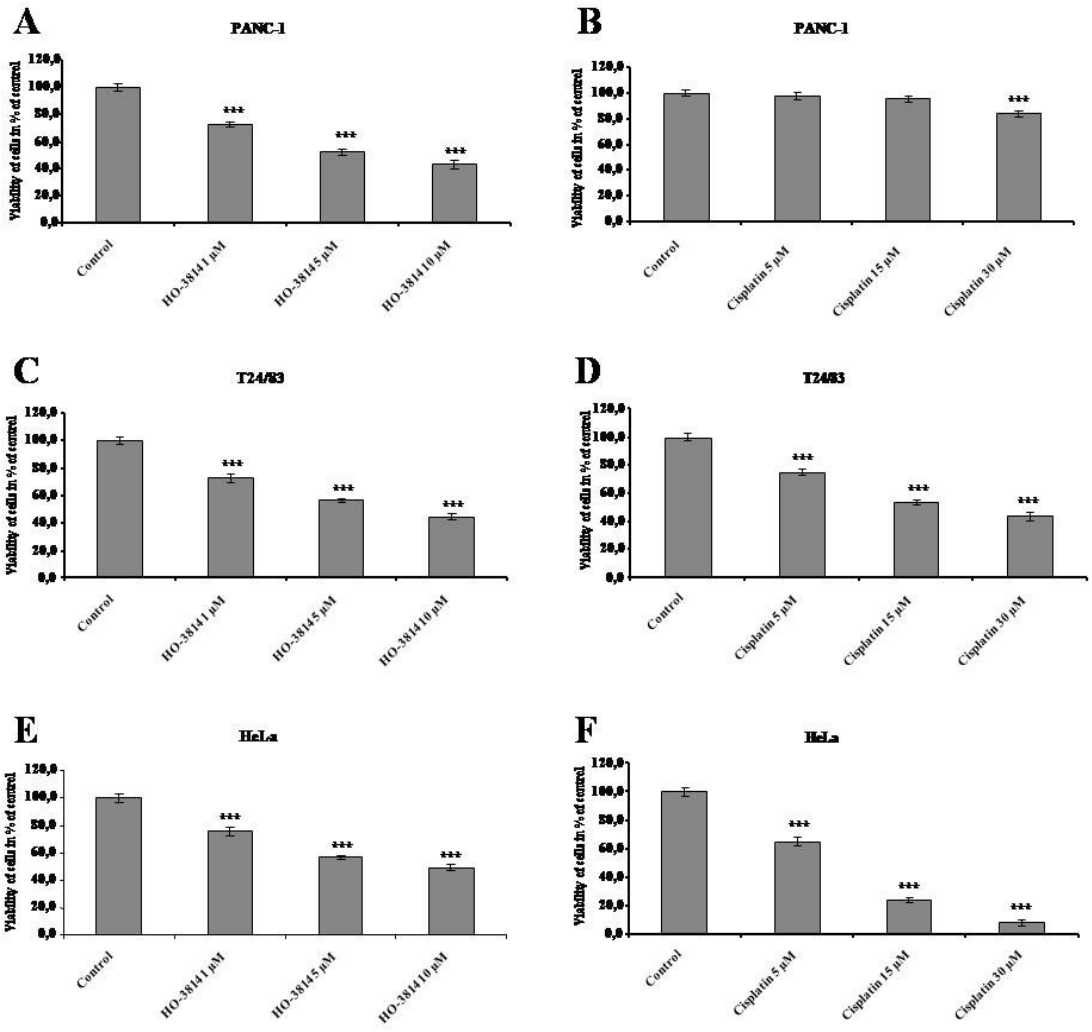


Figure 9: Comparison the effect of HO-3814 and cisplatin treatment on the cancer cell lines. PANC-1 (**A,B**), T24/83 (**C,D**) and HeLa cells (**E,F**) were treated with different concentrations of HO-3814 (**A,C,E**) or cisplatin (**B,D,F**) for 24 h. Cell viabilities were detected by the MTT assay and are expressed as % of untreated (control) cells; results are the mean ± S.E.M. of eight independent experiments. ***P<0.001 compared to control cells.

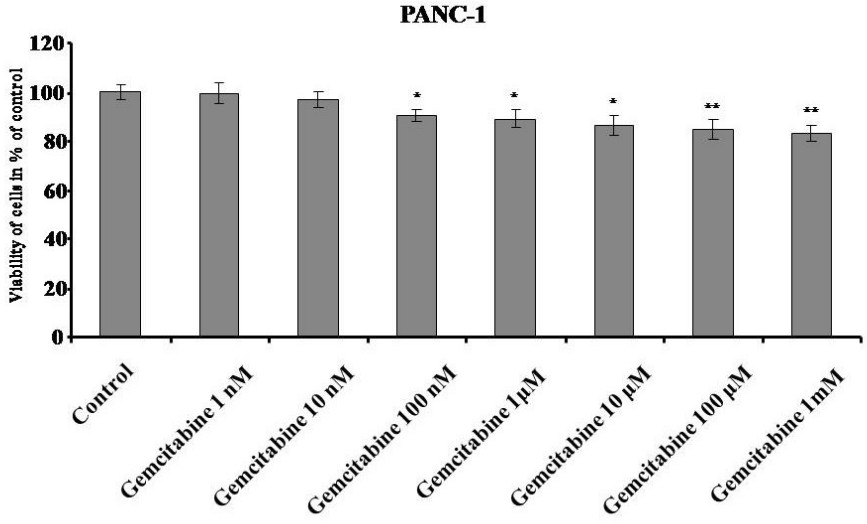


Figure 10: Effect of gemcitabine treatment on PANC-1 cells. PANC-1 cells were treated with different concentrations of gemcitabine for 24 h. Cell viabilities were detected by the MTT assay and are expressed as % of untreated (control) cells; results are the mean ± S.E.M. of eight independent experiments. *P<0.05, **P<0.01 compared to control cells.

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

To establish the type of cell death induced by HO-3814, untreated as well as HO-3814- and HO-4223 treated PANC-1 cells were dual-labeled with Annexin V-FITC and propidium iodide (PI). Annexin V binds to phosphatidylserine, which is located predominantly on the internal, cytoplasmic side of the plasma membrane of intact cells, but becomes exposed in apoptotic cells. Disruption of membrane integrity makes the cell interior accessible to both PI and Annexin V in necrosis. Untreated and HO-4223 treated cells were PI and Annexin V negative (Figure 11A and 11C). However, treatment with 10 µM HO-3814 for 3 hours resulted in propidium iodide positive staining, indicating that HO-3814 induces predominantly necrotic cell death (Figure 11B). Exposing the cells to 250 µg/mL etoposide (a compound which induces apoptosis) resulted in Annexin V positive staining (Figure 11D), verifying that the staining protocol used is capable of identifying the type of cell death. The pictures (Figure 11E, F, G and H) are made with light microscope in the same field verifying that fluorescent microscopic images A and C also contain cells.

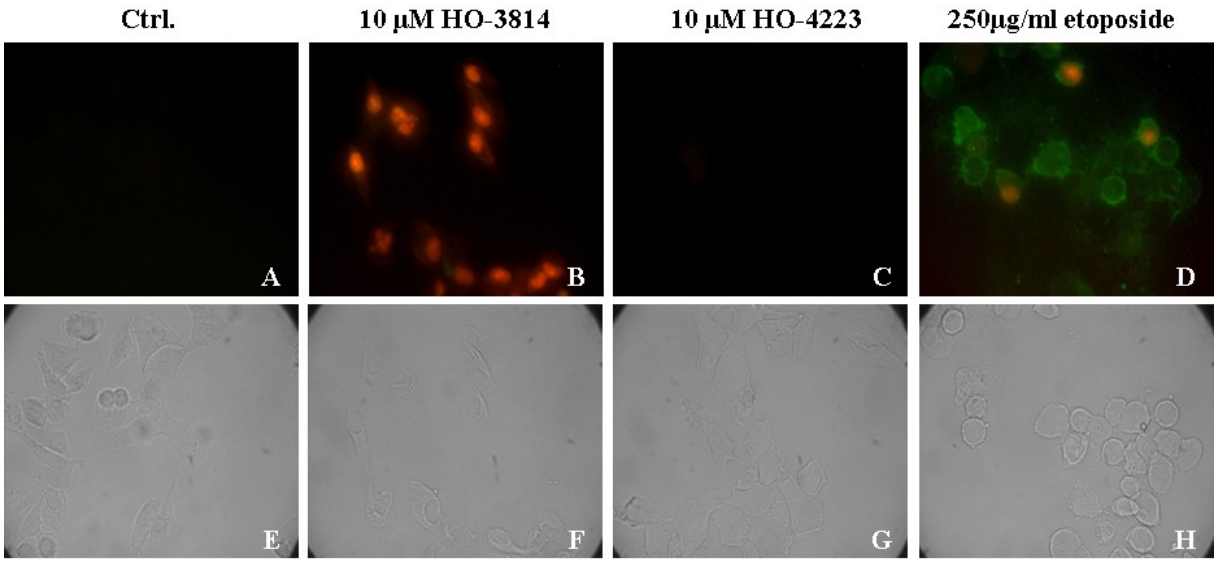


Figure 11: Effect of HO-3814- and HO-4223-treatment on apoptotic and necrotic cell death in PANC-1 cells. PANC-1 cells were treated with 10 µM HO-3814, 10 µM HO-4223 or 250µg/mL etoposide for 3h and double-stained with fluorescein labelled Annexin V (green) and propidium iodide (red). Fluorescent microscopic images taken using the blue and green filters were merged. Representative images are presented for untreated **(A)**, HO-3814 treated (**B**), HO-4223 treated **(C)** and etoposide treated (**D**) cells. In the second line are shown the pictures of the same field **(E,F,G,H)** are made with light microscope. Experiments were repeated three times.

6.2.5. Effect of different inhibitors on HO-3814 induced cell death

To establish the molecular mechanism of the cytotoxic effect of HO-3814, we analysed the effect of the mitochondrial permeability transition inhibitor, cyclosporine A (CSA), 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 exposed to 5 µM HO-3814 for 24 h. Cell viabilities were determined using the MTT^{+} assay and expressed as the % viability of untreated cells. None of the compounds tested had any effect on HO-3814 induced cell death (Figure 12) indicating that most likely, none of these intracellular pathways were involved.

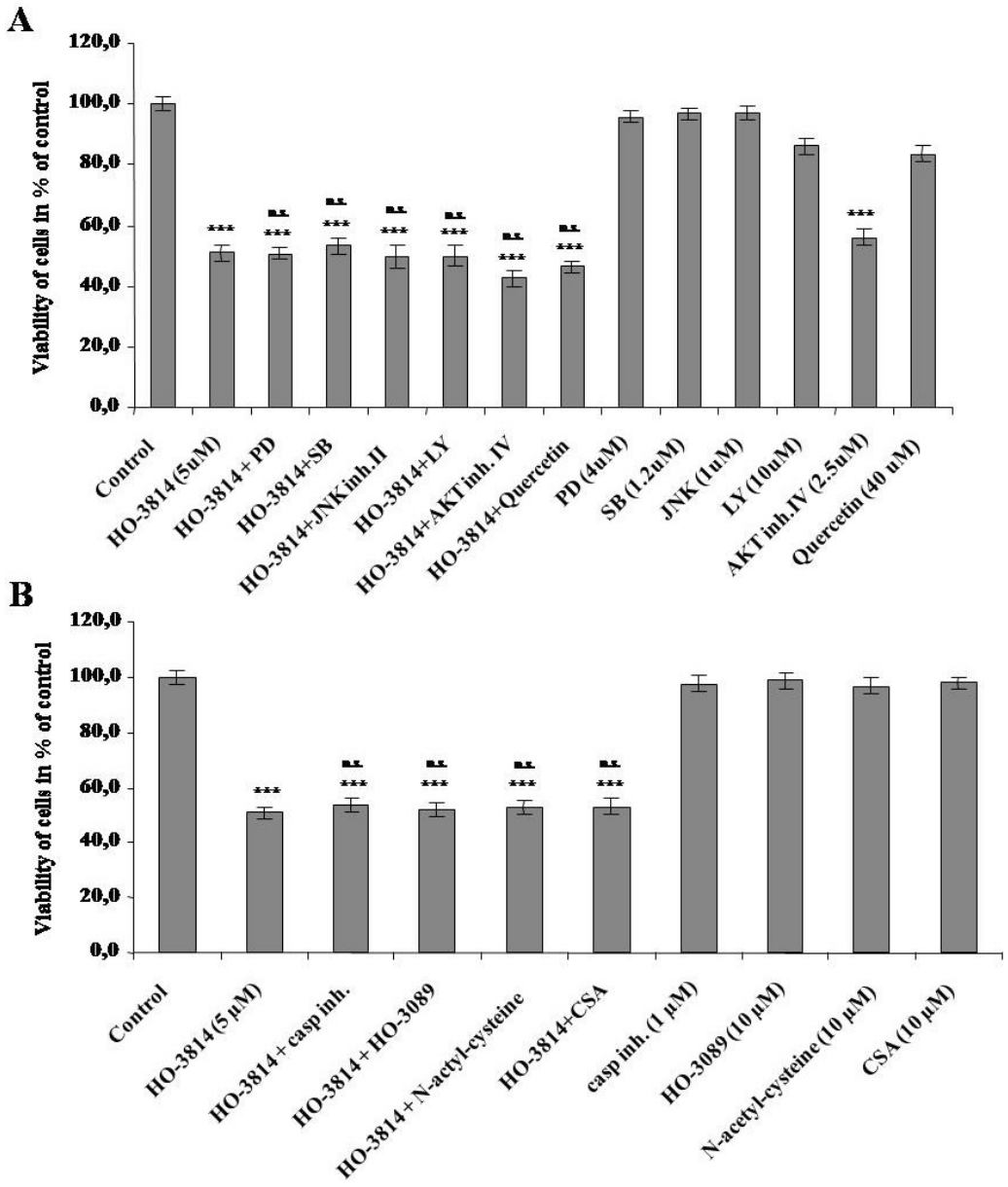


Figure 12: Effect of various inhibitors on HO-3814 induced death in PANC-1 cells. Cells were exposed to 4 µM ERK kinase inhibitor (PD), 1.2 µM p38 inhibitor (SB), 1 µM JNK inhibitor (inh.) II, 10 µM phosphatidylinositol-3 kinase inhibitor (LY), 2.5 µM Akt inhibitor IV and 40 µM quercetin (**A**); as well as 1 µM of caspase 3 inhibitor III (casp. inh.), 10 µM poly(ADP-ribose) polymerase inhibitor (HO-3089), 10 µM N-acetyl cysteine and 10 µM CSA (**B**) in the absence or presence of 5 µM HO-3814. Cell viabilities were detected by MTT assay and are expressed as % of untreated (control) cells; results are the mean ± S.E.M. of eight independent experiments. ***P<0.001 compared to control cells. $^{n.s.}$not different from HO-3814 alone group.

6.2.6. Effect of HO-3814 on isolated mitochondria

We studied whether HO-3814 treatment had any direct effect on mitochondrial membrane systems. To this end, we monitored dissipation of mitochondrial membrane potential and mitochondrial swelling from isolated Percoll-gradient purified rat liver mitochondria.

Depolarization of the mitochondrial membrane was demonstrated by release of a membrane-potential sensitive mitochondrial dye, Rh123. Depolarization was induced by 150 μM Ca^{2+} (Figure 13A, line 2), and was completely inhibited by 2.5 μM CSA (data not shown). HO-3814 induced depolarization in isolated mitochondria in a concentration-dependent manner (data not shown) that was not prevented by the presence of 2.5 mM CSA (data not shown). In fact, at a concentration of 40 μM, the level of depolarization induced by HO-3814 (Figure 13A, line 3) surpassed that of induced by 150 μM Ca^{2+}. In contrast, HO-4223, even at the highest concentration, had only a slight effect on mitochondrial depolarization (Figure 13A, line 4).

High-amplitude mitochondrial swelling was monitored by the decrease in reflectance at 540 nm. Similarly to 150 μM Ca^{2+} treatment (Figure 13B, line 2), HO-3814 caused swelling of the mitochondria (Figure 13B, line 3) in a concentration-dependent manner (data not shown). However, in contrast to 150 μM Ca^{2+}, HO-3814 induced swelling was not inhibited by 2.5 mM CsA (data not shown). HO-4223 did not have any effect on mitochondrial swelling (Figure 13B, line 4).

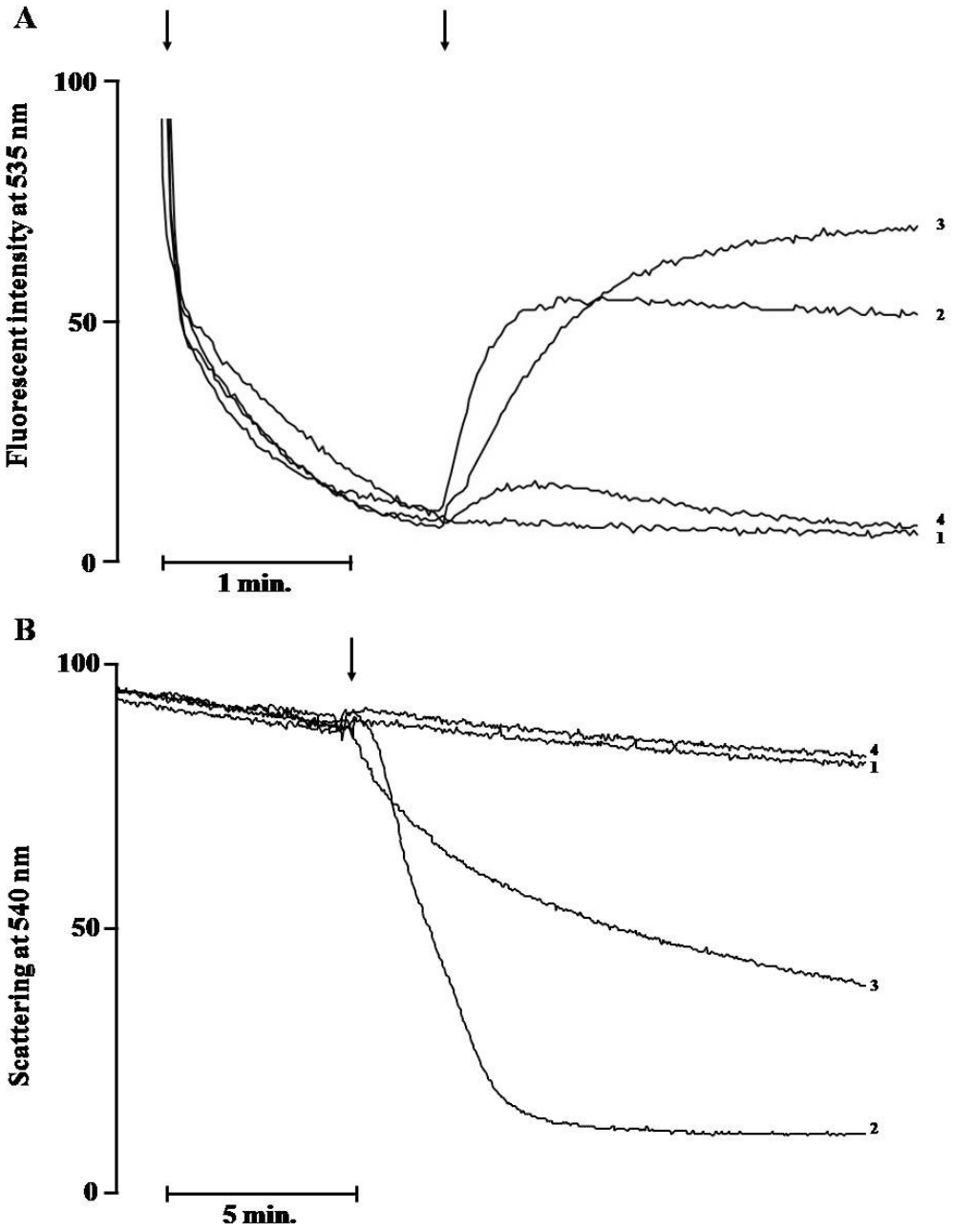


Figure 13: Direct effects of HO-3814 and HO-4223 treatment on isolated rat liver mitochondria. Mitochondrial membrane potential (**A**) was monitored by measuring the fluorescence intensity of the cationic fluorescent dye, Rh123. Isolated rat liver mitochondria, added at the first arrow, took up the dye in a voltage-dependent manner, resulting in fluorescent quenching. At the second arrow, no agent (line 1), 150 µM Ca^{2+} (line 2), 40 µM HO-3814 (line 3) or 40 µM HO-4223 (line 4) was added. A representative plot of three independent experiments running in parallel is presented.
Mitochondrial swelling (**B**) was demonstrated by observing light scattering at 540 nm in isolated rat liver mitochondria. At the arrow, no agent (line 1), 150 µM Ca^{2+} (line 2), 40 µM HO-3814 (line 3) or 40 µM HO-4223 (line 4) was added. A representative plot of three independent experiments running in parallel is presented.

6.2.7. Effect of siRNA Cyclophilin D silencing on HO-3814 induced cell death and mitochondrial depolarization

To confirm that HO-3814 induces cyclophilin D independent mitochondrial permeabilization, we suppressed expression of cyclophilin D using siRNA. PANC-1 cells were mock-transfected or transfected with a construct expressing an siRNA sequence designed against cyclophilin D mRNA. Silencing efficiency was assessed by immuno-blotting with anti-cyclophilin D antibodies (Figure 14A). PANC-1 cells were treated with 1, 5, or 10 μM HO-3814 or HO-4223 for 24 h, and cell viability was tested using the MTT^{+} assay. We found that the viability of cyclophilin D silenced PANC-1 cells did not differ from empty vector transfected cells (Figure 14B) upon exposure to HO-3814. Thus, HO-3814 induced cell death to approximately the same extent in both of these cell backgrounds. In contrast, HO-4223 did not have any effect on cell viability (Figure 14B).

To investigate the effect of cyclophilin D siRNA suppression on mitochondrial membrane potential, cells were exposed to 5 μM HO-3814 for 3h. Treated cells were then stained with JC-1; a specific mitochondrial membrane potential-dependent fluorescent dye, and images were acquired using the blue and green filters of a fluorescent microscope. In intact mitochondria, JC-1 aggregates emit both red and green fluorescence. Upon depolarization, the red fluorescent component is lost, and only the green fluorescent component of JC-1 monomers remains. In addition, a complete loss of fluorescence occurs following collapse of $\Delta\psi$. Merged images of both untreated and mock transfected cells with intact mitochondria were rich in red and green components, with resulting images appearing yellow (Figure 14C, upper left panel). However, in a concentration-dependent manner (not shown), HO-3814 treatment induced a dramatic decrease in the red fluorescence component, while the green fluorescence component in the cytoplasm increased (Figure 14C, upper right panel), consistent with mitochondrial depolarization. Cyclophilin D siRNA silencing did not have any effect on HO-3814 induced mitochondrial depolarization (Figure 14C, lower right panel), confirming our results for isolated mitochondria.

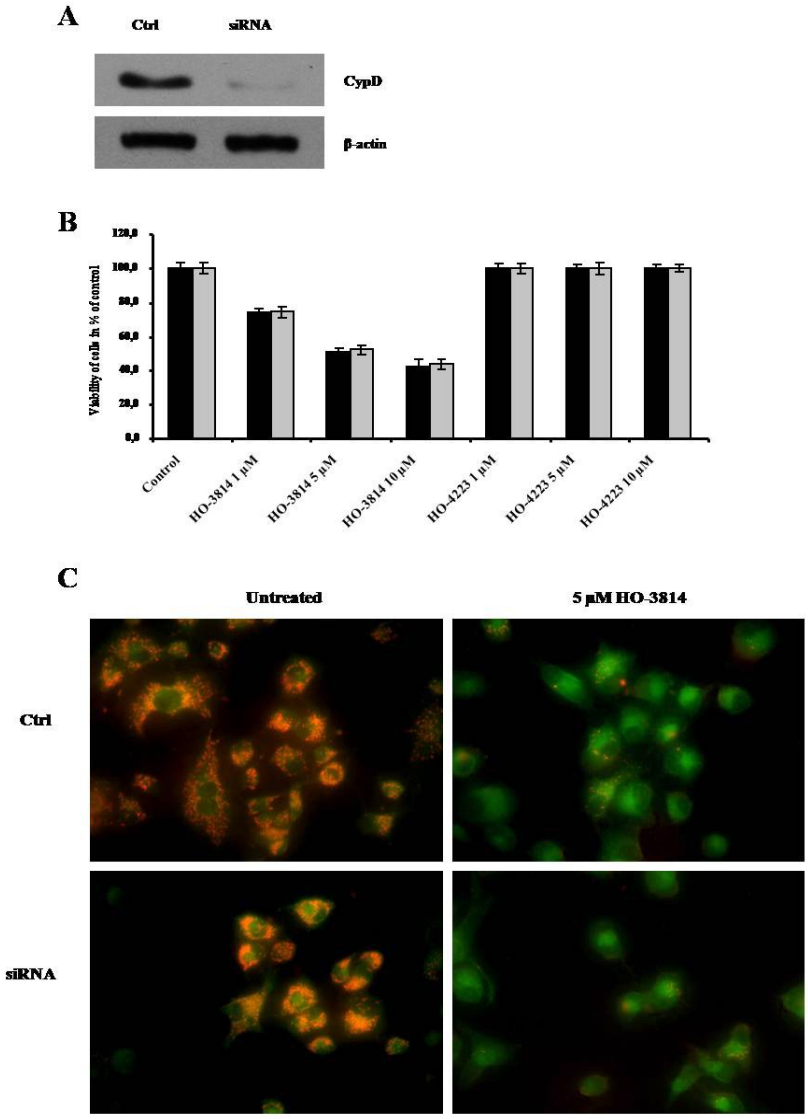


Figure 14: Effect of siRNA Cyclophilin D silencing on HO-3814 induced cell death and mitochondrial membrane depolarisation in PANC-1 cells. Expression of Cyclophilin D was assessed by immunoblot analysis (**A**) with an anti-Cyclophilin D antibody in mock-transfected (Ctrl) and Cyclophilin D silenced (siRNA) cells. β-actin was used as a loading control. Mock-transfected (black bars) and Cyclophilin D silenced (gray bars) cells were exposed to 1, 5, or 10 µM HO-3814 or HO-4223 for 24 h. Cell viabilities (**B**) were detected by MTT assay and are expressed as % of untreated mock-transfected cells; results are the mean ± S.E.M. of eight independent experiments.
Mock-transfected (Ctrl) and Cyclophilin D silenced (siRNA) cells were treated with 5 µM HO-3814 for 3h or not, and then stained with 1µM of JC-1, a membrane potential-sensitive fluorescent dye. After 15 min dye loading, green and red fluorescence images of the same field were acquired using a fluorescent microscope equipped with a digital camera. Representative merged images (**C**) of three independent experiments are presented.

6.2.8. Effect of BCL-2 overexpression on HO-3814 induced mitochondrial depolarization and cell death

All previous data presented thus far suggest that cell death induced by the hydrophobic mitochondria-directed SOD-mimetic, HO-3814, involves permeabilization of mitochondrial membrane systems. In order to verify this notion, we tried to antagonize HO-3814 induced cell death and mitochondrial membrane depolarization by Bcl-2 overexpression. PANC-1 cells were transfected with empty pcDNA3.1 vector, or a construct containing Bcl-2 cDNA. Transfection efficiency was assessed by immuno-blotting with anti-Bcl-2 antibodies (Figure 15A). Cells were treated with 1, 5, or 10 μM HO-3814 or HO-4223 for 24 h, and cell viability was assessed using the MTT assay. Overexpression of Bcl-2 decreased HO-3814 induced cell death at all concentrations tested (Figure 15B) indicating that the effect of HO-3814 observed was at least partially mediated by permeabilizing mitochondrial membrane systems. As before, HO-4223 did not have any effect on cell viability (Figure 15B).

Next we investigated the effect of Bcl-2 overexpression on HO-3814 induced mitochondrial depolarization, and found that Bcl-2 overexpressing cells were more resistant to HO-3814 treatment, as demonstrated by preservation of the red fluorescence component of JC-1 (Figure 15C, lower right panel).

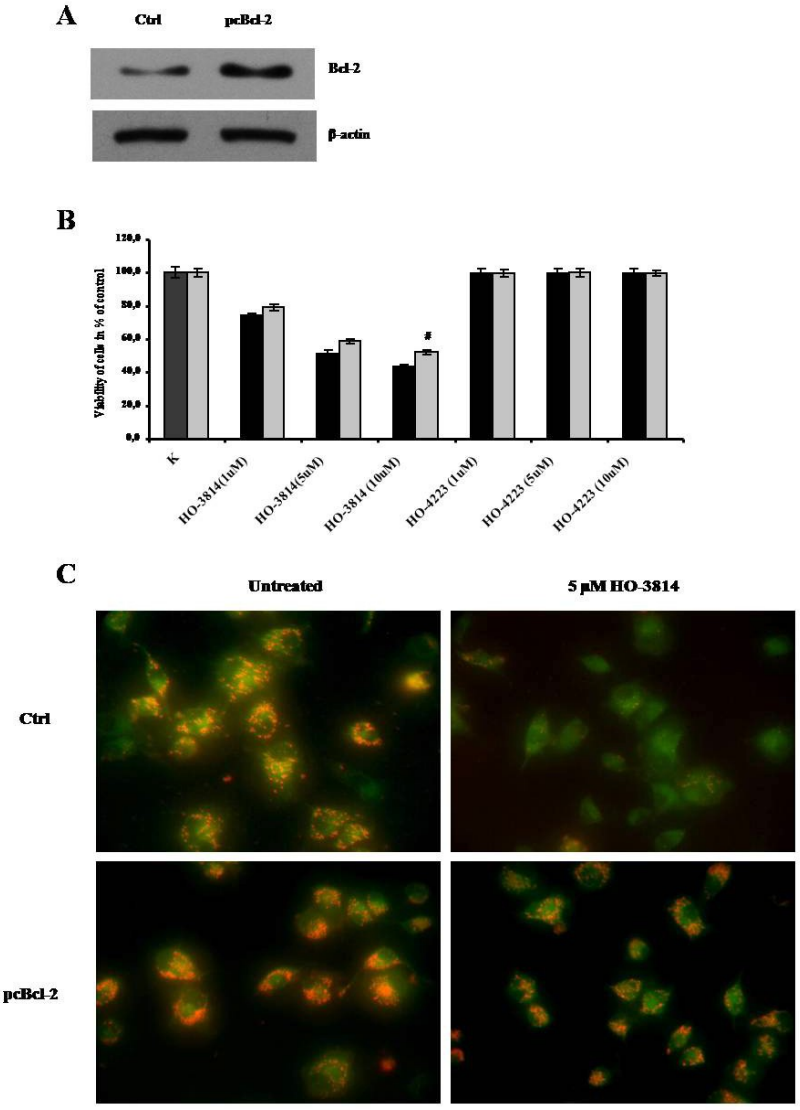


Figure 15: Effect of Bcl-2 overexpression on HO-3814 induced cell death and mitochondrial membrane depolarisation in PANC-1 cells. Expression of Bcl-2 was assessed by immunoblot analysis (**A**) using an anti-Bcl-2 antibody in mock-transfected (Ctrl) and Bcl-2 overexpressing (pCBcl-2) cells. β-actin was used as a loading control. Mock-transfected (black bars) and Bcl-2 overexpressing (gray bars) cells were exposed to 1, 5, or 10 µM HO-3814 or HO-4223 for 24 h. Cell viabilities (**B**) were detected by MTT assay and are expressed as % of untreated, mock-transfected cells; results are the mean ± S.E.M. of eight independent experiments. $^{\#}P<0.05$ compared to identically treated mock-transfected cells.
Mock-transfected (Ctrl) and Bcl-2 overexpressing (pCBcl-2) cells were treated or not with 5 µM HO-3814 for 3h, and then stained with 1µM of JC-1, a membrane potential-sensitive fluorescent dye. After 15 min dye loading, green and red fluorescence images of the same field were acquired using a fluorescent microscope equipped with a digital camera. Representative merged images (**C**) of three independent experiments are presented.

7. Discussion

In our first study we synthesized and studied a series of 4-carboxamidobenzimidazole PARP-1 inhibitors carrying nitroxides and their precursors in the 2-position of the benzimidazole ring. In structure-activity studies we found that five- or six-membered rings without substituents on the nitroxide ring and without spacer between benzimidazole and nitroxide moieties were the most efficient PARP-1 inhibitors (data not shown). The results of PARP inhibition and antioxidant studies did not correlate because the cell death inhibition is based not only on the PARP enzyme inhibition but probably on the ROS scavenging activity as well. Compound **4h** was found to be a potent PARP inhibitor (IC50 = 14 nM) with antiapoptotic (IC50 = 98 nM) activity and an acceptable therapy index LD50 > (500 mg/kg). The structure of **4h** is a six-membered, sterically hindered amine connected directly to the 2-position of 4-carboxamidobenzimidazole and lacking substituents on the cyclic amino moiety. On a rat model we evaluated the metabolism of compound **4h** and found that during its oxidative metabolism a nitroxide is formed that is partially reduced to hydroxylamine. It is interesting to note that this structure is very similar to one that was synthesized independently by an American research group in the 4-carboxamidobenzimidazole series, namely, 2-(1-propynylpiperidin-4-yl)-1*H*-benzimidazole-4-carboxamide (ABT-472) (Pennign et al., 2008). Further biological studies of compound **4h** are currently underway.

In our second study we showed that changing the hydrophobicity of mitochondria-directed SOD-mimetic nitroxides, by attaching a bulky apolar side-chain, reversed their cytoprotective effects, resulting in compounds which induce cell death even at micromolar concentrations. Previously, a number of molecules have been targeted to mitochondria by coupling with a triphenylphosphonium-group, including: lipoic acid (Brown et al., 2007), SOD mimetics (Dhanasekaran et al., 2005), tocopherol (Smith et al., 1999), spin-trap (Murphy et al., 2003), quercetin (Biasutto et al., 2010) and peroxidase mimetic-ebselen (Filipovska et al., 2005). In all of these studies, mitochondria-targeted antioxidants were more effective, even at lower concentrations, then their unsubstituted counterparts, indicating an important advantage and therapeutic potential for such molecules (Hoye et al., 2008).

We tested two apolar, mitochondria-directed SOD-mimetics (HO-3814 and Mito-CP) and their respective unsubstituted counterparts (HO-4223 and HO-435) on a PANC-1 human pancreatic ductal tumor cell line that is noted for its resistance to various drugs used in the

clinical cancer therapeutics (Huanwen et. al., 2009). We observed that at a concentration of 5 μM, both apolar compounds decreased the viability of PANC-1 cells by approximately 50 %, whereas their unsubstituted counterparts did not cause any effect (Figure 7C). To check whether this cytotoxic effect by the apolar mitochondria-directed SOD-mimetics was restricted to the PANC-1 cell line, we tested the effect of HO-3814 at 1, 5 and 10 μM concentrations on T24/83 and HeLa cell lines. Although the sensitivities of these cell lines toward HO-3814 were different, the highest concentration used could induce death in 50-60% of the cells during a 24 h incubation period (Figure 8), indicating that the cytotoxic effect of HO-3814 could be the result of a general, rather than a cell-specific mechanism.

We then compared the cytotoxic effect of HO-3814 with cisplatin, a widely used anti-cancer agent in these cancer cell lines. We applied cisplatin at 5, 15 and 30 μM concentrations, since these concentrations caused 40-95 % death in HeLa cells, the most cisplatin sensitive cell line. While HeLa cells were observed to be more resistant to HO-3814 than cisplatin, T24/83 and PANC-1 cells were much more sensitive to HO-3814 (Figure 9). Because gemcitabine (rather than cisplatin) is the first line drug in pancreatic tumor therapy (Huanwen et al., 2009), we tested the sensitivity of PANC-1 cells toward gemcitabine using the same experimental setup used to compare HO-3814 and cisplatin. We found that gemcitabine, even at the highest concentration used, caused only an approximately 15 % decrease in the viability of PANC-1 cells under our experimental conditions (Figure 10), indicating that HO-3814 was much more effective in inducing cell death in PANC-1 cells than either of the anti-cancer drugs tested. Using mass spectrometric detection after cellular subfractionation (Bognar et al., 2006), we demonstrated that HO-3814 was enriched in the mitochondria (Figure 7B). Recently, it was shown that because of the differences in mitochondrial membrane potential in tumor versus normal cells, radioactive triphenylphosphonium cations (Yang et al, 2008) can be used to monitor tumors in mouse xenografts. Specifically, triphenylphosphonium cations can be preferentially enriched in tumor tissues (Krause et al., 1994; Madar et al., 2006). Therefore, the increased sensitivity of PANC-1 cells to HO-3814 versus gemcitabine and cisplatin suggests that apolar mitochondria-directed SOD mimetics may have therapeutic potential in the management of pancreatic tumors.

We identified the type of cell death caused by HO-3814 as predominantly necrotic, using a fluorescent microscopic assay based on propidium iodide and fluorescent annexin V staining (Figure 11). Furthermore, inhibition of the effector caspase, caspase-3, did not affect HO-3814 induced cell death (Figure 12), in agreement with the previous finding. To identify

mechanisms involved in HO-3814 induced cell death, we used various inhibitors to counteract its effect. We found that inhibitors of MAP kinase, or PI-3-kinase-Akt pathways did not have any significant effect on HO-3814 induced cell death, indicating that these kinases were, most likely, not involved in the mechanisms of HO-3814 mediated death process (Figure 12). Because of the cyclic nitroxide group responsible for its SOD-mimetic activity, HO-3814 exhibits substantial antioxidant activity (data not shown). However, the finding that neither the antioxidants N-acetyl-cysteine or quercetin, nor inhibition of PARP, influenced HO-3814 induced cell death (Figure 12) indicates that significant involvement of free radical mediated cell death mechanisms are unlikely. Finally, an inhibitor of the mitochondrial permeability transition pore complex was also ineffective (Figure 12).

Because HO-3814 is accumulated in the mitochondria (Figure 7B), we studied its effect on isolated, Percoll-gradient purified mitochondria. In a concentration dependent manner, HO-3814 caused a massive decrease in mitochondrial membrane potential and excessive swelling, whereas treatment with its non-hydrophobic analogue resulted in only a slight transient depolarization and no swelling (Figure 13). At the highest concentration used, both the depolarization and swelling induced by HO-3814 was comparable in extent to that induced by 150 μM Ca^{2+}. However, unlike Ca^{2+}, both effects were insensitive to the permeability transition pore complex inhibitor CSA. These data indicate that HO-3814 directly affects mitochondrial membrane systems by changing their permeability. However, this effect was not mediated through the permeability transition pore complex. Destabilization of mitochondrial membrane systems can result in cell death, possibly caused by a decreased effectiveness of the mitochondrial respiratory chain, leading to decreased energy charge. When applied at higher concentrations, HO-3814 could induce mitochondrial depolarization within minutes of application. Because this effect was concentration dependent, even partial mitochondrial depolarization induced by lower concentrations of HO-3814 could result in cell death on a longer time scale, such as the 24 h incubation period used in our cell viability experiments.

To confirm our findings on isolated mitochondria, we siRNA silenced cyclophilin D, an integral protein of the permeability transition pore complex (Tsujimoto et al., 2006; Tsujimoto and Shimizu, 2007) in a PANC-1 cell line. We then exposed wild type PANC-1 and cyclophilin D silenced cells to HO-3814, determined the viability of the cells and assessed mitochondrial depolarization by fluorescent microscopy after staining the cells with a membrane potential sensitive mitochondrial dye. We found that in both cell lines, HO-3814

induced cell death and mitochondrial depolarization to approximately the same extent (Figure 14).

All 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. Increased mitochondrial permeability is considered to be one of the key events in both apoptotic and necrotic death (Tsujimoto et al., 2006). Permeabilization can affect the outer membrane only, leading to predominantly CSA-insensitive apoptosis (Lemasters et al., 2009). Alternatively, it can affect both membranes, with or without opening of the permeability transition pore, leading mainly to CSA-sensitive or CSA-insensitive necrotic death (Lemasters et al., 2009; Tsujimoto et al., 2006). Because anti-apoptotic members of the B cell lymphoma-2 (Bcl-2) protein family have been shown to stabilize mitochondrial membrane systems, and thereby protect cells against both apoptotic and necrotic death (Galuzzi et al., 2009; Sasi et al., 2009), we attempted to antagonize the effects of HO-3814 by overexpressing Bcl-2 protein in the PANC-1 line. We found that Bcl-2 overexpression increased the viability of HO-3814 treated cells, and reduced mitochondrial depolarization (Figure 15), further supporting the above proposed mechanism for HO-3814 induced cell death.

8. 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. This observation inspired us to study hydroxyl radical scavenging activity of the new PARP inhibitors in a Fenton reaction.

Their antioxidant effect/ hydroxyl radical scavenging ability

On the basis of nitrogen oxidative status, obvious structure-activity relationships cannot be drawn yet; 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 compared the effect of HO-3814 on cell viability in three tumor cell lines with that of cisplatin, a widely used anti-tumor agent. 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 using a fluorescent microscopic assay based on propidium iodide and fluorescent annexin V staining.

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 (Sasi N. et al., 2009). In this respect, hydrophobic mitochondria directed SOD mimetics may have therapeutic potential in the future.

9. Acknowledgements

This work was supported by AOKKA-34039-1/2009 and 34039-23/2009 as well as Hungarian National Research Grants OTKA 68469, K-73738 and K81123.

I would like to thank Prof. Balázs Sümegi and Prof. Ferenc Gallyas for supporting my work with smart advices and thought-provoking ideas and giving me the opportunity to work in the Institute of Biochemistry and Medical Chemistry.

I thank my colleagues Krisztina Kovács, Zita Bognár, Eszter Bognár, Enikő Hocsák and László Mester for their co-operation and for the friendly and convivially atmosphere in our lab.

I thank Helena Halász, Istvánné Pásztor, László Girán and Bertalan Horváth for their excellent technical help.

I also thank Prof. Kálmán Hideg, Prof. Tamás Kálai and Mária Balog, Department of Organic and Pharmacological Chemistry, Faculty of Medicine, University of Pecs for synthesizing and conveying in this study presented compounds.

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11. List of Publications

This work is based on the following articles:

Aliz Szabo, Maria Balog, Laszlo Mark, Gergely Montsko, Zsuzsanna Turi, Ferenc Gallyas Jr., Balazs Sumegi, Tamas Kalai, Kalman Hideg, Krisztina Kovacs. Induction of mitochondrial destabilization and necrotic cell death by apolar mitochondria-directed SOD mimetics. Mitochondrion. 2011 May; 11(3):476-487.

IF: 4,145 (2010)

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Tamas Kalai, Maria Balog, **Aliz Szabo**, Gergely Gulyas, Jozsef Jeko, Balazs Sumegi, Kalman Hideg. New Poly(ADP-ribose) Polymerase-1 Inhibitors with Antioxidant Activity Based on 4-Carboxamidobenzimidazole-2-ylpyrroline and -tetrahydropyridine Nitroxides and Their Precursors. J Med Chem. 2009 Mar 26; 52(6):1619-1629.

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Further publications:

Arpad Szanto, Eva E Hellebrand, Zita Bognar, Zsuzsanna Tucsek, **Aliz Szabo**, Ferenc Gallyas Jr., Balazs Sumegi, Gabor Varbiro. PARP-1 inhibition-induced activation of PI-3-kinase-Akt pathway promotes resistance to taxol. Biochem Pharmacol. 2009 Apr 15; 77(8):1348-1357.

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Arpad Szanto, Zita Bognar, Andras Szigeti, **Aliz Szabo,** Laszlo Farkas, Ferenc Gallyas Jr. Critical role of bad phosphorylation by Akt in cytostatic resistance of human bladder cancer cells. Anticancer Res. 2009 Jan; (1):159-164.

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