Novel compounds targeting the mitochondrial protein VDAC1 inhibit apoptosis and protect against mitochondria dysfunction

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Running title: A new inhibitor of VDAC1 oligomerization and apoptosis

Key words: Apoptosis, bioluminescence resonance energy transfer (BRET), drug development, mitochondria, oligomerization, small molecules, VDAC

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ABSTRACT

Apoptosis is thought to play a critical role in several pathological processes, such as neurodegenerative diseases (i.e., Parkinson's and Alzheimer's diseases) and various cardiovascular diseases. Despite the fact that apoptotic mechanisms are well defined, there is still no substantial therapeutic strategy to stop or even slow this process. Thus, there is an unmet need for therapeutic agents that are able to block or slow apoptosis in neurodegenerative and cardiovascular diseases. The outer mitochondrial membrane protein voltage-dependent anion channel 1 (VDAC1) is a convergence point for a variety of cell survival and death signals, including apoptosis. Recently, we demonstrated that VDAC1 oligomerization is involved in mitochondria-mediated apoptosis. Thus, VDAC1 oligomerization represents a prime target for agents designed to modulate apoptosis. Here, high-throughput compound screening and medicinal chemistry were employed to develop compounds that directly interact with VDAC1 and prevent VDAC1 oligomerization, concomitant with an inhibition of apoptosis as induced by various means and in various cell lines. The compounds protected against apoptosis-associated mitochondria dysfunction, restoring dissipated mitochondria membrane potential, and thus cell energy and metabolism, decreasing ROS production, and preventing detachment of hexokinase bound to mitochondria and disruption of intracellular Ca²⁺ levels. Thus, this study describes novel drug candidates with a defined mechanism of action that involves inhibition of VDAC1 oligomerization, apoptosis and mitochondria dysfunction. The compounds VBIT-3 and VBIT-4 offer a therapeutic strategy for treating different diseases associated with enhanced apoptosis and points to VDAC1 as a promising target for therapeutic intervention.
INTRODUCTION

Mitochondria play crucial roles in cellular energy generation and metabolism, maintenance of the cell redox potential, calcium homeostasis, pH control, and fatty acid oxidation; cell signaling, proliferation, differentiation, aging and death (1). It is, therefore, not surprising that mitochondrial dysfunction is associated with various human diseases (1,2).

Located at the outer mitochondrial membrane (OMM), the voltage-dependent channel VDAC, serves as a mitochondrial gatekeeper. Three VDAC isoforms have been discovered (3) but only for VDAC1 are a whole set of structural and functional information available. VDAC1 controls the metabolic and energy cross-talk between mitochondria and the rest of the cell, mediating the fluxes of ions, nucleotides and other metabolites across the OMM (4-7). VDAC1 is composed of 19 transmembrane β-strands connected by flexible loops to form a β-barrel, along with a 26-residue-long N-terminal region that lies inside the pore (8-10).

Mitochondria also function in apoptosis, mediating the intrinsic pathway (11). This pathway is initiated in response to various stimuli, such as high cytoplasmic Ca²⁺, oxygen radicals, activation of pro-apoptotic Bcl-2 family proteins, UV damage and various drugs (11,12). These stimuli provoke permeabilization of the OMM, allowing the release of pro-apoptotic proteins, such as cytochrome c (Cyto c) and apoptosis inducing factor (AIF), from the intermembrane space (IMS) to the cytosol (13). This leads to the activation of caspases, which cleave targeted proteins, and subsequently, to apoptosis. Defects in the regulation of apoptosis are associated with cancer and neurodegenerative diseases (14), with evasion of apoptosis being a hallmark of cancer (15) and enhancement of apoptosis being seen in neurodegenerative diseases (16,17).

VDAC1 has also been recognized as a key protein in mitochondria-mediated apoptosis, regulating the release of apoptogenic proteins, as well as interacting with anti-apoptotic proteins (5,18-20). Release of Cyto c from mitochondria is considered a key initial step in the apoptotic process, although the precise mechanisms regulating Cyto c release remain unknown. To date, all of the mitochondrial components known to translocate to the cytoplasm following an apoptotic stimulus reside in the IMS. Therefore, only the permeability of the OMM needs to be modified.

Among the many models that have been put forward for the release of apoptotic proteins, some suggest that release involves the formation of a channel large enough to allow the passage of apoptogenic proteins (1,5,6,21-23), while others suggest disruption of OMM integrity (24-26).

Recently, we demonstrated that apoptosis induction leads to the oligomerization of VDAC1 into dimers, trimers, tetramers and higher-order oligomers (23,27-35). We have also demonstrated that VDAC1 oligomerization is a general mechanism common to numerous apoptogens acting via different initiating cascades (30,36,37). Furthermore, apoptosis inhibitors (30,38) and recently identified VDAC1-interacting molecules (e.g. diphenylamine-2-carboxylate (DPC) (39), inhibited VDAC oligomerization.

These results led us to propose a novel model in which VDAC1 exists in a dynamic equilibrium between monomeric and oligomeric states, with apoptosis inducers shifting the equilibrium towards oligomers, forming a large channel that enables Cyto c release, leading to cell death.
Our results thus suggest not only that VDAC1 oligomerization is a molecular focal point in cellular life or death decision processes but that VDAC1 may also offer a prime target for therapeutic agents designed to modulate apoptosis. As such, targeting the oligomeric status of VDAC1, and hence apoptosis, offers a strategy for combating cancers and neurodegenerative diseases. Currently, there are no known specific inhibitors of VDAC1-mediated apoptosis. We have, however, developed new molecules that interact with VDAC1, inhibiting VDAC1 oligomerization and preventing apoptosis at pharmacologically-relevant concentrations. These novel drug candidates, with defined modes of action, can serve to treat diseases associated with enhanced apoptosis and point to VDAC1 as a promising target for therapeutic intervention. Indeed, several studies have identified pharmacological agents that target VDAC1 so as to induce cancer cell death or protect against apoptosis (6). However, none of these molecules are specific to VDAC1 and could instead affect the cell via different biochemical pathways.

To identify a molecule with higher specificity towards VDAC, we screened a library of 1,468 drug-like compounds in living mammalian cells using a bioluminescence resonance energy transfer (BRET2)-based VDAC1 oligomerization assay. Several hit compounds were identified as inhibitors of VDAC1 oligomerization. Using medicinal chemistry approaches, these active compounds were structurally optimized to develop two novel active molecules, VBIT-3 and VBIT-4. These novel VDAC1 inhibitors represent drug candidates targeting apoptosis in a variety of diseases.

RESULTS

This study addressed the need for inhibitors of apoptosis that act at an early stage of the apoptotic pathway, inhibiting the release of Cyto c from mitochondria. Our approach considered VDAC1-based compounds able to inhibit VDAC1 oligomerization, thereby protecting cells against apoptotic cell death. Validation of the BRET-2 assay used for high-throughput screening (HTS)

To directly monitor the oligomeric state of VDAC1 molecules in the native membrane, we used BRET2 technology (30). In BRET2 technology, VDAC1 is tagged with either Renilla luciferase (RLuc) as donor or a variant of GFP (GFP2) as acceptor and expressed in T-REx cells stably expressing shRNA-hVDAC1 and containing low levels of endogenous hVDAC1. Energy transfer between the two only occurs when the donor and acceptor are in spatial proximity to each other (<10 nm apart), making this an ideal technique for monitoring protein-protein interactions in biological systems (40). The detection of a BRET2 signal in this case corresponds to VDAC1 oligomerization, while attenuation of an apoptosis-enhanced BRET2 signal indicates an inhibition of VDAC1 oligomerization (Fig. 1A).

To validate the assay, selenite, a known inducer of apoptosis and VDAC1 oligomerization (30), was used to enhance VDAC1 oligomerization. To inhibit the selenite-induced BRET2 signal, 4,4-diisothiocyanostilbene-trans-2,2-disulfonic acid (DNDS), an inhibitor of VDAC1 channel conductance and apoptosis (38,39), was used (Fig. 1B). Chemical cross-linking using the cell-permeable cross-linker ethylene-glycolbis (succinimidylsuccinate) (EGS) and Western blot analysis also served to demonstrate any enhancement or inhibition of VDAC1 oligomerization (Fig. 1C). The results show that selenite provoked oligomerization, as reflected in the enhanced BRET2 signal (Fig. 1B) and in the appearance of protein bands.
corresponding to VDAC1 dimers (72 kDa) and multimers (Fig. 1C). The obtained results suggest that VDAC1 oligomerization is a dynamic process and can be activated or inhibited and thus can be used to search for compounds modulating this process. Here, we focused on inhibition of VDAC1 oligomerization, apoptosis and protection against dysfunction associated with apoptosis induction.

HTS for inhibitors of VDAC1 oligomerization

To search for active molecules that inhibit apoptosis via blocking VDAC1 oligomerization, we screened a drug-like compounds library (1,468 molecules) provided by the National Cancer Institute (NCI) (see Materials and Methods) using BRET2 technology (19,30). The compound library was robotically screened for inhibitors of BRET2 signaling as induced by STS, selenite or As$_2$O$_3$ in VDAC1-Luc- and VDAC1-GFP2-expressing cells (Table 1). Seventy-one compounds caused a 40% decrease in BRET2 signaling induced by the apoptotic agents. The STS-enhanced BRET2 signal was reduced by 22 molecules, while the BRET2 signal generated upon As$_2$O$_3$- and selenite-induced apoptosis was decreased by 69 and 54 molecules, respectively. Twelve compounds inhibited the BRET2 signal induced by all three inducers (Fig. 1D,E). Thus, the hit rate from the primary screen was 0.8% (i.e., 12 out of 1,468 compounds).

Structure-based analysis of hits from the first round of screening for oligomerization inhibitors

The 12 active compounds identified were divided in 3 structurally-related groups (Fig. 2A) and, based on these structures, a 3D pharmacophore model was generated from the lowest energy conformation of all 12 active compounds (Fig. 2B). The resulting structure consisted of aromatic ring areas, a hydrophobic region, and hydrogen bond donor and acceptor points. Next, based on this proposed pharmacophore model, we identified 34 commercially available compounds as potential inhibitors of VDAC1 oligomerization that were subsequently tested for such activity (Fig. 3).

Second round of compound screening for inhibitors of VDAC1 oligomerization

The ability of the 34 compounds, selected on the basis of the pharmacophore model, to inhibit VDAC1 oligomerization as induced by selenite or cisplatin in HeLa cells, was analyzed by following chemical cross-linking with EGS and Western blot analysis using anti-VDAC1 antibodies. The inhibition of dimeric VDAC1 formation by the compounds is presented (Fig. 3A).

Of the compounds tested, four showed high inhibition of VDAC1 oligomerization as induced by selenite or cisplatin (Fig. 3A, boxed in red) and shared several structural aspects. For example, in all four compounds, substituted or non-substituted benzyl rings were bound to a nitrogen-containing ring either directly (DIV03322, DIV02963, DIV00996) or via a thiourea linker (DIV00284) (Fig. 3B). These structural similarities served as the basis for designing compounds for the third round of screening.

Third round of screening for inhibitors of VDAC1 oligomerization

A third round of screening was carried out with 13 compounds designed based on the results obtained in the second round screen. These compounds were commercially available. The active molecules in this screen were identified in terms of their ability to inhibit VDAC1 oligomerization as induced by selenite or cisplatin. The level of oligomerization was estimated by the chemical cross-linking method (Fig. 3C-E). One compound, AKOS-022075291 (AKOS-022), was able to almost completely prevent selenite- and cisplatin-induced VDAC1 oligomerization at low micromolar concentrations
Thus, this molecule was further studied with respect to its ability to interact with VDAC1 and inhibit apoptosis.

**AKOS-022 prevents VDAC1 oligomerization and apoptosis**

AKOS-22 was found to inhibit VDAC1 oligomerization and apoptosis, as analyzed by annexin-V/propidium iodide staining and flow cytometry, regardless if induced by selenite (Fig. 4A,B,C) or cisplatin (Fig. 4D,B,E). AKOS-22 inhibited both VDAC1 oligomerization and apoptosis in a concentration-dependent manner, with 50% inhibition of both apoptosis and VDAC1 oligomerization (Fig. 4C,E) obtained at a similar concentration (7.5 µM). Moreover, quantitative analysis showed a linear relationship between the decrease in the amounts of VDAC1 dimers and the extent of apoptotic cell death (Fig. 4F), as analyzed at the identical AKOS-022 concentration, revealing the tight relationship between these processes.

**Development of new VDAC1-interacting molecules inhibiting oligomerization, HK detachment, cytochrome c release and apoptosis**

AKOS-022 provided the structural basis for the design of several novel compounds. Two such molecules, VBIT-3 and VBIT-4, were successfully synthesized by ChemPartner (Supplemental Chemical Methods). Two main changes were made to the core of AKOS-022 to obtain VBIT-3 and VBIT-4. First, the piperazine ring was conjugated with an aniline moiety in both molecules instead of a piperidine in AKOS-022 and secondly, the central part of the AKOS-022 core corresponding to a pyrrolidine-2,5-dione ring was linearized to obtain the butanamide linear moiety found in VBIT-4.

The parent molecule (AKOS-022) and its two derivatives (Fig. 5A) were tested for their ability to directly interact with VDAC1 and inhibit VDAC1 oligomerization and apoptosis as induced by a known pro-apoptotic agent (Figs. 5 and 6). The direct interaction between purified VDAC1 and the tested compounds was measured by assessing VDAC1 channel conductance, following its reconstitution into a planar lipid bilayer (PLB). AKOS-022, VBIT-3 and VBIT-4 interacted with purified VDAC1 and reduced its channel conductance, especially at voltages between -40 and +40 mV (Fig. 5B,C). To obtain a quantitative analysis of the interaction of the compounds with VDAC1 and derive dissociation constants, a microscale thermophoresis (MST) interaction assay was performed. The fraction of VDAC1 bound to the compound was analyzed as a function of their concentration (Fig. 5D). The dissociation values were derived from the curves showing that AKOS-022 and VBIT-4 interacted with VDAC1 with similar affinity, but two-fold higher than that of VBIT-3 (Fig. 5D,E).

The interaction of VBIT-4 with recombinant purified VDAC1, VDAC2 and VDAC3 was analyzed using the MST method (Fig. 5F). VBIT-4 bound to the three recombinant isoforms with a similar binding affinity, although 3-fold lower than that of VDAC1 purified from rat liver mitochondria.

The effects of AKOS-022 and its two synthetic derivatives, VBIT-3 and VBIT-4, on VDAC1 oligomerization, Cyto c release from mitochondria and apoptosis in HEK-293 cells, as induced by selenite, were analyzed (Fig. 6A-E). The results indicated that VBIT-4 was more potent and more effective than VBIT-3 and AKOS-022 in inhibiting all three apoptosis-related activities. Importantly, the IC_{50} values of VBIT-4 in all three assays were in the same range of concentrations (about 1.8-2.9 µM) (Fig. 6F).

VBIT-4 and AKOS-022 also inhibited apoptosis as induced by STS (Fig. 6G). However, when apoptosis was induced by As_{2}O_{3}, addition of...
VBIT-4 or AKOS-022 resulted in slightly enhanced apoptosis rather than inhibition (Fig. 6H). The reasons for this effect is not clear (see Discussion).

Finally, we also analyzed the effect of VBIT-4 on mitochondria-bound HK (Fig. 6I). Apoptosis induction by selenite resulted in the detachment of mitochondria-bound HK that was subsequently detected in the supernatant of digitonin-treated cells. This selenite-induced HK detachment was strongly inhibited in the presence of VBIT-4. The results also show that in the absence of apoptosis induction, 80% of the HK pool is bound to the mitochondria.

Separation and activity evaluation of two VBIT-4 enantiomers

VBIT-4 contains a chiral carbon, allowing for two enantiomers (Fig. 7A). VBIT-4 was synthesized as a racemate and racemic VBIT-4 was used in all of the experiments described above. Given the possible lack of activity of one of the enantiomers, the two VBIT-4 enantiomers were separated by chiral column chromatography and their effects on VDAC1 oligomerization and apoptosis were investigated in HEK-293 and HeLa cells (Fig. 7B,C). Both enantiomers inhibited VDAC1 oligomerization and apoptosis as induced by selenite in an identical manner.

To demonstrate that VBIT-4 and AKOS-022 inhibited VDAC1 oligomerization and apoptosis regardless of the cell type used, we also tested the compound-mediated inhibition of cisplatin-induced apoptosis in the neuroblastoma cell line SH-SY5Y, in addition to HEK-293 and HeLa cells (Fig. 8A,B). Both compounds inhibited cisplatin-induced VDAC1 oligomerization and apoptosis in a similar manner.

VBIT-4-mediated inhibition of VDAC1 oligomerization in Bax/Bak-lacking cells

As the pro-apoptotic Bax and Bak proteins are considered central to apoptosis, we asked whether the protection against apoptosis conferred by the molecules tested was VDAC1-but not Bax/Bak-dependent (Fig. 8C-E). The results showed that AKOS-022 and VBIT-4 were effective in inhibiting cisplatin-induced VDAC1 oligomerization and Cyto c release in Bax+/Bak- mouse embryonic fibroblasts (MEFs), with VBIT-4 being the most effective inhibitor.

Compounds providing protection against mitochondrial depolarization, ROS production and [Ca^{2+}]_i increase, as induced by apoptosis induction

Apoptosis induction was shown to disrupt cellular Ca^{2+} homeostasis and energy production (41). Indeed, many anti-cancer drugs and other cytotoxic agents, such as thapsigargin, staurosporine, As_2O_3, and selenite, induce apoptotic cell death, as well as disrupt cell Ca^{2+} homeostasis (36,37). Thus, we investigated a possible protective effect of VBIT-4 and AKOS-022 on the increased intracellular [Ca^{2+}]_i elicited by selenite in HEK-293 cells. As expected, selenite increased [Ca^{2+}]_i, as monitored using Fluo-4 and FACS analysis (Fig. 9A). [Ca^{2+}]_i was, however, maintained at the basal level when the cells were pre-incubated with AKOS-022 or VBIT-4.

Apoptosis induction may affect the mitochondria membrane potential (ΔΨm) directly or via increased [Ca^{2+}]_i which led to an increase in mitochondrial Ca^{2+}, a process expected to lead to dissipation of ΔΨm (42). We thus tested the effect of VBIT-4 and AKOS-022 on ΔΨm. Both compounds prevented the decrease in ΔΨm as measured using tetramethylrhodamine methylester (TMRM) (Fig. 9B). The compounds were further tested for their ability to inhibit overall cellular reactive oxidative species (ROS) production, as analyzed by DCF fluorescence, and in mitochondria as
measured by MitoSOX Red, a mitochondrial superoxide indicator (Fig. 9C,D). The increases in both cellular and mitochondrial ROS levels as induced by selenite were completely prevented by VBIT-4 and AKOS-022.

These results showed that the compounds inhibited not only VDAC1 oligomerization, Cyto c release and apoptosis but also protected against the mitochondrial dysfunction associated with apoptosis induction and compromised cell energy production.

**DISCUSSION**

Accumulating evidence points to VDAC1 as functioning in mitochondria-mediated apoptosis-involving in the release of apoptogenic proteins, such as Cyto c, leading to caspase activation (4,5,43,44). Based on our previous studies (19,23,30,36,37,45), we proposed that Cyto c is transported across the OMM via a large flexible pore formed within oligomeric VDAC1 upon apoptosis induction. Thus, shifting the equilibrium of oligomeric toward monomeric VDAC1, thus preventing Cyto c release, is an effective approach for blocking apoptosis at an early stage.

Despite the fact that apoptotic mechanisms are well defined, there is still no substantial therapeutic strategy to stop or even slow this process. Thus, there is an unmet need for therapeutic agents able to block or slow apoptosis in neurodegenerative and cardiovascular diseases. Currently, the majority of known apoptosis inhibitors directly target end steps in the apoptosis pathway, such as blocking caspase activity (16,46,47). At the same time, known VDAC1 inhibitors are non-specific and were effective in inhibiting apoptosis at comparatively high concentrations (19,30,38). Thus, we searched for a more effective VDAC-specific apoptosis inhibitor. Specifically, we sought anti-apoptotic drugs targeting VDAC1 to prevent its oligomerization, an early and critical step in the progression of apoptosis. These molecules would be potential candidates for treating neurodegenerative disorders (16,17) and various cardiovascular diseases, where enhanced apoptosis also occurs (48-50).

A BRET-based method was used to follow VDAC1 oligomerization in living cells as part of a screen of a library of small compounds, with the aim of identifying possible potent and effective inhibitors of VDAC1 oligomerization and thus, apoptosis. Following three cycles of structure-activity relationship studies, a commercially available molecule, AKOS-022, was identified as a compound interacting with VDAC1 and inhibiting both VDAC1 oligomerization and apoptosis (Fig. 10). AKOS-022 inhibited VDAC1 oligomerization and apoptosis at a pharmacological range of concentrations (Fig. 4). Furthermore, AKOS-022 interacts directly with purified VDAC1, as revealed by MST and its effect on bilayer-reconstituted channel conductance (Fig. 5).

Based on the structure of AKOS-022, we designed several new compounds of which two, VBIT-3 and VBIT-4, directly interacted with VDAC1 and strongly inhibited VDAC1 oligomerization and apoptosis as induced by various means in several cell types (Figs. 5-8). VBIT-4 was more potent and more effective than VBIT-3 and AKOS-022 in inhibiting VDAC1 oligomerization, Cyto c release and apoptosis as induced by cisplatin, STS and selenite. Interestingly, VBIT-4 enhanced As2O3-induced rather than inhibited apoptosis. This could be associated with one or more defined molecular targets of As2O3 and molecular mechanisms of action that are not affected or may be enhanced by VBIT-4 inhibition of VDAC1 oligomerization.
These including induction of ROS formation, down-regulation of Bcl-2 expression (51), direct binding to promyelocytic leukemia (PML), a tumor suppressor protein, and induction of its oligomerization (52), interaction with sulphhydryl groups (53), induction of VDAC homo-dimerization, which can be prevented by Bcl-xL (54), and VDAC1 oligomers (30), and the formation of Bax and VDAC hetero- and homo-oligomers (55).

The linear relationship between inhibition of VDAC1 oligomerization and inhibition of apoptosis by AKOS-022 (Figs. 4 and 6) and VBIT-4 (Fig. 6) offers further correlation for the involvement of VDAC1 oligomerization in the induction of apoptosis.

VBIT-4 interacted with the three recombinant VDAC isoforms with a similar affinity of 53 ± 3 μM. This is about 3-fold lower than that of VDAC1 purified from rat liver mitochondria. The lower affinity of the recombinant proteins may result from the unfolding and refolding process resulting in modified conformations. The three VDAC isoforms share high homology and structural similarities (3). This can explain their capacity to bind VBIT-4 with similar affinity. In addition, the three recombinant isoforms share a short tail of histidines that was shown to be of no importance for VDAC activity in reconstituted membranes (56) but can influence the binding of VBIT-4 to the proteins.

VBIT-4 had no effect on cell growth and viability. Moreover, mice that were exposed to the compound for 4 months showed no toxic signs, as reflected by weight, behavior, organ histo-chemistry and more (results not shown). It thus seems that VBIT-4 interaction with any of the VDAC isoforms has no effect on cell functional homeostasis. As VDAC1 is the major isoform in most cell types and that VBIT-4 inhibits VDAC1 oligomerization while no VDAC2 or VDAC3 oligomerization has been reported, it is reasonable to assume that the anti-apoptotic effect of VBIT is mainly mediated via its interaction with VDAC1.

In addition to inhibition of apoptosis VBIT-4, as well as AKOS-022, prevented the elevation of [Ca\(^{2+}\)i] associated with apoptosis induction, and thus Ca\(^{2+}\) accumulation by the mitochondria, thereby preventing the collapse of the associated ΔΨm and ROS production (57) (Fig. 9) and apoptosis-associated mitochondria dysfunction.

Interestingly, VBIT-4, the more active and potent compound, has only one structural difference that distinguishes it from VBIT-3, namely a linear 4-hydroxybutanamide moiety in place of a pyrrolidine-2,5-dione rigid ring. This change makes VBIT-4 more flexible (due to the presence of a linear moiety versus a cycle group) and hydrophilic (alcohol versus amide). The increased flexibility of VBIT-4 is important when considering its interfering with protein-protein interactions associated with VDAC1 oligomerization. Moreover, VBIT-4 is a chiral molecule, yet the two enantiomers showed identical activity in inhibiting VDAC1 oligomerization (Fig. 7). This suggests that flexible VBIT-4 interferes with the interactions between VDAC1 monomers in areas that are widely accessible for both the VBIT-4 R and S conformations.

The VDAC1-interacting molecule VBIT-4 can be used to further explore the function of VDAC1 in controlling metabolism, energy production, transport of cholesterol and apoptosis. Moreover, VBIT-4, as an apoptosis inhibitor, can be used for therapeutic purposes in apoptosis-associated disorders, such as neurodegenerative and cardiovascular diseases. Indeed, many dying neurons in brains of patients with neurodegenerative diseases appear to display morphological features of apoptosis,
such as chromatin condensation, DNA fragmentation, and activation of caspases (58). There is also evidence suggesting that caspases assume a role in Alzheimer’s and Parkinson’s diseases (59). Thus, the activated cell death that occurs in neurologic diseases makes inhibition of apoptosis with reagents such as VBIT-4 an attractive therapeutic approach.

In other types of human disease, such as heart failure, myocardial infarction, and cardiac ischemia/reperfusion injury, apoptosis, necrosis, and autophagy of cardiac myocytes were detected (48-50). Activation of the mitochondrial apoptotic pathway has also been implicated in ischemia/reperfusion injury, involving the release of Cyto c from mitochondria, followed by activation of caspase-9 in the myocardium (60). In addition, atrial fibrillation (AF), the most common cardiac arrhythmia and associated with high morbidity and mortality rate in adults, was also linked to apoptotic processes in cardiomyocytes (61). Apoptosis is a contributing factor in the initiation and progression of fibrosis, which is a main factor in the occurrence, development and poor prognosis of AF patients (62,63). Thus, VBIT-4 can be used to inhibit apoptosis in cardiac myocytes as induced under pathological conditions.

The inhibition of mitochondrial-bound HK detachment by VBIT-4 (Fig. 6H) is extremely important. In addition to the metabolic function assigned to mitochondrial-bound HK (HK-I and HK-II), namely the coupling of cytosolic glycolysis to mitochondrial oxidative phosphorylation it is now clear that VDAC1-bound HK also prevents the release of pro-apoptotic factors, and subsequent apoptosis accompanied with detachment of HK (31,32,64-71). Indeed, several pro-apoptotic agents have been shown to induce VDAC1-HK complex dissociation (7,72-74). The results presented here show that VBIT-4 inhibited HK detachment, as induced by apoptosis induction. This finding supports the concept of HK detachment being a prerequisite for apoptosis induction. VBIT-4 inhibition of HK detachment suggests that such detachment is associated with VDAC1 oligomerization, with VBIT-4 inhibiting oligomerization and preventing HK detachment.

In this respect, several pathological conditions, such as Parkinson’s disease (75), mood and psychotic disorders (76), AD (77), and schizophrenia (78) were related to HK detachment from the mitochondria. Thus VBIT-4, may be a valuable in treating these pathological conditions.

Finally, in this respect, VDAC1 over-expression was shown to lead to apoptotic cell death by shifting monomeric to oligomeric VDAC1, enabling Cyto c release and hence apoptosis in the absence of any apoptosis stimuli (37,79-82). VDAC was shown to be over-expressed in brains of Alzheimer’s disease patients (83-85), beta cells of type 2 diabetes patients (86) and in cardiovascular diseases (87). This VDAC over-expression may be responsible for the apoptosis observed in these diseases. Thus, VBIT-4 interacting with VDAC and inhibiting apoptosis, HK detachment from the mitochondria and the associated increase in ROS and [Ca^{2+}]_i may also protect against cell death in these diseases.

**EXPERIMENTAL PROCEDURES**

**Materials**

Carbonyl cyanide m-chlorophenyl hydrazone (CCCP), carboxymethyl-cellulose (CMC), cisplatin, cytochalasin B, dimethyl sulfoxide (DMSO), DL-dithiothreitol (DTT), EDTA, HEPES, leupeptine, phenylmethylsulfonyl fluoride (PMSF), N-decane, sodium selenite,
soybean asolectin, staurosporine (STS),
tetramethylrhodamine methylester (TMRM) and
Tris were purchased from Sigma (St. Louis, MO). N-N-Lauryl-(dimethyl)-amino-oxide
(LDAO) was obtained from Fluka (Buchs,
Switzerland). Coelenterazine (DeepBlueC
[DBC]) was obtained from Bioline (Taunton,
MA). Hydroxyapatite (Bio-Gel HTP) was
procured from Bio-Rad Laboratories (Hercules,
CA). Digitonin came from Calbiochem-
NovoBiochem (Nottingham, UK). Celite was
purchased from the British Drug Houses
(London, UK). Rabbit monoclonal antibodies
against VDAC1 (ab154856) and against HK-I
(ab150423) and mouse monoclonal antibodies
against GAPDH (ab9484) were obtained from
Abcam (Cambridge, UK). Monoclonal
antibodies against actin were obtained from
Millipore (Billerica, MA) and anti-Cyto c
antibodies (556433) were obtained from BD
Bioscience (San Jose, CA). Fluo-4 AM,
carboxy-H2DCFDA and MitoSOX Red were
acquired from Invitrogen (Grand Island, NY).
Horseradish peroxidase (HRP)-conjugated anti-
mouse and anti-rabbit antibodies were obtained
from Promega (Madison, WI). Ethylene glycol
bis[succinimidylsuccinate] (EGS) was obtained
from Pierce (Rockford, IL). Annexin V-
fluorescein isothiocyanate (FITC) was from
Enzo Life Sciences (Lausen, Switzerland).
Dulbecco’s modified Eagle’s medium (DMEM)
and the supplements fetal bovine serum (FBS),
L-glutamine and penicillin-streptomycin were
purchased from Biological Industries (Beit-
Haemek, Israel).

The diversity set compound library was
provided by the National Cancer Institute (NCI)
in frozen bar-coded 96-well plates (465030-
466730), each well containing 20 μl of 10 mM
stock solution in DMSO with plate map
accession numbers. Compounds used for the
second and third round of screenings were
purchased from Diverchim (France) and AKos
Consulting & Solutions (Germany). VBIT-3 and
VBIT-4 were synthesized by ChemPartner
(Chengdu, China). The synthetic pathway and
the analytical data for each compound (including
two enantiomers of VBIT-4) are presented in
Supplemental Experimental Procedures.

**Plasmids**

Plasmids encoding the fusion proteins
rat(r)VDAC1-GFP2 and rVDAC1-luc were
constructed using the BRET2 plasmids (Perkin
Elmer, Waltham, MA). The rVDAC1 gene was
cloned into *BamH*I and *Hind*III sites of the
BRET2 plasmids (*N2* variants) and amplified
using the forward primer CCAAGCTT
TGGCTGTGCCACCCACGTATGCC and the
reverse primer GGAATCCGCCGCCGCCGCCGA
GCCGCGGCCGCTGCTTGAAAT-TC. The
reverse primer was designed to contain a double
linker sequence (*GGGS*2) connecting
VDAC1 and the *RLuc* or *GFP2* genes that introduced
flexibility to the region (88).

Plasmids encoding shRNA against human
VDAC1 (hVDAC1) for specific silencing of
endogenous human (h)VDAC1 were introduced
into a shRNA-expressing vector. The hVDAC1-
shRNA-encoding sequence was created using
the two complimentary oligonucleotides
indicated below, each containing the 19
nucleotide target sequence of hVDAC1 (337–
355), followed by a short spacer and an anti-
sense sequence of the target: oligonucleotide 1,
AGCTTAAAAACACTAGGCACCCAGATTA
TCTCTGAAAATATCTCGGTGCCTAGT GTG
and oligonucleotide 2, GATCCACACTAGGCA
CCCGAGATTATTCAGAGATAATCTCGGT
GCCTA GTGTTTTTAA, with the VDAC1-
derived sequence being underlined. The
hVDAC1-shRNA-encoding sequence was
cloned into the *Bgl*II and *Hind*III sites of the
pSUPERretro plasmid (OligoEngine, Seattle, WA), containing a puromycin-resistance gene. Transcription of this sequence under the control of the H1 RNA promoter of RNA Polymerase III produces a hairpin (hVDAC1-shRNA).

**Tissue Culture**

HEK, HeLa, SH-SY5Y and K-Ras-transformed Bax<sup>-/-</sup>/Bak<sup>-/-</sup> MEF cell lines were grown at 37°C under an atmosphere of 95% air and 5% CO<sub>2</sub> in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1000 U/ml penicillin and 1 mg/ml streptomycin. T-REx-293 cells (HEK cells stably containing the pcDNA6/TR regulatory vector and thus expressing the tetracycline repressor; Invitrogen) stably expressing hVDAC1-shRNA and showing low (10-20%) endogenous VDAC1 expression (referred to as T-REx-pS10) were grown under the same conditions as HEK cells, except with an addition of 5 μg/ml blasticidin.

**Cell transfection**

In the BRET experiments, T-REx-pS10 cells were transfected using calcium phosphate. Transfections were carried out with 0.2 μg of a plasmid coding for rVDAC1-Rluc and with 0.8 μg of a plasmid coding for rVDAC1–GFP2, respectively. Cells were analyzed for induction of apoptosis and BRET2 48 to 72 h post-transfection. For negative controls, cells were either transfected with plasmids encoding for rVDAC1–RLuc (0.2 μg DNA) and GFP2 (0.8 μg) or with a plasmid encoding for rVDAC1–Rluc (0.2 μg) and plasmid pcDNA4/TO (0.8 μg).

**Compound library testing**

The drug-like compound library used in this study was provided by the National Cancer Institute (NCI) in frozen bar-coded 96-well plates, with each well (with a plate map accession number) containing 20 μl of a 10 mM stock solution in 100% DMSO diluted with DMSO to yield 2 mM of the tested compound and stored frozen. The screen was conducted using the cells in a 96-well format for enhancement of BRET2 signals to identify inhibitors of VDAC1 oligomerization. Compounds (1 μl of 2 mM stock solutions) were added to a final concentration of 10 μM in 100 μl (1% final DMSO concentration).

The compounds used for the second and third rounds of screening were analyzed for their effects on VDAC1 oligomerization by chemical cross-linking, as described below.

**Bioluminescence resonance energy transfer (BRET) assay**

DNA encoding genetically engineered fusion proteins rVDAC1-Rluc (in which RLuc was connected to rVDAC1 at the C terminal position through a linker (GGGS)) and rVDAC1-GFP2 (in which the GFP2 was fused to the rVDAC1 C terminus) was cloned into BRET2 vectors. rVDAC1-GFP2 and rVDAC1-Rluc were expressed in T-REx cells stably expressing shRNA-hVDAC1 and a low level of endogenous hVDAC1 (79). shRNA-VDAC1, being specific to human VDAC1, allowed the expression of rVDAC1 and decreased the participation of endogenous hVDAC in oligomerization, thereby enhancing the BRET2 signal. rVDAC1-GFP2 and rVDAC1-Rluc expression levels were correlated with the amount of plasmids used. Specifically, 0.8 μg rVDAC1-GFP2 and 0.1 μg rVDAC1-Rluc were found to give the best signal.

Following incubation, cells were harvested, washed twice with PBS, resuspended in 200 μl of PBS and divided between two wells of a 96-well clear-bottom plate (Grenier). Luciferase activity was assayed using the membrane-permeable substrate DeepBlueC coelentrazine (DBC) in PBS supplemented with MgCl<sub>2</sub> (1 g/L) and glucose (1 g/L), with DBC being added to a
final concentration of 5 μM just before luminescence was measured. Appropriate conditions for resolving VDAC1 oligomerization in mammalian living cells using BRET2 technology were developed. These considered the number of cells to be plated, amounts and ratios of rVDAC1-Rluc and rVDAC1-GFP2 plasmids, apoptosis inducer concentration, the time of incubation and concentration of the luciferase substrate coelentrazine to be used. Cells transiently expressing rVDAC1-Rluc and rVDAC1-GFP2, as well as control cells, were incubated with the apoptosis inducer.

The BRET2 signal represents the ratio of the GFP2 fluorescence, measured at its emission wavelength (510 nm), over the light intensity (luminescence) emitted at 395 nm. All measurements were performed using the Infinite 200 ELISA reader (Tecan). BRET2 signals were defined as GFP2/Rluc intensity ratio and calculated as follows:

(a) The BRET2 signals obtained in VDAC1-RLuc/pcDNA4/TO cells (control cells) were subtracted from the signals obtained in cells expressing VDAC1-Rluc and VDAC1-GFP2.

(b) The net ratios of *Renilla* luciferase and GFP2 activities (GFP2/luciferase ratio after the subtraction of the BRET2 signals from control cells) were calculated.

(c) The ratios of BRET2 signals between different cells exposed and not exposed to apoptosis inducers were compared.

**High-throughput screening to identify inhibitors of VDAC1 oligomerization**

The screen was conducted using the cells in a 96-well format for enhancement of BRET2 signals to identify inhibitors of VDAC1 oligomerization. T-REx cells with low VDAC1 levels were transfected to express rVDAC1-GFP2 and rVDAC1-Rluc and seeded at a density of 9,000 cells/well in a 96-well plate. Compounds (1 μl of 2 mM stock solutions) were added to a final concentration of 10 μM in 100 μl (1% final DMSO concentration). The cells were pre-incubated for 1 h with the NCI compounds, and then incubated with the apoptosis inducers for an additional 3 h (STS, 1 μM; selenite, 30 μM; As2O3, 60 μM). The tested NCI compounds were dispensed by a robotic system into the 96-well plates. After treatment, the medium was removed and assayed for BRET2 signals as described above. Liquid handling was done with the Tecan (Männedorf, Switzerland) Freedom 150 Robotic & MCA Liquid Handling System, while luciferase luminescence and fluorescence readings were obtained a robot-integrated Tecan Infinite M1000 reader.

**Cross-linking experiments**

Cells (2.5-3 mg/ml) in PBS, were harvested after the appropriate treatment and incubated with the cross-linking reagent EGS (pH 8.3) for 15 minutes. Samples (60-80 µg protein) were subjected to SDS-PAGE and immunoblotting using anti-VDAC1 antibodies. Quantitative analysis of immuno-reactive VDAC1 dimer, trimer and multimer bands was performed using FUSION-FX (Vilber Lourmat, France).

**Gel electrophoresis and immunoblot analyses**

Samples (10-40 μg of protein) were subjected to SDS-PAGE and immunoblotting using monoclonal anti-VDAC1, anti-Cyto c or anti-actin antibodies, followed by HRP-conjugated anti-mouse or anti-rabbit IgG, serving as secondary antibodies and detected by chemiluminescence. To detect VDAC1 oligomers, membranes were treated with 0.1 M glycine, pH 2.0, prior to immunoblotting and washed several times with 0.1% Tween-20 in Tris-buffered saline. Band intensities were
imaged and quantified using FUSION-FX (Vilber Lourmat, France).

Flow cytometry using propidium iodide (PI) and annexin V-FITC staining

Cells (2×10⁵), untreated or treated with apoptosis-inducing reagents, were analyzed for apoptotic cell death using PI, annexin V-FITC and flow cytometer analysis. Cells were collected (1500 × g for 10 min), washed, and resuspended in 200 μl binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl₂). Annexin V–FITC was added according to the recommended Protocol (Enzo Life Sciences, Switzerland), and the cells were incubated in the dark for 15 min. Cells were then washed with binding buffer and resuspended in 200 μl binding buffer, to which PI was added immediately before flow cytometry analysis. At least 10,000 events were collected, recorded on a dot plot, and analyzed by the FACSCalibur flow cytometer software (BD Biosciences, Franklin Lakes, NJ).

Cytochrome c release and HK detachment from mitochondria

Cells treated with apoptosis inducers in the absence or presence of the indicated reagent were harvested, washed twice with PBS, pH 7.4 and gently resuspended at 6 mg/ml in ice-cold buffer (100 mM KCl, 2.5 mM MgCl₂, 250 mM sucrose, 20 mM HEPES/KOH pH 7.5, 0.2 mM EDTA, 1 mM dithiothreitol, 1 μg/ml leupeptin, 5 mg/ml cytochalasin B and 0.1 mM PMSF) containing 0.025% digitonin and incubated for 10 min on ice. Samples were centrifuged at 10,000xg at 4°C for 5 min to obtain supernatants (cytosolic extracts) and pellets (contains mitochondria). HK and Cyto c released to the cytosol was analyzed by immunoblotting using HK and Cyto c–specific antibodies. Anti-VDAC1 and anti-GAPDH antibodies were used to verify that the cytosolic extracts are mitochondria-free.

VDAC1 purification

VDAC1 protein was purified from rat liver mitochondria as previously described (89). Briefly, rat liver mitochondria (5 mg/ml) in 10 mM Tris-HCl, pH 7.2, were incubated with 2% LDAO at 0°C for 20 min, followed by centrifugation (30 min, 14,000 g) and the obtained supernatant was loaded onto a dry celite:hydroxyapatite (2:1) column. VDAC1 was eluted with a solution containing 2% LDAO, 10 mM Tris-HCl, pH 7.2, 50 mM NaCl, and 22 mM NaH₂PO₄. The VDAC1-containing fractions were dialyzed against 10 mM Tris-HCl, pH 7.2, and subjected to a second chromatography step on a carboxymethyl-cellulose (CMC) column from which VDAC1 was eluted with a solution containing 10 mM Tris-HCl, pH 7.2, 0.1 % LDAO and 500 mM NaCl. The VDAC1-containing fractions were collected and used for VDAC1 channel conductance and MST assays.

Expression, purification and refolding of 6xHis-tagged VDAC isoforms

The preparation of refolded VDAC isoforms was performed essentially as described (90). In brief, Escherichia coli BL21(DE3) cells were transformed with plasmid pET21a containing the VDAC isoforms coding sequences. Protein expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma) at an optical density (λ = 595 nm) of ~0.6 at 37°C for 3 h, as reported (91). The cells were resuspended in 8 M urea, phosphate buffer, pH 8.0, and shaken overnight at 4 °C. After pelleting cell debris by centrifugation, the clear lysate was loaded onto a Ni-NTA agarose-packed column (Qiagen), pre-equilibrated with 10 column volumes of the same buffer. The column was then washed twice with 5 volumes of the same solution at pH 6.2 and the purified
proteins were eluted with 5 volumes of the same solution at pH 3.5. The denatured protein mixture was added drop-wise to a refolding buffer (25 mM Tris-HCl, pH 7.0, 100 mM NaCl, 1 mM EDTA, 1% (v/v) lauryldimethylamine-oxide (LDAO, Sigma)) to obtain a ten-fold dilution of the urea concentration, and gently stirred overnight at 4°C. The protein solution was dialyzed against 100 volumes of a dialysis buffer (25 mM Tris-HCl, pH 7.0, 1 mM EDTA, 0.1% LDAO) in a Thermo Scientific Slide-A-Lyzer Dialysis Cassettes (3.5 K MWCO) changing the dialysis buffer two times after 2 h stirring and once more 24 h later, at 4°C. Samples were then subjected to additional purification and concentration using a CMC column (89). Protein purity was verified by SDS-PAGE and Coomassie staining. Purified samples were stored at -20°C. VDAC protein concentration was determined using SDS-PAGE and Commassie staining with ovalbumin as a standard.

**VDAC1 channel conductance**

The reconstitution of purified rat VDAC1 into a PLB and subsequent single and multiple channel current recordings and data analysis were carried out as previously described (92). Briefly, the PLB was prepared from soybean asolectin dissolved in n-decane (30 mg/ml). Purified VDAC1 (1 ng) was added to the chamber defined as the *cis* side containing 1 M NaCl, 10 mM Hepes, pH 7.4. Currents were recorded under voltage-clamp using a Bilayer Clamp BC-535B amplifier (Warner Instrument, Hamden, CT). The currents, measured with respect to the trans side of the membrane (ground), were low-pass-filtered at 1 kHz and digitized online using a Digidata1440-interface board and pClampex 10.2 software (Axon Instruments, Union City, CA).

**Measurement of superoxide generation**

ROS production was monitored using the oxidant sensitive dye DCFDA fluorescent probe, a cell-permeable indicator of ROS, which is converted by H$_2$O$_2$ and peroxidases to the DCF fluorescent derivate. Briefly, incubated with DCFDA (4 µM) for 30 minutes. For mitochondrial accumulated ROS, MitoSOX Red (4 µM), mitochondrial superoxide indicator for live-cell imaging was used according to the manufacturer's protocol (Invitrogen, Grand Island, NY). Fluorescence was measured using a FACScalibur flow cytometer software (BD Biosciences, Franklin Lakes, NJ).

**MST analysis**

MST analysis was performed using a NanoTemper Monolith NT.115 apparatus as described previously (93). Briefly, purified VDAC1 was fluorescently labeled using NanoTemper's Protein labeling kit BLUE (L001, NanoTemper Technologies). A constant concentration of the protein was incubated with different concentrations of the tested inhibitor in PBS. Afterwards, 3-5 µl of the samples were loaded into a glass capillary (Monolith NT Capillaries) and thermophoresis analysis was performed (LED 20%, IR laser 20%).

**Mitochondrial membrane potential determination**

Mitochondrial membrane potential (ΔΨ) was determined using TMRM, a potential-sensitive dye, and a plate reader. HEK-293 cells were treated with the compounds considered here and an apoptotic inducer and subsequently incubated with TMRM (0.5 µM, 20 min). The cells were then washed twice with PBS and examined with FACScalibur flow cytometer software (BD Biosciences, Franklin Lakes, NJ). CCCP-mediated ΔΨ dissipation served as control.

**Cellular Ca$^{2+}$ analysis**
Fluo-4-AM was used to monitor changes in cytosolic Ca\(^{2+}\) levels. HeLa cells (1x10\(^6\) cells/ml) were harvested after the appropriate treatment, collected (1,500xg for 10 min) washed with HBSS buffer (5.33 mM KCl, 0.44 mM KH\(_2\)PO\(_4\), 138 mM NaCl, 4 mM NaHCO\(_3\), 0.3 mM Na\(_2\)HPO\(_4\), 5.6 mM glucose, 0.03 mM phenol red) supplemented with 1.8 mM CaCl\(_2\) and incubated with 2.5 \(\mu\)M Fluo-4 in 200 \(\mu\)l HBSS(+) buffer in the dark for 30 min at 37\(^\circ\)C. After washing the remaining dye, the cells were incubated with 200 \(\mu\)l HBSS(+) buffer and changes in cellular free Ca\(^{2+}\) concentration were measured immediately via FACS analysis. At least 10,000 events were recorded on the FL1 detector, represented as a histogram, and analyzed with FACSCalibur flow cytometer software. Positive cells showed a shift to an enhanced level of green fluorescence (FL1).

**Statistical analysis**

Data are expressed as means ± SD. Statistical evaluation was carried out using Student’s t test (two-tailed) to test for differences between control and experimental results. P values less than 0.05 were considered as significant.

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Table 1. Summary of BRET2-based screen results of the anti-VDAC1 oligomerization activity of compounds from the NCI library. T-Rex cells expressing hVDAC1 shRNA were co-transfected with plasmids encoding rVDAC1-Rluc (0.1 μg) and rVDAC1-GFP2 (0.8 μg) and treated with As$_2$O$_3$ (60 μM, 3 h), selenite (30 μM, 3 h), or STS (1 μM, 3 h), with or without pre-treatment with the indicated compounds (10 μM, 1 h), followed by BRET2 signal measurement. Results are presented as percent inhibition of the BRET2 signal induced by the indicated pro-apoptotic agent. The highlighted numbers indicate compounds which inhibited BRET2 signals induced by selenite and STS (orange), by As$_2$O$_3$ (blue), by As$_2$O$_3$ and selenite (green), and by As$_2$O$_3$, selenite, and STS (yellow). The 12 most active compounds are highlighted in red.

| #  | NSC   | As$_2$O$_3$ | Selenite | STS   | #  | NSC   | As$_2$O$_3$ | Selenite | STS   |
|----|-------|-------------|----------|-------|----|-------|-------------|----------|-------|
| 1  | 16631 | 0           | 41.5     | 30.5  | 37 | 335048| 76.5        | 35       | 17    |
| 2  | 48422 | 0           | 38.6     | 36.8  | 38 | 19637 | 76.2        | 61.8     | 0     |
| 3  | 308849| 99.5        | 0        | 0     | 39 | 404057| 74.6        | 37.1     | 0     |
| 4  | 42537 | 84.9        | 0        | 0     | 40 | 15571 | 72.3        | 77.5     | 1.3   |
| 5  | 324623| 78          | 16.3     | 0     | 41 | 672441| 69.6        | 41.8     | 0     |
| 6  | 667251| 77.5        | 0        | 1.4   | 42 | 40275 | 64.8        | 35.2     | 16.8  |
| 7  | 109292| 75.4        | 0        | 0     | 43 | 41377 | 60.4        | 27.3     | 0     |
| 8  | 31069 | 74.9        | 0        | 0     | 44 | 31703 | 56.7        | 33.3     | 16.6  |
| 9  | 13151 | 67          | 0        | 0     | 45 | 132868| 55.5        | 41.4     | 0     |
| 10 | 163802| 65.8        | 1.2      | 0     | 46 | 341956| 51.5        | 24       | 0     |
| 11 | 605333| 63.7        | 16       | 0     | 47 | 8816  | 49.6        | 20.1     | 0     |
| 12 | 30205 | 62.7        | 0        | 0     | 48 | 31672 | 46.4        | 32.3     | 17.3  |
| 13 | 205968| 58.5        | 0        | 0     | 49 | 317605| 46         | 52       | 0.3   |
| 14 | 32892 | 55.4        | 16.4     | 0     | 50 | 338042| 44.7        | 80.2     | 0     |
| 15 | 10768 | 52          | 9.6      | 0     | 51 | 343966| 39.4        | 20.1     | 0     |
| 16 | 31698 | 51.3        | 2.1      | 0     | 52 | 15362 | 98         | 41       | 39.7  |
| 17 | 36586 | 49          | 0        | 0     | 53 | 601359| 97.7        | 74.4     | 46    |
| 18 | 41066 | 48.8        | 18.8     | 0     | 54 | 42199 | 97         | 78.7     | 31    |
| 19 | 39938 | 48.7        | 18.8     | 0     | 55 | 10428 | 96.4        | 75.7     | 38.2  |
| 20 | 151252| 100         | 29.8     | 0     | 56 | 154389| 90.6        | 90.6     | 45.9  |
| 21 | 146554| 100         | 71.8     | 0     | 57 | 19487 | 88.8        | 68.6     | 32.6  |
| 22 | 23247 | 97.3        | 52.1     | 0     | 58 | 680515| 87.8        | 66.3     | 33.4  |
| 23 | 11130 | 95.2        | 67       | 11.6  | 59 | 15364 | 85.4        | 75.9     | 40.9  |
| 24 | 204232| 90.3        | 32.5     | 0     | 60 | 146771| 70.7        | 68.1     | 41.2  |
| 25 | 135618| 88.9        | 64.9     | 0     | 61 | 39047 | 70.2        | 54       | 39.1  |
| 26 | 657149| 88.3        | 25.3     | 0     | 62 | 36815 | 67.7        | 50       | 34.5  |
| 27 | 20045 | 88          | 77.4     | 0     | 63 | 19115 | 64.2        | 62       | 36.4  |
| 28 | 268487| 86.7        | 69.4     | 0     | 64 | 319990| 96.2        | 43.2     | 22    |
| 29 | 522131| 86.8        | 47.6     | 0     | 65 | 43678 | 95.1        | 78.3     | 34.6  |
| 30 | 191029| 86.5        | 36.4     | 0     | 66 | 252172| 83.1        | 50       | 44.1  |
| 31 | 331208| 86.4        | 28.3     | 0     | 67 | 103520| 82.5        | 74       | 22.7  |
| 32 | 28837 | 85.2        | 48.3     | 0.3   | 68 | 43344 | 80.7        | 50.1     | 23.8  |
| 33 | 329249| 82.8        | 22.4     | 12.1  | 69 | 372275| 72.5        | 46.5     | 28.5  |
| 34 | 12262 | 81.5        | 67.4     | 0     | 70 | 41376 | 71.6        | 41.8     | 29.7  |
| 35 | 67436 | 78.1        | 65.7     | 14.8  | 71 | 321502| 67.3        | 46.5     | 22.4  |
| 36 | 372767| 77.3        | 26.6     | 0     |    |       |             |          |       |
Figure legends

Figure 1. BRET assay for monitoring VDAC1 oligomerization and inhibition

A. Schematic representation showing energy transfer between VDAC1-luciferase (RLuc, a light-producing enzyme) as donor and VDAC1-GFP2 (fluorophore) as acceptor. Energy transfer only occurs when the donor and the acceptor are in spatial proximity or physically interact. Compounds enhancing apoptosis lead to VDAC1 oligomerization and thus enhance the BRET2 signal, while apoptosis inhibitors inhibit VDAC1 oligomerization and, therefore, decrease the BRET2 signal. The luciferase substrate DBC emits light upon cleavage and thus causes excitation of the proximal GFP2 protein, thereby generating a BRET2 signal.

B. DNDS inhibits the BRET2 signal induced by selenite. T-REx cells expressing hVDAC1 shRNA were co-transfected with plasmids encoding rVDAC1-Rluc (0.1 μg) and rVDAC1-GFP2 (0.8 μg). The BRET2 signals obtained in cells, treated with or without pre-treatment with DNDS (200 μM, 1 h) and selenite (30 μM, 3 h), were measured. A representative of three independent repeats is presented.

C. T-REx cells were treated as described in B, harvested, cross-linked with EGS, and then analyzed by immunoblotting using anti-VDAC1 antibodies. The positions of VDAC1 monomers, dimers and multimers (multi) are indicated. The white star indicates monomeric VDAC1 with modified electrophoretic mobility, representing intra-molecular crossed-linked monomeric VDAC1.

D. Inhibition of BRET2 enhancement by the 12 most active compounds from the NCI library. BRET2 signals were obtained in cells treated with As$_2$O$_3$ (60 μM, 3 h; purple bars), selenite (30 μM, 3 h; green bars) or STS (1 μM, 3 h; red bars) with or without pre-treatment with the indicated compounds (10 μM, 1 h). Results are presented as percent inhibition of the BRET signal and correspond to mean ± SEM (n=2), p <0.05(*).

E. A summary of BRET assay. Out of an NCI library of 1,468 compounds, 71 were able to inhibit the BRET signal (30-100%) induced by treatment with As$_2$O$_3$, selenite or STS (the list is presented in Table S1). These inhibited BRET induced by either As$_2$O$_3$ or selenite or by selenite or STS (overlapping areas). Twelve out of the 71 compounds (see Fig. 2A) were able to inhibit the BRET2 signal elicited by all 3 inducers (orange).

Figure 2. Structure-activity analysis from the first round of screening for inhibitors of VDAC1 oligomerization

A. Chemical structure of the 12 most active inhibitors of VDAC1 oligomerization from the NCI library. B. Pharmacophore Search was applied to the compounds shown in A. Pharmacophore and the four anchoring points are presented (orange, aromatic points; purple, hydrogen bond donor points; blue, hydrogen bonds acceptor point; green, hydrophobic points). The six aligned active compounds that led to the pharmacophore are also presented.

Figure 3. Second round of screening for inhibitors of VDAC1 oligomerization

A. HeLa cells were incubated with the indicated compounds obtained from Diverchim (10 μM, 1 h). The cells were then exposed to selenite (30 μM, 3 h; gray bars) or cisplatin (15 μM, 20 h; blue bars), harvested, analyzed for VDAC1 oligomerization by chemical cross-linking as described in the legend to Fig. 1C and VDAC1 dimer levels were quantified. The results are presented as percent inhibition. The red squares indicate the most active compounds selected for analysis in round 3 (medicinal chemistry-based compound design). The results shown correspond to means ± SEM.
B. Chemical structure of the four most active inhibitors of VDAC1 oligomerization are shown. The yellow background highlights similar structural moieties in all four molecules. C–D. HeLa cells were incubated with the indicated compounds (20 μM, 1 h) and then with or without selenite (30 μM, 3 h) (C) or cisplatin (15 μM, 20 h) (D). The cells were harvested, cross-linked with EGS (300 μM, 15 min), and analyzed by immunoblotting using anti-VDAC1 antibodies. The positions of VDAC1 monomers, dimers and multimers (multi) are indicated. The white star corresponds to monomeric VDAC1 with modified electrophoretic mobility, representing intra-molecular cross-linked monomeric VDAC1. The positions of molecular size protein standards are provided. The source of the molecules (AKOS or Diverchim) is indicated. E. Quantitative analysis of the amount of the VDAC1 dimers obtained in C and D is shown. Gray and blue bars represent results obtained with selenite and cisplatin, respectively. The data are presented as percent inhibition of dimer formation. The results shown correspond to means ± SEM (n=3), p <0.05(*)

Figure 4. Correlation between the extent of AKOS-022 inhibition of apoptosis and VDAC1 oligomerization

HeLa cells were incubated with the indicated concentrations of AKOS-022 for 2 h and then with or without selenite (30 μM, 3 h) (A,B,C) or cisplatin (15 μM, 20 h) (B,D,E). Cells were harvested and either cross-linked with EGS (300 μM, 15 min) and analyzed for VDAC1 oligomerization by immunoblot using anti-VDAC1 antibodies (A,D), or assayed for apoptotic cell death using annexin V-FITC/PI staining and FACS analysis (B,C,E). Representative FACS results are presented (B). Quantitative analysis of the inhibition of VDAC1 dimer levels and cells undergoing apoptosis (annexin-positive cells) as induced by selenite (C) or cisplatin (E) are presented as a function of AKOS-022 concentration. The results reflect means ± SD (n=3). F. Quantitative analysis of the extent of apoptosis inhibition as a function of the inhibition of VDAC1 dimers formation as induced by selenite (■) or cisplatin (□), and as analyzed at the identical AKOS-022 concentration.

Figure 5. Compounds directly interact with purified VDAC1 and reduce channel activity

A. Chemical structure of the parent molecule (AKOS-022) and two newly synthesized molecules (VBIT-3 and VBIT-4). B. Purified VDAC1 was reconstituted into a PLB and currents through VDAC1, in response to a voltage step from 0 to 10 mV, were recorded before and 30 min after the addition of 40 μM of AKOS-022, VBIT-3 or VBIT-4. C. Multi-channel recordings of VDAC1 conductance as a function of voltage, and the average steady-state conductance of VDAC1 before (■) and 30 min after the addition of AKOS-022 (○), VBIT-3 (□), or VBIT-4 (●). Relative conductance (G/Go, conductance/maximal conductance) was determined at a given voltage. The data were normalized according to the conductance at -10 mV (maximal conductance). D. Purified VDAC1 (133 nM), labeled using the NanoTemper fluorescent protein-labeling Kit BLUE, was incubated with increasing concentrations of AKOS-022 (○), VBIT-3 (□), or VBIT-4 (●) (0.3 μM to 100 μM). After 20 min of incubation, 3-5 μl of the samples were loaded into MST-grade glass capillaries and the thermophoresis process was measured using the Monolith-NT115 apparatus. The results are presented as % of the bound fraction calculated as:

\[
\text{Fraction bound} = 100 \times \frac{F-F_{\text{min}}}{F_{\text{max}}-F_{\text{min}}}
\]
E. VDAC1 binding affinities of the tested compounds, derived from the MST measurements presented in C. The results shown correspond to means ± SD (n=3). F. VBIT-4 binding to recombinant purified VDAC isoforms as revealed using the MST method. (○), (●) and (□) indicate binding by VDAC1, VDAC2 and VDAC3, respectively. Inset show Commassie blue staining of the purified proteins used.

Figure 6. Inhibition of selenite-induced VDAC1 oligomerization by the tested compounds
A. HEK-293 cells were incubated with the indicated concentration of AKOS-022, VBIT-3 or VBIT-4 for 2 h and then with or without selenite (15 µM, 4 h), harvested, cross-linked with EGS (300 µM, 15 min), and analyzed by immunoblot using anti-VDAC1 antibodies. The positions of VDAC1 monomers and multimers are indicated. The star indicates monomeric VDAC1 with modified electrophoretic mobility, representing intra-molecular cross-linked monomeric VDAC1. The positions of molecular size protein standards are provided. B. Quantitative analysis of selenite-induced VDAC1 dimer formation by the various compounds, presented as percent of inhibition. The results show means ± SD (n=3). C. Inhibition of selenite-induced apoptosis by the compounds as analyzed using annexin V-FITC/PI staining and FACS. (●), (○) and (□) indicate VBIT-4, AKOS-022 and VBIT-3, respectively. D. Inhibition of Cyto c release from the mitochondria as induced by selenite. To assess Cyto c release, cells were incubated on ice for 10 min with 0.025% digitonin, centrifuged, and the pellet (mitochondria) and supernatants (cytosol) were subjected to SDS-PAGE and immunoblotting, using anti-Cyto c, antibodies. Anti-VDAC1 and anti-GAPDH antibodies were used to verify that the cytosolic extracts are mitochondria-free. E. Quantitative analysis of selenite-induced Cyto c release to the cytosol by the tested compounds. The data are presented as percent inhibition. The results shown correspond to means ± SD (n=3). (●), (○) and (□) indicate VBIT-4, AKOS-022 and VBIT-3, respectively. F. IC_{50} values (µM) of the compounds as derived from B, C, and E. The results shown correspond to means ± SD (n=3). G. VBIT-4 and AKOS-022 protection against STS-induced cell death. HEK cells were incubated with VBIT-4 or AKOS-022 (15 µM, 2h) and then apoptosis was induced by STS (0.2 µM, 3 h). Apoptotic cell death was analyzed by acridine orange and ethidium bromide staining. It should be noted that with STS, the apoptotic cells undergo fragmentation and FACS analysis was problematic. H. VBIT-4 effect on As\textsubscript{2}O\textsubscript{3}-induced cell death. HEK cells were incubated with VBIT-4 (15 µM, 2h) and then apoptosis was induced by incubation with As\textsubscript{2}O\textsubscript{3} (16 h, 30 µM). Cell death was analyzed by PI staining and FACS analysis. I. Inhibition of HK-I detachment from the mitochondria as induced by selenite. To assess HK-I detachment, cells were treated as in D and subjected to SDS-PAGE and immunoblotting, using anti-HK-I, antibodies. Anti-VDAC1 and anti-GAPDH antibodies were used to verify that the cytosolic extracts are mitochondria-free. The levels of HK in the supernatants are presented as percentages of selenite-induced HK detachment (after accounting for the amount of cytosolic HK present in untreated cells).

Figure 7. VBIT-4 and its enantiomers are equally effective in inhibiting selenite-induced VDAC oligomerization and cell death
A. Chemical structure of the VBIT-4 two enantiomers, VBIT-4-1 and VBIT-4-2. B. HEK-293 cells were incubated with VBIT-4, VBIT-4-1 or VBIT-4-2 (10 µM) for 2 h and then with or without
selenite (15 µM, 4 h). The cells were harvested, cross-linked with EGS (300 µM, 15 min), and analyzed by immunoblot using anti-VDAC1 antibodies. The positions of VDAC1 monomers and multimers are indicated. The star indicates monomeric VDAC1 with modified electrophoretic mobility, representing intra-molecular cross-linked monomeric VDAC1. Cell death was measured using PI staining and FACS analysis and is indicated in the bottom of the gel. C. HeLa cells were incubated with the indicated concentrations of the tested compounds for 1 h and then with or without selenite (25 µM, 3 h). Cells were harvested and assayed for cell death, using PI staining and FACS analysis. The results shown correspond to means ± SD (n=3).

Figure 8. Compounds inhibition of VDAC1 oligomerization and apoptosis in neuronal cells and Bax/Bak-lacking MEFs cells
A,B SH-SY5Y cells were incubated with AKOS-022 or VBIT-4 (30 µM, 2 h) and then with or without cisplatin (20 µM, 20 h). The cells were harvested, cross-linked with EGS (200 µM, 15 min), and analyzed by immunoblot using anti-VDAC1 antibodies (A) or apoptosis (B) as analyzed using annexin V-FITC/ PI staining and FACS. B. Quantitative analysis of cisplatin-induced VDAC1 dimer formation (gray columns) and apoptosis (black columns). The results shown correspond to mean ± SD (n=3), p <0.001(***).

C. Bax−/−/Bak−/− MEFs cells were incubated with AKOS-022 or VBIT-4 (20 µM, 2 h) and then with or without cisplatin (20 µM, 20 h). The cells were harvested, cross-linked with EGS (200 µM, 15 min). D. Bax−/−/Bak−/− MEFs cells were treated as in C and assessed for Cyto c release. Cells were incubated on ice for 10 min with 0.025% digitonin, centrifuged, and the pellet (mitochondria - Mito) and supernatants (cytosol) were subjected to SDS-PAGE and immunoblotting, using anti-Cyto c, antibodies. Anti-VDAC1 and anti-GAPDH antibodies were used to verify that the cytosolic extracts are mitochondria-free. E. Quantitative analysis of cisplatin-induced VDAC1 dimer formation (gray bars) and Cyto c release (black bars). The results shown correspond to mean ± SD (n=3), p <0.01 (**) or <0.001(***). The positions of molecular size protein standards are provided.

Figure 9. Compounds inhibition of the selenite-induced increases in intracellular calcium and ROS levels and decreases in membrane potential mediated
HEK-293 cells were incubated with AKOS-022 or VBIT-4 (30 µM, 2 h) and then with or without selenite (15 µM, 4 h). The cells were harvested and intracellular calcium ([Ca2+]i) levels were measured using Fluo-4 and FACS analysis and representative FACS results are presented. Quantitative analysis of the results are presented as percent maximal [Ca2+]i at the bottom. B. Mitochondrial membrane potential (ΔΨm) was analyzed with TMRM and FACS analysis. Representative FACS results are presented. CCCP (25 µM, 30 min) served as a positive control for ΔΨm dissipation and the CCCP-sensitive TMRM fluorescence was considered as 100% and the results are presented at the bottom. C. Cellular ROS levels were analyzed with carboxy-H2DCFDA and FACS analysis, with representative FACS results and quantitative analysis (bottom) are shown. D. Mitochondrial superoxide was detected with MitoSOX Red and flow cytometry with representative FACS result and quantitative analysis (bottom) are shown. The results shown (A-D) correspond to mean ± SD (n=3), p <0.05(*) or <0.01(***).
A. Lead compound development:

i. First screen - HTS of 1,468 small molecules by BRET2 resulted in the identification of 12 compounds that inhibited VDAC1 oligomerization.

ii. Second round of screening of 34 molecules, selected based on the 12 compounds identified in the first screen. The screen identified 4 compounds displaying strong inhibition of VDAC1 oligomerization.

iii. Third round of screening addressed 13 molecules designed based on the four compounds identified in the second screen. The most potent compound, AKOS-022, was identified.

iv. AKOS-022 was used for the design and synthesis of the novel derivatives, VBIT-3 and VBIT-4.

v. VBIT-4 enantiomers

B. Proposed model for VDAC1-based apoptosis and its inhibition by VBIT-4 and AKOS-022.

Apoptotic stimuli or pathological conditions lead to VDAC1 oligomerization, forming a pathway for the release of apoptogenic proteins (e.g., Cyto c and AIF). VBIT-4 and AKOS-022 directly interact with VDAC1 and inhibit its oligomerization, the subsequent HK detachment and release of apoptogenic proteins from the mitochondrial inter-membrane space, such that apoptosis is inhibited.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

| Compound      | Kd, µM   |
|---------------|----------|
| AKOS-022      | 15.4 ± 2.9 |
| VBIT-3        | 31.3 ± 1.7 |
| VBIT-4        | 17 ± 5.3  |
Figure 6
Figure 7
Figure 8
Figure 9
Figure 10
Novel Compounds Targeting the Mitochondrial Protein VDAC1 Inhibit Apoptosis and Protect Against Mitochondria Dysfunction
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