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Voltage dependent anion channel-1 (VDAC-1) as an anti-cancer target

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Voltage dependent anion channels (VDACs) are pore-forming proteins (approximately 30 kDa) associated with mitochondria, the membrane-bound organelles found in almost all eukaryotes. Commonly referred to as the “power-generators of cells”, mitochondria are composed of binary membranes, an outer membrane (OMM; outer mitochondrial membrane) and an inner membrane (IMM; inner mitochondrial membrane). While the latter is essentially impermeable to hydrophilic solutes of any kind, the OMM is made “porous” by the VDACs, which are integral membrane proteins that facilitate the free diffusion of molecules of approximately 5 kDa or less. Thus, the presence of VDACs (aptly named the “porins”),¹ were used to explain the high solute permeability of the OMM, and its unresponsiveness to osmotic pressures. Electron micrographs of the OMM also display a porous appearance (reviewed by Mannella and Kinnaly, 2008)² with a near-crystalline array of pores visible in lipid depleted OMMs. It should be noted that larger proteins encoded by nuclear DNA (and destined for the mitochondrion via an N-terminal signal sequence), bind to specialized “translocating protein complexes” on the OMM and are then transported across. The OMM also associates with the endoplasmic reticulum (ER) to facilitate transport of specific solutes, including lipids.

Discovered over three decades ago in mitochondrial extracts, the VDACs were first thought to be exclusively mitochondrial entities.^{3,4} However, at present, the VDACs have been characterized not only in the endoplasmic reticulum,⁵ but also in the plasma membrane as well (reviewed by Pinto, 2010).⁶ In fact, VDACs are the most abundant of proteins in the OMM.⁷ Currently, three isoforms of VDACs have been characterized primarily from mammalian tissues, with the VDAC-1 isoform being the most abundant by approximately an order of magnitude in comparison to the next most abundant VDAC isoform, VDAC-2 and over two orders of magnitude more abundant than the VDAC-3 (100-fold). VDACs 2- and 3- have been isolated primarily from testicular tissue and spermatozoa,^{8,9} while VDAC-1 is universally abundant across all other tissues types. Thus, it can be inferred that VDAC-1 is the most prevalent and physiologically and metabolically the most important isoform. In fact, almost all functional studies used to characterize VDACs have utilized VDAC-1 and form the basis for this commentary.

Functional Characterization of VDACs

The “pores” in the OMM were re-designated as VDACs primarily due to their electrophysiological properties when reconstituted in vitro within phospholipid bilayers or in liposomal vesicles. Although first thought to be “just a pore”, these in vitro functional studies indicated that the porins had the capacity to respond to an electrochemical potential.⁴ In essence, the OMM pores were found to be biased toward anion-selectivity among charged solutes (or metabolites), i.e., the VDAC channels display ion-selectivity that depends on the voltage across the membrane.¹⁰ In low-voltage states (~10 mV across the lipid bilayer) VDAC maintains an open-state, while at high-voltage states of either polarity (~40 mV or greater), VDAC manifests altered ion-selection and permeability levels.

Whether these in vitro phospholipid bilayer based studies faithfully relate to the function of VDACs in mitochondria (in their true physiological environment) is still being debated by various research groups. One possibility is that, at least at the “contact” sites between the OMM and IMM, the electrochemical potential generated across the inner-mitochondrial membrane (IMM) may directly induce a voltage potential across the OMM to regulate the “open” and “closed” states of the VDACs. Also, high cytoplasmic concentrations of NADH or a more reduced or bioenergetically sufficient state, have been found to promote VDAC closure, with the pore being more sensitive to closure at higher voltage and higher NADH levels.^{11,12}

Which solutes are actually regulated by this pore? It is presumed that larger metabolites, i.e., nucleotides are regulated by the voltage-gated activity of VDAC. Which protein sub-structures facilitate this formation of a voltage-gated pore? Computer modeling and crystallography studies with bacterially expressed reconstitution systems indicate a barrel formed by 19 lipid bilayer spanning β -strands that form a membrane spanning β -barrel structure and one alpha-helical structure,^{13–15} while peptide loops that interconnect the β -strands and are exposed to the cytoplasm or the transmembrane space of mitochondria are thought to interact with other proteins or enzymes. A well-characterized example is the VDAC-hexokinase interaction on the cytoplasmic face of the OMM in tumor cell mitochondria.^{16,17}

VDAC in Programmed Cell Death

Programmed cell death or apoptosis is primarily initiated via release of pro-apoptotic proteins from the inter-membrane space of the mitochondria to the cytosol, i.e., release of *Cytochrome c* from the inter-membrane space to the cytosol via permeabilization of the OMM.¹⁸ While the hexokinase-VDAC interaction described above facilitates preferential access to mitochondrial generated ATP to maintain the high glycolytic flux of such tumors,^{19,20} it also prevents initiation of such apoptotic cascades.²¹ Here, hexokinase acts as a “damper” against formation of the yet-to-be fully characterized mitochondrial permeability transition pore (MPTP),²² thought to be necessary for induction of OMM permeabilization. This and other similar molecular interactions of VDAC with OMM associated pro- and anti-apoptotic proteins are multi-faceted and help promote or prevent cell death. This phenotype is critical for the development of successful anti-cancer strategies. Thus, the mitochondrially expressed VDAC presents itself as a core component that can be targeted in tumors, which has the potential to debilitate the aberrant metabolic fluxes of tumors and initiate apoptotic signaling cascades.²³

It should be noted that transgenic VDAC-1 knockout mice display normal behavior and functionality,²⁴ while VDAC-3 “knockout” males are infertile.²⁵ VDAC-2 “knockouts” appear to be embryonically lethal, as that isoform was found to manifest an anti-apoptotic

effect by interacting with (and sequestering away) pro-apoptotic members of the Bcl-2 family of proteins.²⁶

VDAC as an Anti-Cancer Target

Since several studies have indicated that the hexokinase-VDAC interaction on the OMM can inhibit apoptosis in several mammalian cell systems including tumor cells, an obvious cancer-targeting strategy would be to disrupt the complex or force the release of hexokinase from VDAC.^{21,27,28} In fact, several approaches to force the separation have been tested and shown to promote apoptosis in the targeted cells; in one, peptides that correspond to the N-terminal of hexokinase (necessary to anchor hexokinase to VDAC) or those that are on the putative hexokinase binding site on VDAC were used to interfere with the binding interaction.²⁸ In another strategy, small-molecule drugs, i.e., methyl jasmonate (a stress hormone of botanicals)²⁹ or clotrimazole³⁰ were used, again to disrupt the hexokinase-VDAC binding. Expression analysis indicates that tumor cells highly overexpress VDAC, a fact borne by other indirect evidence, i.e., tumors display a much greater amount of mitochondrial-bound hexokinase than normal tissues. As such, targeting VDAC is an attractive modality to generate an anti-tumor response as many of the above *in vitro* studies have indicated. However, while either peptide-based or a small-molecule based strategies have been utilized in the past to test for inhibition of VDAC-1 and the effects of inhibition on tumor cell function, the effect of “silencing” VDAC-1 expression and its effects on the bioenergetic functions or apoptotic cascades in a tumor cell line has been tested only recently *in vitro*. These studies demonstrated that both mitochondrial bioenergetic functions and cell proliferation were affected by VDAC-1 silencing, at least in a non-tumorigenic recombinant cell line.³¹

The article by Koren, et al.³² in the current issue of *Cancer Biology and Therapy* now provides the first *in vivo* evidence of the effects of silencing VDAC-1 using a cervical cancer cell line (HeLa). To test the efficacy of such an “anti-VDAC” tumor targeting strategy, Koren et al. have utilized an inducible shRNA expression system (a doxycycline-based “Tet-On” system by Clontech) to first engineer cell-clones capable of regulated expression of RNAi against VDAC-1. After screening a series of RNAi templates (designed *in silico*) for the efficacy of VDAC-1 “knockdown” by western blotting, the authors then tested the same clonal cell isolates in a nude mouse model by sub-cutaneous implantation of the tumors followed by activation of RNAi via provision of “doxycycline-laced” water to the animals *ad libitum*.

The authors’ goals were to establish HeLa cell-lines that can express VDAC-1 in a regulated manner via exogenously applied doxycycline and test the cells for changes in proliferation rate *in vitro*, followed by implantation of the clonal isolates in the murine model to verify the effects of doxycycline-regulated silencing of VDAC-1 knockdown on tumor proliferation. Although the selected RNAi targets within VDAC-1 mRNA showed significant efficacy in reducing VDAC-1 expression, the “Tet-On” system employed also displayed the inherent difficulties sometimes encountered by researchers in getting this “molecular-switch” to work in a true “on/off” manner. While the authors were able to silence VDAC-1 expression by almost a magnitude, the test cells that were not exposed to doxycycline also displayed significant silencing of VDAC-1, most likely due to “leak-through” of the tet-regulated promoter elements employed by the system. Also surprising was the fact that doxycycline by itself silenced VDAC-1 expression by approximately 30% in control cells that harbored just the empty vector devoid of an shRNA template against VDAC-1; a facet of the authors’ results that need to be followed up in future studies. Perhaps the tetracycline mediated transcription silencing factors (Tts) that are utilized in the

Tet-regulated system by themselves influence or alter the mitochondrial proteome (by as yet unrecognized methods).

Despite the above difficulties in employing these “cutting-edge” molecular biology tools to better elucidate the phenotypic changes due to VDAC-1 silencing of tumors, both the in vitro and in vivo data presented in the article display a clear correlation between silencing of VDAC-1 expression and inhibition of tumor proliferation. The in vivo data indicate an approximately 40-fold reduction in tumor size (as measured by tumor volume) in targeted cells, in comparison to that in controls. Interestingly, the studies also demonstrate that while tumors initially became established in both control and test animals, further development of the tumors were retarded in the test animals. This indicates that continued silencing of VDAC-1 inhibited growth of the tumors.

It would be interesting to continue these in vivo studies with longer-term analyses of the results obtained. For example, since both pro- and anti-apoptotic phenotypes have been associated with VDAC-1, does silencing of VDAC-1 expression (which should deprive hexokinase of a mitochondrial anchor and negate the anti-apoptotic effects of the hexokinase-VDAC binding) promote apoptosis? Or, would the strategy suppress apoptosis in tumors (as the latter is possible if pro-apoptotic members of the Bcl-2 family of proteins are prevented from forming hetero-oligomers with VDAC-1)? Also, does silencing of VDAC-1 prevent the formation of the still-elusive MPTP complex (as recent data in ref. 33 indicate that VDAC-1 may form multi-mers in order to form or support the MPTP)?

With the RNAi templates that were developed by Koren et al. to target VDAC-1 and displayed near total silencing, the above questions can now be answered at the pre-clinical level. This should reveal whether targeted-therapy against VDAC-1 will be an effective strategy to pursue in continuing efforts to harness the aberrant metabolic flux pathways of tumors and debilitate them.

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Abbreviations

Tet	tetracycline
RNAi	interference RNA
shRNA	short-hairpin RNA
NADH	reduced nicotinamide adenine dinucleotide

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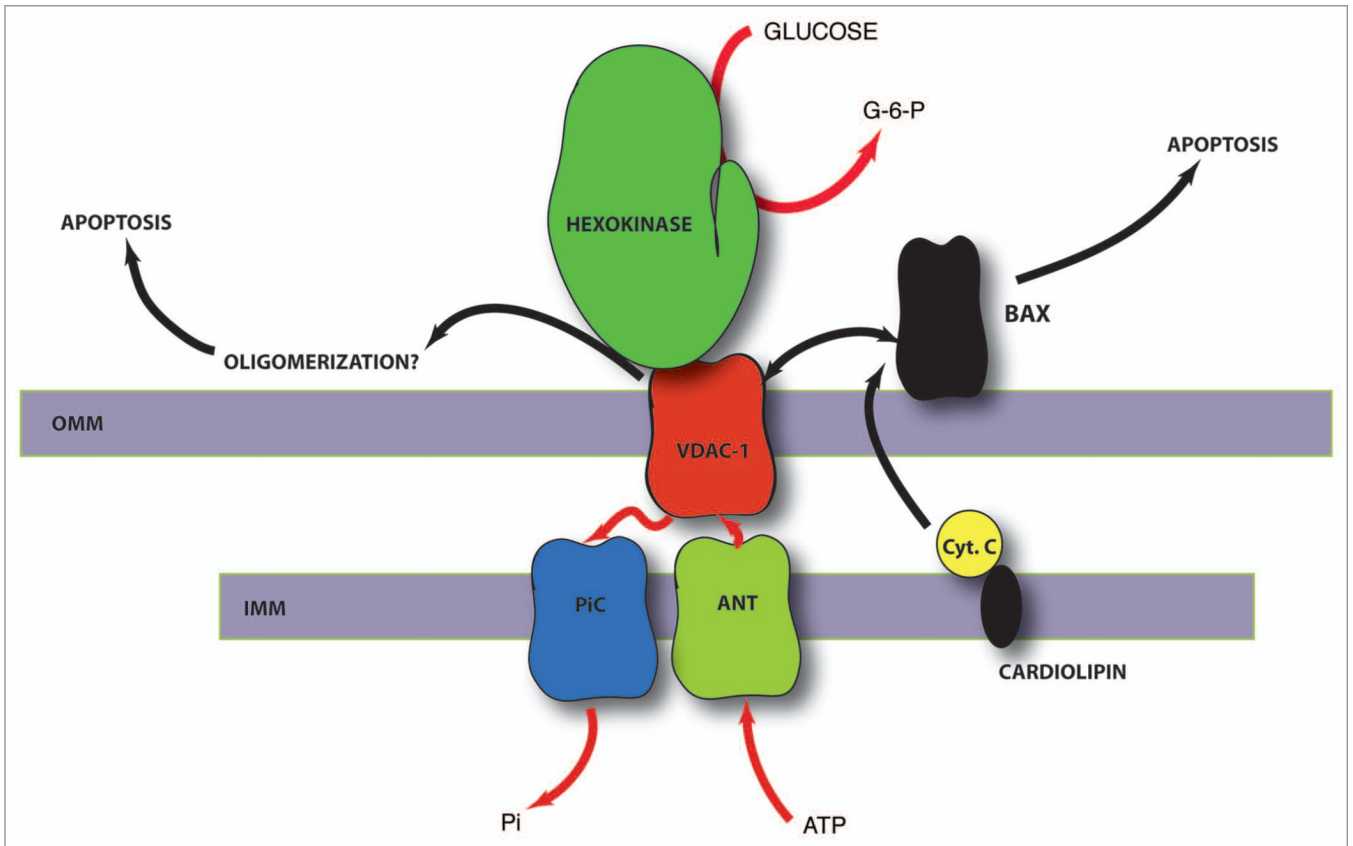


Figure 1.

Silencing of VDAC-1 expression via RNAi may result in opposing physiological effects on the tumor cell. Dispossessing of an “anchor” for hexokinase on the outer mitochondrial membrane may deprive the tumor of a high flux rate of glycolysis, thus inhibiting tumor proliferation; Pro-apoptotic—the same can relieve the anti-apoptotic effects that result from the hexokinase-VDAC interaction. Anti-apoptotic—if oligomers of VDAC are involved in forming the mitochondrial permeability transition pore complex (MPTP), silencing VDAC may inhibit apoptosis; the same may occur if VDAC is involved in hetero-multimer formation with Bax or other pro-apoptotic members of the Bcl-2 family of proteins. Despite the opposing effects on apoptosis, the data presented by Koren et al. indicate that silencing VDAC-1 inhibits tumor metabolism significantly, most likely via disruption of both the enhanced glycolytic flux and the nucleotide/metabolite/ion shuttles across mitochondria.