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Specific VDAC inhibitors: phosphorothioate oligonucleotides

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Abstract VDAC channels are ancient, highly-conserved voltage-gated channels in the mitochondrial outer membrane. They are the pathways by which metabolites travel between the cytosol and mitochondria. They are involved in the apoptotic process and probably other functions as well. The lack of specific inhibitors has hampered research in the past but now phosphothioate oligonucleotides can serve this function. These molecules were generated to be stable in the cytosol of cells but, unlike the oligonucleotides with the physiological phosphodiester linkage, these have the ability to bind to and block VDAC channels. They are potent, specific, and available commercially. At 1 μM concentration they block VDAC channels in mitochondria but do not affect the respiration complexes, the adenine nucleotide translocator or the ATP synthase.

Keywords Mitochondria · Outer membrane · Blocker · G3139 · Randomer

The study of the VDAC-blocking ability of phosphorothioate (PS) oligonucleotides grew out of their use as antisense silencing molecules. First synthesized chemically by Stec et al. (1984) a PS oligonucleotide contains a sulfur atom substituted for an oxygen at a non-bridging location at each phosphorus in the chain. Therefore, an 18 mer oligonucleotide, such as G3139, contains 17 phosphorothioate linkages. These molecules were designed to be significantly more nuclease resistant than isosequential phosphodiester oligonucleotides, and hence have been evaluated as drug candidates, completing Phase III trials in chronic lymphocytic leukemia (O'Brien et al. 2007) and advanced melanoma (Bedikian et al. 2006).

Each phosphorothioate linkage can occur in one of two optically active diastereomers, known as Rp and Sp. When PS oligonucleotides are produced by DNA synthesizers (the method employed for all clinical trials with PS oligonucleotides, e.g., G3139), they are done so as mixtures of Rp and Sp diastereoisomers at each phosphorus. Optically pure PS oligonucleotides (i.e., either all Rp or all Sp, or mixtures with defined sense of chirality) have also been synthesized (Stec et al. 1991). The Rp form, which hybridizes to its complementary mRNA with higher melting temperature than the Sp form, is unfortunately more nuclease resistant than the Sp diastereomer (Guga et al. 1998).

G3139 is a PS oligonucleotide targeted to the initiation codon of the Bcl-2 mRNA, and numerous experiments (Klasa et al. 2002) have demonstrated that this oligonucleotide, in a variety of cellular contexts, can silence Bcl-2 mRNA and protein expression. Since Bcl-2 is a strongly anti-apoptotic protein, silencing its expression should lead to increased sensitivity to cytotoxic chemotherapy. However, because G3139 is a polyanion, and cannot pass through hydrophobic cell membranes, several lipidic carrier molecules (e.g., Lipofectin, Lipofectamine 2000, Oligofectamine) have been employed to complex G3139 and to promote its passage from the bulk phase into the cytosol and cell nucleus. In several relatively recent experiments performed in 518A2 and other melanoma cells lines, G3139/Lipofectin complexes silenced Bcl-2 expression at the same time and concentration as they initiated cellular

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apoptosis (Benimetsksya et al. 2004). Moreover, apoptosis was shown to be dependent on the mitochondrial release of cytochrome c, and on caspase-3 activity (Lai et al. 2005). Since an siRNA/Lipofectin complex targeted to Bcl-2 also silenced but did not induce apoptosis, it was reasoned that G3139 treatment itself, not Bcl-2 silencing, was the apoptosis inducer.

It has also been long recognized that PS oligonucleotides are able to bind to the heparin-binding site of numerous heparin-binding proteins, including FGF2, PDGF BB, VEGF165, αMβ2 integrin, laminin and fibronectin, among others (Guvakova et al. 1995; Khaled et al. 1996; Ma et al. 2000). This binding is length dependent, but virtually totally sequence independent. Given that VDAC is a polyanion-interacting protein whose closure is believed to result in the release of cytochome c from the mitochondria, we hypothesized that the interaction of G3139 with VDAC could have led to the observed cellular apoptosis. Lai et al. (2006) isolated mitochondria from 518A2 cells by differential centrifugation and by flow cytometry, demonstrated the ability of FITC-G3139 to bind to them. This was confirmed in intact cells by double staining with Mitotracker Red. When isolated mitochondria were directly treated with 20 μM G3139 in an energizing buffer, cytochrome c was released into the supernatants, as detected by Western blotting. The release of cytochrome c from the mitochondria was not accompanied by inner mitochondrial membrane permeabilization, because no detectable fumarase activity, which is present in the mitochondrial matrix, was released. Cytochrome c release induced by homopolymers of thymidine with PS linkages, SdT18 and SdT14, was almost equivalent to the G3139-induced release, demonstrating the non-sequence specificity of the process. The release induced by SdT12 was somewhat diminished vs. SdT18, and little or no release was observed with SdT10. An 18 mer PS oligonucleotide with an equal mixture of each base at each position was also just as effective at releasing cytochrome c as is G3139. Thus, PS oligonucleotides of sufficient length effectively permeabilize the mitochondrial outer membrane to cytochrome c. How does this occur?

The role of VDAC in the permeabilization of the mitochondrial outer membrane is controversial. Evidence exists that VDAC closure somehow leads to protein release (Vander Heiden et al. 2000, 2001). Others (for example: Shimizu et al. 2000) believe that VDAC can itself serve as a conduit for protein release. Thus we sought to determine if PS oligonucleotides influence the function of VDAC.

Inhibition of metabolite flux in mitochondria

In order to study the influence of oligonucleotides on the ability of VDAC to permeabilize membranes to metabolites in its natural setting, experiments were performed on isolated mammalian mitochondria (Tan et al. 2007a, b). A great deal of evidence exists that the permeability of the mitochondrial outer membrane to metabolites is primarily, if not exclusively, due to VDAC channels (see for example: Benz et al. 1988; Liu and Colombini 1992; Lee et al. 1998; Xu et al. 1999). The evidence includes VDAC knock-out, selective VDAC expression, and VDAC block or closure by VDAC inhibitors. Thus, measurement of outer membrane permeability is equivalent to measurement of VDAC permeability.

The measurement of the permeability of the outer membrane to metabolites is challenging because of the small volume of the intermembrane space. Another obvious problem is the presence of the inner membrane and the much larger matrix space. Thus it is critical to distinguish between flux through the outer and inner membranes. The methods that have been developed all take advantage of enzymatic activity in the intermembrane space or on the outer surface of the inner membrane. By consuming the substrate as it enters, metabolite flux reaches a steady state, allowing the estimation of the permeability of the outer membrane to the metabolite. A detailed description of methods developed to measure the permeability of the outer membrane to metabolites in isolated mitochondria, has been published recently (Colombini 2007).

A convenient method, developed by Anchin Lee (Lee and Colombini 1997), measures the permeability of the outer membrane to ADP by taking advantage of the curvature in the state III respiration trace just prior to the exhaustion of ADP in the medium leading to state IV.

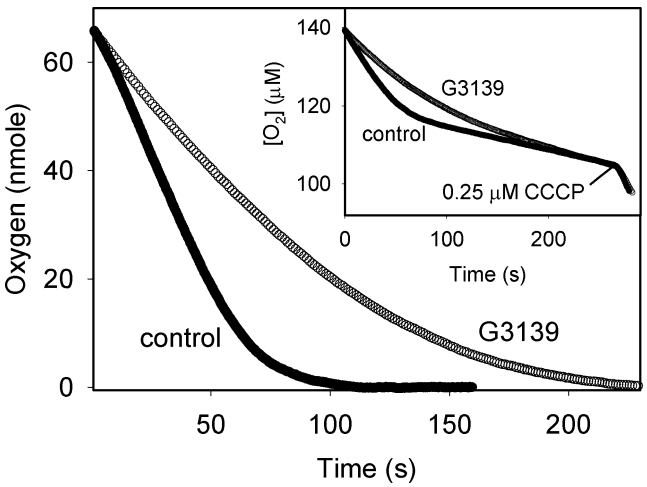

Fig. 1 G3139 reduced state III respiration and induced a pronounced curvature at the state III/state IV interface. O_2 consumption was monitored following addition of 80 μM ADP to rat liver mitochondria in the presence or absence of 1 μM G3139 (*inset*). The rate of state IV respiration was subtracted from the respiration curve (*main figure*). The mitochondrial protein concentration was 320 μg/ml. The figure was reproduced with permission from Tan et al. 2007a

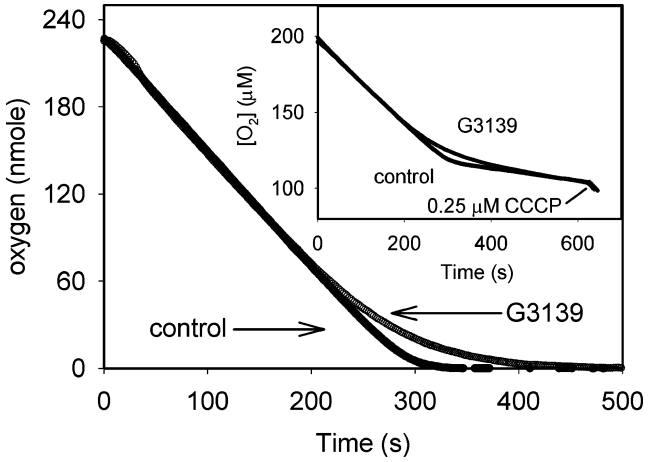

Fig. 2 G3139 does not alter state III respiration at high [ADP]. O_2 consumption was measured following addition of 270 μM ADP to mitochondria in the presence or absence of 1 μM G3139 (*inset*). The rate of state IV respiration was subtracted from the respiration curve (*main figure*). The figure was reproduced with permission from Tan et al. 2007a

At high [ADP], the rate of respiration is independent of [ADP] because the adenine nucleotide transporter (ANT) is saturated and flux through VDAC in the outer membrane is not rate limiting. Since the flux of ATP through VDAC increases linearly with [ATP] until about 100 mM (Rostovtseva and Colombini 1997), at the high [ADP] typically used in respiration experiments the flux is much higher than the rate of ADP phosphorylation as measured by the rate of respiration in coupled mitochondria. However, as the [ADP] declines into the physiological range (low micromolar), flux through the outer membrane can become rate limiting, depending on the permeability of the VDAC channels. This was first observed when NADH was added to isolated potato mitochondria (Lee et al. 1994). NADH reduced the outer membrane permeability whereas NAD was not effective.

The application of Anchin's method to the PS oligonucleotide, G3139, resulted in not only pronounced curvature in state III respiration but also a much shallower rate of respiration even at high [ADP] (Fig. 1; Tan et al. 2007a). This was not due to inhibition of processes such as ANT or ATP-synthase because damaging the outer membrane with a mild hypotonic shock relieved the inhibitory effect of G3139. In addition, by starting at higher [ADP], 270 μM, the initial rate of state III respiration was not affected by G3139 and curvature was only seen at lower ADP levels (Fig. 2). By fitting to a steady-state kinetic model (Tan et al. 2007a) the permeability of the outer membrane to ADP was calculated. G3139 reduced the permeability by as much as 85% with a K_I of 0.2 μM. The inhibition was close to maximal at 1 μM. By contrast, an oligonucleotide of identical sequence but with phosphodiester linkages had no significant effect event at 10 μM (Fig. 3).

At 1 μM G3139, there was no significant inhibition of respiration or ANT activity. There was also no significant swelling and the inhibitory effect of G3139 was not sensitive to cyclosporine A (Tan et al. 2007a). Thus, at 1 μM, G3139 seems to be highly specific for inhibition of VDAC permeability in isolated mitochondria.

Random sequences of nucleotides connected with phosphorothioate linkages (randomers) also inhibited mitochondrial outer membrane permeability (Table 1). The longer chains (18-mers) were more effective than the shorter chains (12-mers). Thus the inhibition does not arise from any particular base sequence.

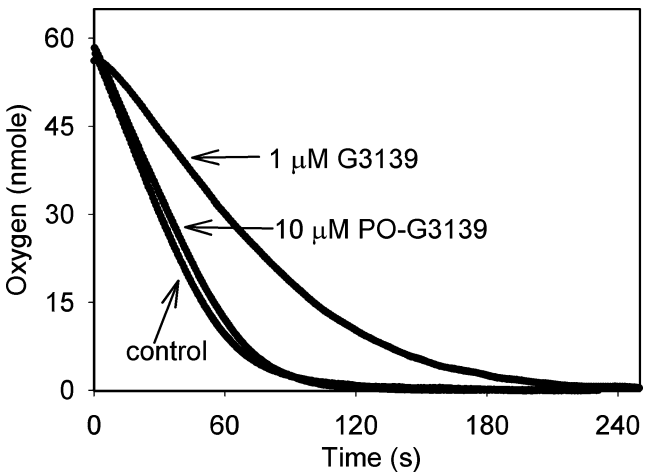

Fig. 3 The phosphodiester version of G3139 does not reduce the permeability of the mitochondrial outer membrane. State IV respiration was subtracted as indicated in Fig. 1. The figure was reproduced with permission from Tan et al. 2007a

Table 1 PS oligonucleotide length, not the base sequence, determines its ability to block VDAC channels

	Permeability (cm^3/s/g prot.)	% Inhibition
Control	244±23	
G3139	135±16**	45
12 mers	168±13*	31
14 mers	135±7**	45
16 mers	132±7**	46
18 mers	116±20**	52

Permeability of mitochondria to ADP following addition of 0.5 μM PS oligonucleotide. *N*-mers were random sequences of length 12, 14, 16, 18. Results are expressed as average±standard deviation (three experiments). Statistical tests indicate significance in the reduction of permeability. All the results of PS oligomers except 12 mers are not significantly different from that of G3139. Results were taken from Tan et al. 2007a.
*$p<0.01$; **$p<0.005$

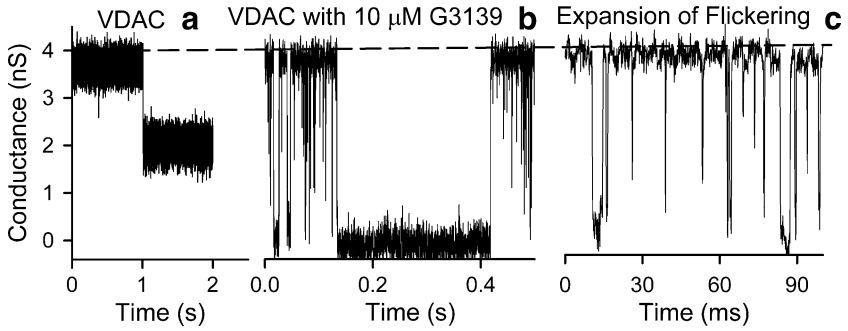

Fig. 4 Comparison of normal VDAC closure and G3139 induced VDAC closure. These recordings were made on the same single VDAC channel before and after addition of 10 μM G3139. The applied voltage was −50 mV. **a** An example of voltage-dependent VDAC closure. **b** An example of G3139 induced rapid flickering and complete conductance loss. **c** An expanded scale to show G3139 induced rapid flickering. The figure was reproduced with permission from Tan et al. 2007b

Inhibition of VDAC channels reconstituted into planar membranes

That PS oligonucleotides act directly on VDAC, as opposed to an indirect action, was demonstrated by performing experiments in VDAC channels reconstituted into planar phospholipid membranes (Tan et al. 2007b). G3139 induced transient blocking of VDAC channels that can become persistent (Fig. 4). Blocking of the channel over extended periods likely involves a search over conformational space for optimal interaction between G3139 and VDAC. At times the blockage appears to be almost permanent but then can be easily reversed by washing away G3139 (Fig. 5).

Blocking occurs preferentially when an electrical potential is applied making the side of the membrane to which G3139 was added, negative. The potential clearly favors the entry in the pore of the channel of this highly negatively-charged oligomer. Yet it is not merely the entry of the oligomer that occludes the pore otherwise the oligomer with phosphodiester bonds would act in the same way. On the contrary, no blockage is seen with the phosphodiester congener. Thus, G3139 must physically bind to the wall of the channel, perhaps to the positively-charged voltage sensor, so that its presence within the channel lasts for some time resulting in the measured interference of ion flow. The sulfur groups on the phosphorothioate oligomers are leading candidates for the sites that are responsible for binding to VDAC.

As expected, the formation of the block is dependent on the medium concentration of G3139 and on the applied electrical potential. The dissociation is not. The concentration dependence is consistent with a 1:1 complex. The voltage dependence has an effective charge valence of 3, so that the steepness can be explained if three charges moved through the entire potential difference when G3139 bound to VDAC. Considering partial translocation through the transmembrane electric field, the results are consistent with a good portion of the oligonucleotide entering the pore of the channel.

This effect of G3139 and other phosphorothioates on VDAC is distinctly different from the general polyanion effect described for a variety of anionic polymers such as dextran sulfate and polyaspartate (Mangan and Colombini 1987). The latter increase the propensity for channel closure without altering the closed-state conductance (the closed state of VDAC is still permeable to small ions but not to ATP). It does this by dramatically increasing the voltage

Fig. 5 A single VDAC channel reconstituted into a planar phospholipid membrane is closed by G3139. The voltage was alternated every 50 s between +50 and −50 mV to show VDAC gating. The zero current level is indicated as a long horizontal line. G3139 (40 μM final) was added where indicated. The side of the membrane containing G3139 was perfused with six chamber volumes of buffer to remove the G3139. Following perfusion the VDAC channel reopened. The figure was reproduced with permission from Tan et al. 2007a

dependence of the gating process producing a state referred to as ultra-steep voltage dependence. The polyanion partitions into the access resistance region of the channel where the transmembrane potential results in a more positive potential in that region as compared to the bulk phase. Electrostatic interaction between the polyanion and the positive voltage sensor on VDAC is believed to result in outward movement of the sensor to the closed position resulting in channel closure. Thus the polyanion does not need to make direct contact with the voltage sensor and 500 kDa dextran sulfate (a size that cannot possibly enter the channel to any significant extent) works very well. Note that G3139 produces a much lower conductance in the blocked state than the conductance range for the closed states of VDAC (Fig. 4). Moreover, the blocked-state conductance depends on the length of the oligonucleotide used (Tan et al. 2007b). This is consistent with a blockage mechanism. That is not the case with the polyanion effect. The length of the polyanion does not change the closed-state conductance but does influence the voltage-dependence of the effect because partitioning, according to the Boltzmann distribution, depends on the valency of the polyanion.

Implications

This research has implications that range from the study of apoptosis and mitochondrial function to the use of PS nucleotides for therapeutics. The lack of a specific inhibitor for VDAC has resulted in the use of agents that, while potent, clearly have other targets. Perhaps the most used and abused VDAC inhibitor is König's polyanion (Colombini et al. 1987). Whereas this polymer is useful as a potent VDAC inhibitor, its use has resulted in erroneous conclusions due to effects on other proteins being attributed to effects on VDAC. The existence of side effects was clear from the beginning in the publications of Tamás König (König et al. 1977; König et al. 1982). At this point, PS oligonucleotides at 1 μM concentration seem to be highly specific for VDAC. In a living cell, G3139 clearly has other targets as its ability to induce apoptosis is much greater than that of the L-stereoisomer or the randomers. Thus, it is recommended that randomers be used rather than G3139. In addition, fluorescently-tagged PS oligonucleotides bind strongly to the nucleus so that other targets do exist. Finally, it is a well-known fact that the specificity of inhibitors decays with time. Nevertheless, at this stage the specificity for VDAC is remarkably high making these compounds the best candidates to use in probing the role of VDAC in mitochondrial function and apoptosis.

Oligonucleotides have been used to suppress the levels of specific proteins in target cells. The phosphorothioate linkage stabilizes them by making them relatively poor substrates for hydrolytic enzymes. The effects on VDAC now compromise this method of stabilization.

Commercial source

The phosphorothioate randomer is available from Trilink Industries. For reasons that are unclear, it is not listed as a VDAC inhibitor. The catalog number is O-30030-50. The catalog number for the phosphodiester control (same random sequence and same charge) is O-30040-50.

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