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Phosphorothioate oligonucleotides reduce mitochondrial outer membrane permeability to ADP

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[1]*Department of Biology, University of Maryland, College Park, Maryland;* [2]*Department of Biomedical Engineering, Columbia University, New York, New York;* [3]*Johns Hopkins University, School of Public Health, Baltimore, Maryland; and* [4]*Albert Einstein-Montefiore Cancer Center, Department of Oncology, Montefiore Medical Center, Bronx, New York*

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Tan W, Lai JC, Miller P, Stein CA, Colombini M. Phosphorothioate oligonucleotides reduce mitochondrial outer membrane permeability to ADP. *Am J Physiol Cell Physiol* 292: C1388–C1397, 2007. First published November 29, 2006; doi:10.1152/ajpcell.00490.2006.—G3139, an antisense Bcl-2 phosphorothioate oligodeoxyribonucleotide, induces apoptosis in melanoma and other cancer cells. This apoptosis happens before and in the absence of the downregulation of Bcl-2 and thus seems to be Bcl-2-independent. Binding of G3139 to mitochondria and its ability to close voltage-dependent anion-selective channel (VDAC) have led to the hypothesis that G3139 acts, in part, by interacting with VDAC channels in the mitochondrial outer membrane (21). In this study, we demonstrate that G3139 is able to reduce the mitochondrial outer membrane permeability to ADP by a factor of 6 or 7 with a K_i between 0.2 and 0.5 μM. Because VDAC is responsible for this permeability, this result strengthens the aforesaid hypothesis. Other mitochondrial respiration components are not affected by [G3139] up to 1 μM. Higher levels begin to inhibit respiration rates, decrease light scattering and increase uncoupled respiration. These results agree with accumulating evidence that VDAC closure favors cytochrome *c* release. The speed of this effect (within 10 min) places it early in the apoptotic cascade with cytochrome *c* release occurring at later times. Other phosphorothioate oligonucleotides are also able to induce VDAC closure, and there is some length dependence. The phosphorothioate linkages are required to induce the reduction of outer membrane permeability. At levels below 1 μM, phosphorothioate oligonucleotides are the first specific tools to restrict mitochondrial outer membrane permeability.

respiration; voltage-dependent anion-selective channel; apoptosis; cell death

G3139 (OBLIMERSEN) IS AN ANTISENSE 18mer phosphorothioate oligonucleotide that is targeted to the initiation codon region of the Bcl-2 mRNA (19). When transfected into melanoma and other cancer cell lines, G3139 downregulates the anti-apoptotic Bcl-2 mRNA and protein expression (20, 24, 31). However, G3139 induces cytochrome *c* release from mitochondria more than 10 h before the downregulation of Bcl-2 proteins (20), and there is no strict sequence dependence of its proapoptotic effect (21). The phenotype of G3139-treated prostate and melanoma cell lines is Bcl-2 independent because the siRNA targeted to the Bcl-2 mRNA does not produce apoptosis and has little or no effect on cellular viability (1). Thus G3139 must be exerting its effect in other ways.

Further research has demonstrated direct binding of G3139 to the mitochondrial surface and has correlated its potency to induce cytochrome *c* release with the level of voltage-dependent anion-selective channel (VDAC) expression in a cell-line dependent manner. G3139 has also been shown to inhibit the conductance of VDAC reconstituted into planar phospholipid membranes (21). As VDAC has been widely recognized as a participant in cell apoptosis, these findings suggest a new proapoptotic role for G3139.

VDAC is the major permeability pathway by which metabolites cross the mitochondrial outer membrane (9, 18, 33). There are claims that proapoptotic signals stimulate VDAC (alone or together with proapoptotic Bcl-2 family proteins) to form a larger channel by which cytochrome *c* is released into the cytosol (38, 39, 46). These proposals are in conflict with the physico-chemical properties of VDAC and, where tested, have been found to be incorrect (34, 35). Indeed, VDAC activity seems to be antiapoptotic. VDAC2 inhibits the activation of the proapoptotic protein Bak, suppressing its ability to induce apoptosis (5). An alternative proposal consistent with this view is that VDAC closure favors mitochondria-initiated apoptosis. VDAC, in its closed states, allows small ion flow but virtually excludes large highly charged metabolic anions, such as ADP and ATP (32). VDAC closure results in failure to exchange metabolites between the cytosol and mitochondria. This process, in a still undefined manner, favors the permeabilization of the mitochondrial outer membrane (MOM) and the release of cytochrome *c* and other proteins into the cytosol. Some of these proteins become components of the apoptosome (25), activating caspase-9 and initiating the apoptotic cascade.

The addition of G3139 to pure VDAC channels formed in phospholipid membranes results in channel closure (21). This demonstrates a direct effect of G3139 on VDAC in the absence of other proteins. Thus, G3139 is able to induce VDAC closure, in agreement with the hypothesis that VDAC closure favors apoptosis (44).

In this paper, we report a phosphorothioate-oligonucleotide-specific strong reduction of MOM permeability to ADP by G3139. This is consistent with the inferred in situ reduction of VDAC permeability and supports the hypothesis that G3139 induces cytochrome *c* release by closing VDAC.

MATERIALS AND METHODS

Measurement of MOM permeability and intactness. Mitochondria were isolated from rat liver (30). A portion of the mitochondrial suspension (about 1 mg mitochondrial protein) was diluted into 3 ml of respiration buffer containing 0.3 M mannitol, 10 mM NaH_2PO_4, 5 mM $MgCl_2$, and 10 mM KCl (pH 7.2). The mitochondrial respiration

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was measured according to the method of Lee et al. (22). The respiratory control ratio was typically above 5 (for succinate). Briefly, succinate (5 mM final) was added to the mitochondrial suspension followed by ADP addition (usually 80 μM). The oxygen consumption was measured by using a Clark oxygen electrode, and the MOM permeability was obtained by fitting to the theoretical model developed by Lee et al. (22). Because mitochondria respire at a slow rate (state IV respiration rate) even without ADP addition, the state IV respiration was subtracted from state III respiration to obtain the ADP-stimulated oxygen consumption. This result was converted to [ADP] as a function of time by multiplying the [oxygen] by the P/O ratio (2 for succinate as the substrate).[1] The derivative of the [ADP] vs. time is the rate of ADP consumption at any time. Each of the experiments in Figs. 1, 4*B*, 6*A*, 7*B* has been repeated three times using the same mitochondria. It has also been repeated at least three times using mitochondria isolated on different days.

The intactness of the MOM was calculated (11) from the KCN-sensitive rate of oxygen consumption following addition of 2.5 μM cytochrome *c* and 8 mM sodium ascorbate. This rate was compared with that of hypotonically disrupted mitochondria. Hypotonically disrupted mitochondria were prepared by adding 30 μl mitochondria into 1.5 ml of double-distilled water (on ice) and incubating for 3 min, followed by addition of 1.5-ml double-concentrated respiration medium.

To measure the respiration of mitoplasts, mildly shocked mitochondria were employed (23). These were generated by the addition to the mitochondrial suspension, 2 volumes of cold double-distilled water. The shocked mitochondria were incubated for 10 min on ice followed by the addition of 5 volumes of respiration buffer. Finally, 2 volumes of double-concentrated respiration buffer were added to restore normal osmotic pressure. These steps were used to minimize damage to the inner membrane.

Measurement of MOM intactness by adenylate kinase release assay. Mildly shocked mitochondria containing 1 mg protein were pelleted at 14,000 *g* for 5 min at 4°C, and the supernatant was kept on ice until assayed. Thirty microliters of supernatant was added to 700 μl of adenylate kinase reaction mixture (50 mM Tris·HCl, pH 7.5, 5 mM $MgSO_4$, 10 mM glucose, 5 mM ADP, 0.2 mM NADP, 10 units of hexokinase, and 10 units of glucose-6-phosphate dehydrogenase) (40). The activity of adenylate kinase was detected as an increase in absorbance at 340 nm. Intact mitochondria and mitochondria with lysed outer

[1] P/O ratio is defined as ATP produced per mole of O consumed for a particular substrate.

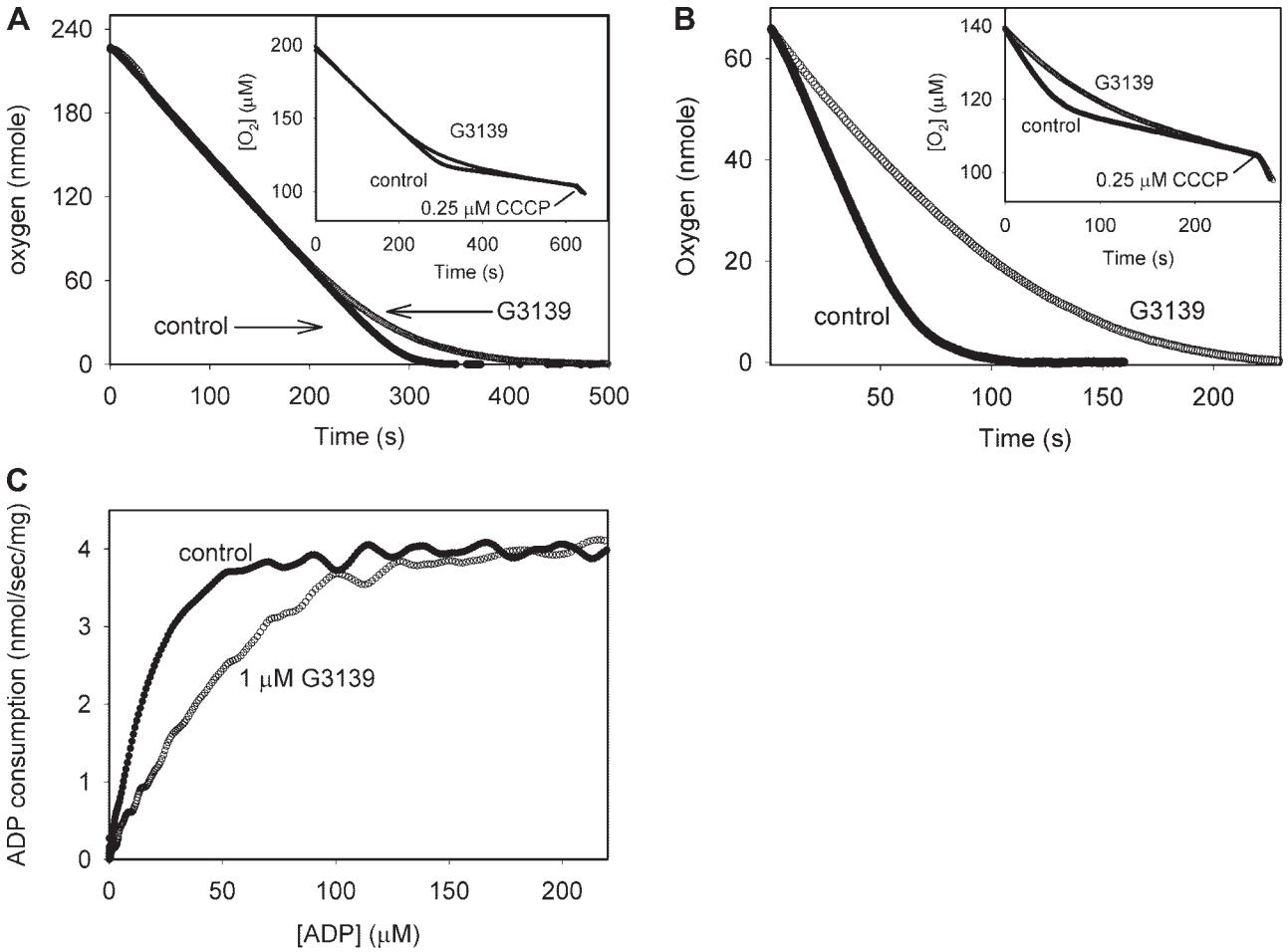

Fig. 1. An example of G3139-induced reduction of mitochondrial respiration between state III and state IV. *A*: O_2 consumption following the addition of 270 μM ADP to mitochondria in the presence or absence of 1 μM G3139 (*inset*). The rate of state IV respiration was subtracted from the respiration curve (main figure). *B*: O_2 consumption following the addition of 80 μM ADP to the 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 in *A* and *B*. *C*: ADP consumption rate vs. [ADP]. The ADP consumption rate was calculated from the oxygen consumption rate and the P/O ratio (2 for succinate as the substrate) and divided by the amount of mitochondrial protein present (1 mg). The O_2 consumption rate, at each point in *A*, was determined by calculating the slope at each point using the linear regression of 13 adjacent points.

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membranes served as negative and positive controls. The mitochondria with lysed outer membranes were hypotonically shocked by adding 50 volumes of cold double-distilled water to the mitochondria.

The assay of protein content. Mitochondrial protein was measured using the BCA method (Pierce, Rockford, IL) following the addition of Triton X-100 to the mitochondrial suspension (1% vol/vol final). BSA was the standard.

Oligonucleotides. G3139 was kindly donated by Dr. R. Brown, Genta (Berkeley Heights, NJ). The N-mers, the oligonucleotides of random sequence, were a generous gift of Trilink Biotechnologies (San Diego, CA).

Planar phospholipid membrane studies. The planar phospholipid membranes were generated according to standard methods (8, 27). The membranes were formed from phospholipid monolayers consisting of diphytanoyl phosphatidylcholine, asolectin (soybean phospholipid polar extract), and cholesterol (1:1:0.1 mass ratios).

VDAC was purified from mitochondria isolated from rat liver (2, 13). A 1- to 3-μl aliquot of the VDAC-containing solution (2.5% Triton X100, 50 mM KCl, 10 mM Tris, 1 mM EDTA, 15% DMSO, pH 7.0) was stirred into 4–6 ml of aqueous solution containing 1.0 M KCl, 5 mM $CaCl_2$, 1 mM EDTA, and 5 mM HEPES (pH 7.2) on the *cis* side of the chamber. The *trans* side, containing the same aqueous solution, was held at virtual ground by the voltage clamp. All of the experiments were performed at ~23°C.

Cell culture. The mycoplasma-free human melanoma cell line 518A2 was a kind gift of Dr. Volker Wacheck (University of Vienna, Vienna, Austria). Cells were grown in DMEM supplemented with 10% heat-inactivated FBS and 100 U/ml penicillin G (sodium salt) and 100 μg/ml streptomycin sulfate. Stock cultures of all cells were maintained at 37°C in a humidified 5% CO_2 incubator.

Subcellular fractionation and oligonucleotide treatment of mitochondria from melanoma cells. Cells were harvested by trypsinization and were washed with cold PBS. Cell pellets were resuspended in 300 μl of buffer A (250 mM sucrose, 10 mM Tris·HCl, pH 7.4, 1 mM EGTA, 50 μg/ml Pefabloc, and 15 μg/ml leupeptin, aprotinin, and pepstatin). Cells were then homogenized on ice in a dounce homogenizer until ~90% of cells were disrupted, as judged by Trypan blue staining. Crude lysates were centrifuged at 1,000 *g* for 10 min at 4°C twice to remove nuclei and unbroken cells. The supernatant was collected and subjected to a 10,000 *g* centrifugation for 30 min at 4°C. The pelleted mitochondria were resuspended in 20 μl of energizing *buffer B* (250 mM sucrose, 10 mM Tris·HCl, pH 7.4, 1 mM EGTA, 50 μg/ml Pefabloc, 10 mM KCl, 3 mM KH_2PO_4, 5 mM succinate, 100 μM ADP, and 15 μg/ml leupeptin, aprotinin, and pepstatin), which contained increasing concentrations of oligonucleotides (10 to 40 μM). After 2 h of incubation at 10°C water bath, samples were centrifuged to pellet the mitochondria, and the supernatant was colleted and subjected to Western blot analysis for cytochrome *c* release.

Western blot analysis. Aliquots of protein samples, containing 25–40 μg of protein, were resolved by SDS-PAGE, and then transferred to Hybond ECL filter paper (Amersham, Arlington Heights, IL). The filters were incubated at room temperature for 1–2 h in 5% milk in TBS containing 0.5% Tween 20. The filters were then probed with 1:200 dilutions of the anti cytochrome *c* antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in 5% milk in TBS containing 0.5% Tween 20 at 4°C overnight. After being washed in TBS containing 0.5% Tween 20, the filters were incubated for 1 h at room temperature in 5% milk in TBS containing 0.5% Tween with a 1:3,000 dilution of a peroxidase-conjugated secondary antibody (Amersham). After the washing (3 × 10 min), ECL was performed according to the manufacturer's instructions.

RESULTS

G3139 reduces the MOM permeability to ADP by interacting with VDAC. Mitochondrial respiration is coupled to ADP phosphorylation through the protomotive force (PMF). The stimulation of respiration by ADP phosphorylation requires a continuous flow of ADP from the cytosol into the mitochondrial matrix, involving the permeation through the MOM and translocation across the mitochondrial inner membrane (MIM) through the adenine nucleotide translocator (ANT). The phosphorylation of ADP is fast and the two permeation processes are rate limiting. At high extramitochondrial ADP concentrations, the translocation through the MIM is saturated, and the mitochondria undergo state III respiration, which is characterized by a constant rate of oxygen consumption. However, as the ADP level declines, the flux through the MOM becomes rate limiting, and thus mitochondria consume ADP and oxygen more slowly. When ADP is totally depleted, mitochondria undergo state IV respiration. Thus the transition between state III and state IV respiration provides information about the MOM permeability to ADP. (Please note that the permeability to ATP should also be reduced, but this was not measured.) The broader the transition between the states, the lower is the permeability of the outer membrane (22). Following the method of Lee et al. (22), the permeability of the MOM to ADP was calculated from this transition.

As shown in Fig. 1, *A* and *B*, when 1 μM G3139 was added to isolated mitochondria, it reduced the rate of respiration in the transition between state III and state IV. The calculated rates of ADP consumption are shown in Fig. 1*C*. In the presence of G3139, the rate of ADP consumption saturates at more than 100 μM external ADP. Without G3139, the rate of ADP consumption essentially saturates at about 50 μM external ADP. At the lower ADP levels, which are more physiological, 1 μM G3139 reduced the rate of state III respiration by as much as 50% (Fig. 1*C*). The presence of adenylate kinase inhibitor, P1,P5-di(adenosine-5′)pentaphosphate, does not change G3139 inhibition of mitochondrial respiration, showing that adenylate kinase is not involved (data not shown).

As VDAC is the major pathway for metabolite flux across the MOM (7, 9, 33), the reduced permeability should be due to

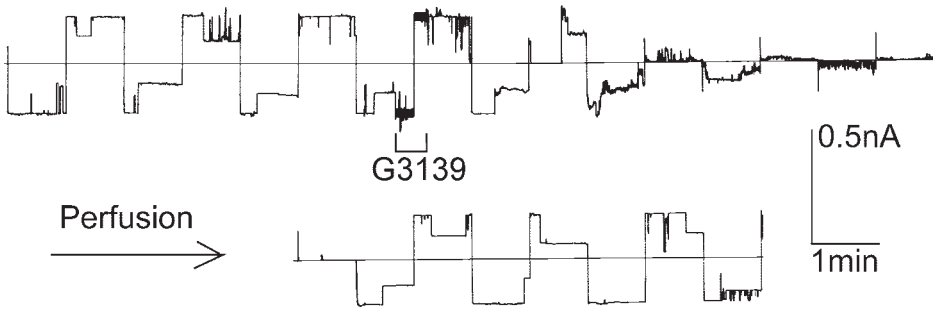

Fig. 2. A single voltage-dependent anion-selective channel (VDAC) channel was reconstituted into a planar phospholipid membrane as described in the MATERIALS AND METHODS. The voltage was held at either +50 mV or −50 mV and alternated every 50 s. The long horizontal line indicates zero current. G3139 (40 μM final) was added where indicated. Before the perfusion, there were 7 min of record (deleted to save space) where the channel was fully closed. The side of addition of G3139 was perfused with 30 ml (6 times of chamber volume) of buffer for 5 min.

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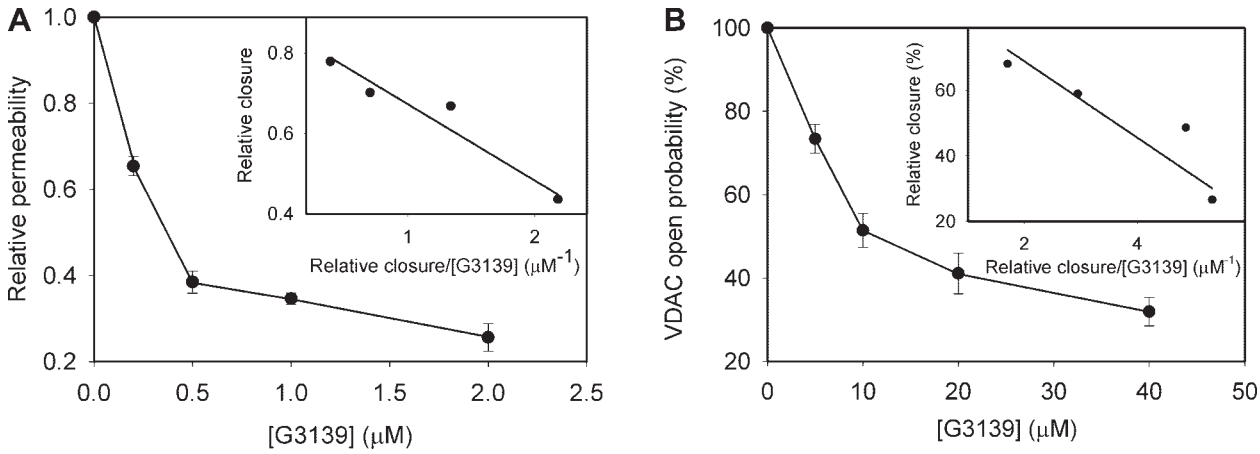

Fig. 3. *A*: concentration dependence of G3139-induced mitochondrial outer membrane (MOM) permeability reduction. This is the average of the results obtained in three independent experiments. Mitochondria were added to 3 ml of respiration buffer with final protein concentration of 370 μg/ml. The results were normalized to the permeability in the absence of G3139. The error bars are the standard deviations of the three independent experiments. *B*: concentration dependence of G3139-induced VDAC closure. Each point is the average of at least three separate experiments. The indicated amount of G3139 was added to the *cis* side of the chamber. The voltage was held at either +50 mV or −50 mV and alternated every 50 s. Relative closure is the fractional decrease in permeability or conductance. The error bars are the SEs of 3–5 independent experiments.

VDAC closure. This was also observed in planar membrane experiments, which assess the direct interaction between G3139 and VDAC. Forty micromolar of G3139 were able to fully close a VDAC channel reconstituted into a planar membrane (Fig. 2). Channels closed in this way remain closed irrespective of the applied voltage. This effect can be reversed by removing G3139 from the system (Fig. 2), demonstrating a reversible interaction. The reversibility was also observed in mitochondrial respiration experiments (data not shown). The direct effect of G3139 on VDAC indicates that VDAC closure is responsible for the lowered respiration rate observed at low ADP levels. Closure of some of the VDAC channels slows down the permeation of ADP and decreases the intermembrane space and matrix ADP levels.

Concentration dependence of the potency of G3139. G3139 reduces MOM permeability in a concentration-dependent manner (Fig. 3*A*). At a concentration of 1 μM, G3139 is able to reduce the permeability by 70%. This concentration of G3139 is similar to the estimated cellular level required to induce cytochrome *c* release from mitochondria (21). The concentration dependence data can be further analyzed to obtain the concentration at half inhibition K_i, and the maximum permeability reduction P_{max}.

Assuming a 1 to 1 stoichiometry between G3139 (G) and a single permeability unit (V), which forms a closed permeability unit (GV) with the equilibrium constant K, the ratio of active open and closed permeability units is $P_{closed}/(P_{max} - P_{closed}) = K[G]$, where P_{closed} is the permeability reduction (i.e., P_{max} minus the remaining permeability). This equation can be rearranged to yield $P_{closed} = -1/K \cdot (P_{closed}/[G]) + P_{max}$.

A linear plot of P_{closed} vs. $P_{closed}/[G]$ was generated to determine K and P_{max} (Fig. 3, *A* and *B*). K_i is equal to 1/K. The inset of Fig. 3*A* shows the linearized results that yield a K_i of 0.18 μM and maximum permeability reduction of 85%.

G3139 also closes VDAC channels in a concentration-dependent manner (Fig. 3*B*). However, a higher concentration is needed for G3139 to induce the same extent of closure as observed in the mitochondrial experiments. The K_i of 9.6 μM is about 50 times greater, but the maximum conductance drop of 86% is indistinguishable in the two types of experiments (Fig. 3*B*, *inset*). The difference in sensitivity could be due to endogenous mitochondrial factors that influence the closure of VDAC, factors eliminated by VDAC purification and reconstitution into phospholipids membranes. One example of such a factor is the presence of modulating proteins in the mitochondrial intermembrane space that induce the VDAC closure by favoring the closed states (9). Despite the quantitative difference, both results are strikingly similar, thus providing evidence that G3139 is actually closing VDAC in the MOM.

G3139-induced mitochondrial respiration reduction is not due to release of cytochrome c. G3139 causes the release of cytochrome *c* from mitochondria, but this release takes time and is evident after 2 h (21). The permeability changes reported here are early and immediate effects. However, we observed that elevated levels of G3139 induced small decreases in light scattering at 600 nm, consistent with some mitochondrial swelling (Fig. 4*A*). Large-scale mitochondrial swelling is usually due to the formation of a permeability transition, and this could lead to outer membrane tearing and cytochrome *c* release[2]. Loss of cytochrome *c* impairs electron transport and therefore respiration. However, at concentrations between 0.5 and 1 μM, G3139 does not induce significant swelling ($n = 3$, 95% confidence). Even at concentrations of 2 or 5 μM of G3139, the apparent swelling is very small compared with swelling induced by calcium ions, associated with the permeability transition. This slight change in light scattering is unlikely to reflect a matrix swelling that would tear the outer membrane and release cytochrome *c* (Fig. 4*A*).

Cyclosporin A is known to prevent outer membrane damage and cytochrome *c* release caused by swelling and permeability transition. We find that it partially inhibits G3139-induced

[2] Even at the low ionic strength used, mild hypotonic shocks that damage the outer membrane result in reductions in the rate of state III respiration that can be reversed by addition of cytochrome *c*.

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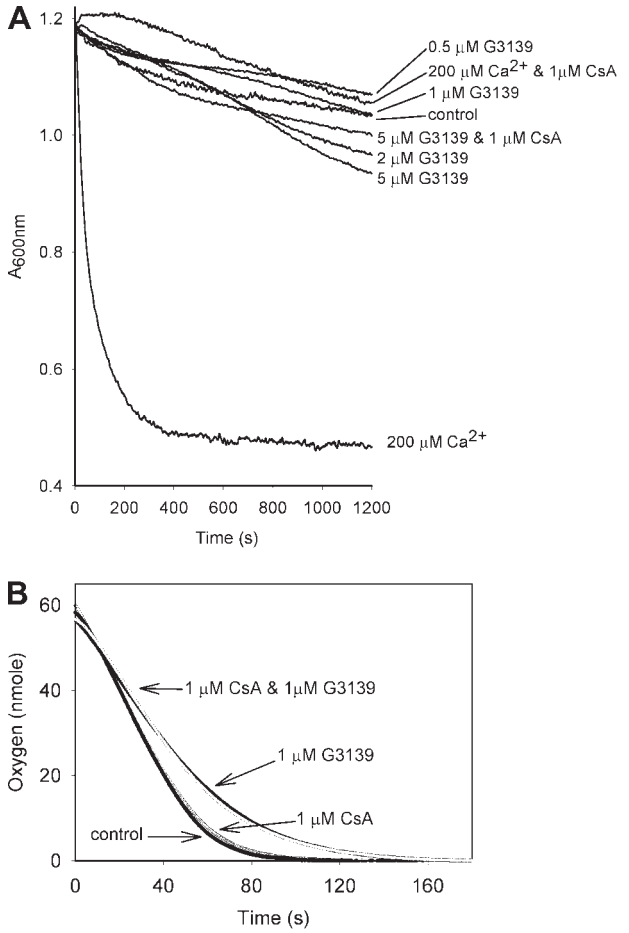

Fig. 4. *A*: mitochondrial swelling in the absence or presence G3139 and/or cyclosporin A. It was measured as light scattering at 600 nm. Mitochondria were added into the respiration buffer containing 5 mM succinate and 80 μM ADP. The final mitochondrial protein concentration was 320 μg/ml. *B*: cyclosporin A does not influence the ability of G3139 to reduce MOM permeability to ADP. Mitochondrial O_2 consumption was recorded following the addition of 80 μM ADP. The mitochondrial protein concentration was 350 μg/ml. The rate of state IV respiration was subtracted from the entire respiration curve.

mitochondrial swelling (Fig. 4*A*). However, treatment with cyclosporin A doesn't change the G3139-induced reduction of outer membrane permeability to ADP. (Fig. 4*B*, Table 1). To confirm that, 2.5 μM of cytochrome *c* was added to the mitochondrial suspension. If the MOM of some of the mitochondria in the population had become permeable to cytochrome *c*, respiration of these mitochondria would have been suppressed due to a functional break in the electron transport chain. (We observe this after mild hypotonic shock of the mitochondria.) Restoring the cytochrome *c* would restore this respiration at least in part. However, the addition of cytochrome *c* did not significantly change the measured respiration rate, the calculated permeability value or the G3139-induced permeability change (Table 1). The measured degree of mitochondrial intactness [96% ± 1% (n = 3)] shows no significant change [1% ± 1% (n = 3)] after treatment with 5 μM of G3139 [95% ± 1% (n = 3)] for 30 min, which is the time range of each experiment. This is consistent with the published finding that cytochrome *c* release takes time and is evident after 2 h of treatment with G3139 (21).

The effects of G3139 on the inner membrane cannot explain the permeability change. Despite the fact that G3139 did not damage the MOM nor release cytochrome *c* during short-term incubations, it might still permeate through VDAC and affect the components of the electron transport chain located in the mitochondrial inner membrane. In this way, it could reduce the enzymatic activities of the electron transport complexes and thus inhibit respiration. It could also inhibit the ANT on the MIM, thus reducing the rate of ADP/ATP translocation through the inner membrane and lower the matrix ADP concentration, inhibiting respiration.

The effects of G3139 on the vital respiratory functions of the inner membrane were tested by measuring state III, state IV, and uncoupled respiration rates as a function of [G3139] (Fig. 5). Please note that true state III is only achieved, in the presence of G3139, at high levels of added ADP. We rationalize that the high levels overcome the reduced outer membrane permeability, allowing maximal ADP-dependent stimulation of respiration. Thus at 80 μM ADP, there is an apparent reduction in ADP-dependent respiration that probably arises from the reduced permeability of the outer membrane. Even at the high ADP levels used (as in Fig. 1*A*), G3139 reduces state III and increases state IV respiration, resulting in a reduced respiration control ratio. However, at 0.5 μM and 1 μM, these effects are not significant. The increase in state IV at higher concentrations is consistent with some uncoupling. The decrease of state III respiration rate was mainly caused by inhibition of the electron transport chain, confirmed by the carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) uncoupled mitochondrial respiration rate (Fig. 5*B*). CCCP eliminates the protomotive force and should result in maximal rates of respiration. At 5 μM G3139 there was a significant drop in the rate of respiration in the presence of CCCP. However, even at a concentration of 5 μM, G3139 only influenced state III/state IV respiration by 20% compared with more than 80% reduction in MOM permeability (Fig. 5). Thus, effects observed at higher concentrations of G3139 cannot account for the permeability change (Fig. 5). Moreover, the ratio of state III to uncoupled rate does not change with increased amounts of G3139, indicating that ANT can fully function at least at high ADP concentrations (Fig. 5*B*).

Table 1. *Cyclosporin A and cytochrome c do not alter the calculated values of the reduction in MOM permeability to ADP induced by G3139*

	Permeability, $cm^3 \cdot s^{-1} \cdot g\ protein^{-1}$	Percent Inhibition
No G3139	242 (17)	
1 μM G3139	116 (6)	52
1 μM cyclosporin A	210 (20)*	13
1 μM cyclosporin A and 1 μM G3139	120 (16)†	50
2.5 μM cytochrome c and 1 μM G3139	126 (5)†	48
1 μM cyclosporin A and 1 μM G3139 and 2.5 μM cytochrome *c*	115 (2)†	52

Values are presented as average (SD) of three of four experiments. The permeability values given here are the permeability per gram mitochondrial protein (at 400 μg/ml). All of the results of the experiments containing 1 μM G3139 are significantly different from control (99.9% confidence). *No significant difference from control (95% confidence). †No significant difference from the result obtained with only 1 μM G3139 (95% confidence).

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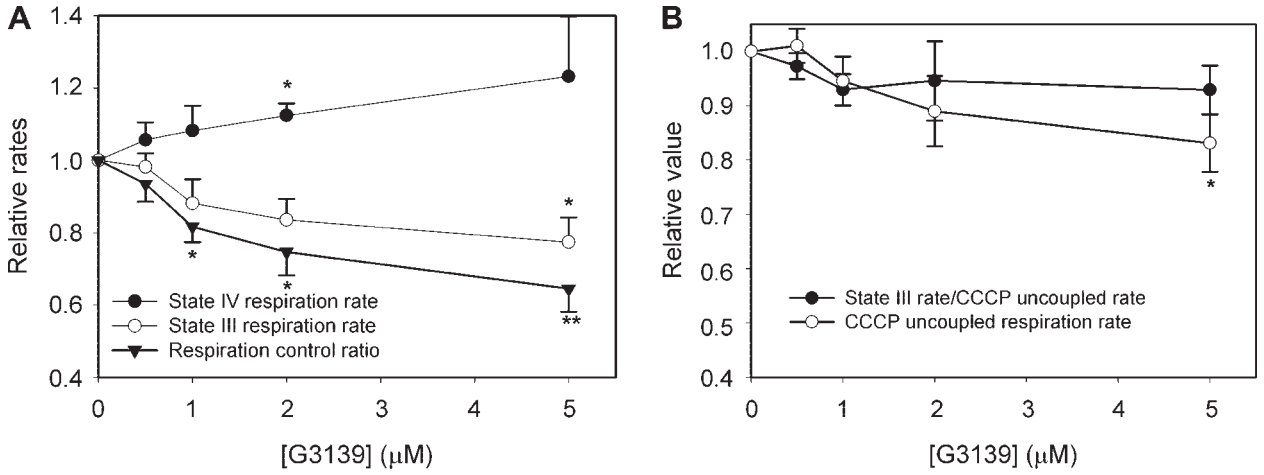

Fig. 5. The effects of G3139 on mitochondrial state III respiration, state IV respiration, respiratory control ratio (RCR), and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) uncoupled respiration. The values were normalized to the value in the absence of G3139 (RCR: 6.0 ± 0.7, state III respiration rate: 1.2 ± 0.1 nmole $O_2 \cdot s^{-1} \cdot$mg mitochondrial protein^{-1}, state IV respiration rate: 0.21 ± 0.03 nmole $O_2 \cdot s^{-1} \cdot$mg mitochondrial protein^{-1}, CCCP uncoupled respiration rate: 1.5 ± 0.2 nmole $O_2 \cdot s^{-1} \cdot$mg mitochondrial protein^{-1}). The values were given as means ± SE of four independent experiments with each experiment performed in triplicate. ADP (270 μM) was added in those experiments to reach the maximum state III respiration. Statistical tests indicate the significance of the difference compared with control experiments. (*$P < 0.05$, **$P < 0.01$).

In isolated mitochondria, one can add high levels of ADP to achieve maximal respiration rates, but, physiologically, ADP levels are much lower and thus G3139 would reduce ADP-dependent respiration. For example, when 80 μM ADP was added to mitochondria pretreated with 1 μM G3139, the initial respiration rate was 48% ± 6% of the uncoupled rate, which is significantly less than the ratio of the control (76% ± 4%) experiments (means ± SE, 4 independent observations; $P <$ 0.05). This reinforces the conclusion that at physiological ADP levels, G3139 can directly inhibit mitochondrial respiration.

Further evidence that G3139 acts by reducing outer membrane permeability, was obtained from mildly shocked mitochondria. The mitochondrial outer membrane was damaged to bypass any permeability barrier of this membrane. Both mild hypotonic shock and carefully titrated amounts of digitonin were used for the initial trials. Of these, the hypotonic shock did the least damage, as assessed by measuring ADP-dependent respiration. There was an unavoidable reduction in respiration following hypotonic shock, and a trade-off was achieved between MOM breakage and respiration reduction. The effects of G3139 are clear despite this limitation. The state III/state IV respiration with and without G3139 is nearly the same, especially compared with the huge difference observed with the intact mitochondria (Fig. 6*A*). Even more persuasive, in the presence of 1 μM G3139, disruption of the outer membrane restored state III respiration to a level close to the rate observed without G3139, indicating that the major factor inhibiting respiration is the limitation of the rate of ADP transport through the outer membrane (Fig. 6*A*). However, it is clear that mildly shocking only partially restores the permeability value

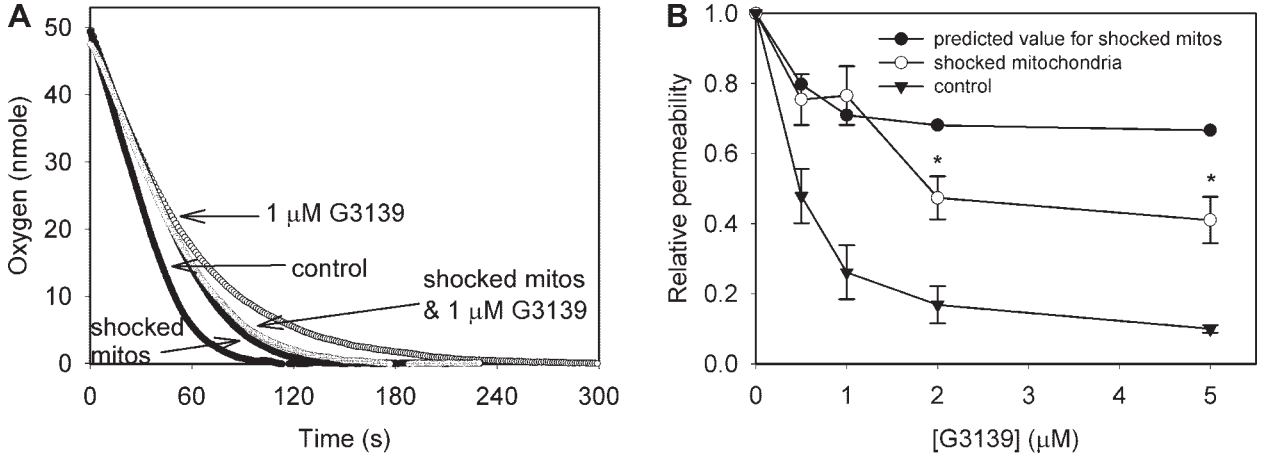

Fig. 6. Low concentrations of G3139 have a minimal effect on mitochondrial respiration after the outer membrane has been broken. *A*: mitochondrial O_2 consumption was recorded as described in Fig. 4*B*. Mildly shocked mitochondria (mitos) were obtained by exposing them to a 100 mOsmolar solution for 10 min on ice. The normal osmotic pressure was restored as described in MATERIALS AND METHODS. *B*: comparison of mitochondrial outer membrane permeability changes induced in mildly shocked and normal mitochondria by G3139. All values were measured after 10 min of treatment with G3139. The error bars are the SE of four independent experiments. The adenylate kinase activity assay showed that after a mild shock, 37% ± 1% ($n = 4$), mitochondria were still intact. This result was used to generate the predicted change in permeability of mildly shocked mitochondria after G3139 treatment. Statistical tests indicate the significance of the difference between predicted values and measured values (*$P < 0.05$).

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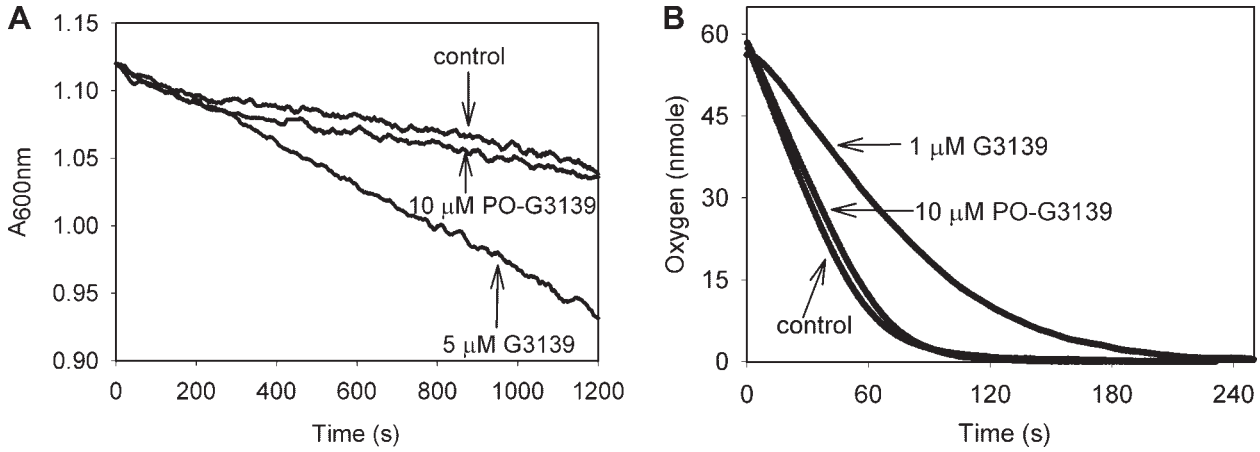

Fig. 7. The phosphodiester version of G3139 does not induce mitochondrial swelling nor does it reduce the permeability of the MOM. Mitochondrial swelling and O_2 consumption were performed as in Fig. 4, *A* and *B*, respectively. The mitochondrial protein concentration was 320 μg/ml. These results are typical of three independent experiments.

(Fig. 6*B*). Is this a sign that G3139 affects ANT? To address this quantitatively, one must compensate for the mitochondria that remained intact following the hypotonic shock. The adenylate kinase activity assay showed that 37% ± 1% ($n = 4$) of mitochondria were still intact after a mild shock. These remaining intact mitochondria should still respond to G3139 and thus there should still be a small reduction in MOM permeability upon addition of G3139. Assuming the remaining intact mitochondria were still inhibited by G3139, we calculated predicted values of changes in relative permeability upon the addition of G3139 (Fig. 6*B*). It is clear that at 0.5 and 1 μM concentrations, the restoration of permeability following hypotonic shock was complete (compare experimental values to predicted values). However, at 2 and 5 μM, G3139 had a greater inhibitory effect than can be accounted for by incomplete MOM damage and therefore, at these higher levels, G3139 does have effects on inner-membrane processes.

These results demonstrate that G3139 does not significantly alter the activity of ANT or the complexes of the respiratory chain at submicromolar concentrations. Even at higher concentrations, the effect of G3139 on the inner membrane cannot explain the reduction of permeability. Thus, the conclusion we draw is that G3139 can interact with VDAC channels in the MOM and reduce their ability to translocate ADP.

Important features of the oligonucleotide. The isosequential phosphodiester analog of G3139 does not induce mitochondrial swelling, and it does not inhibit the respiration of mitochondria (Fig. 7). This agrees with our previous findings that this single-stranded oligodeoxyribonucleotide does not interact with VDAC and does not induce cytochrome *c* release from mitochondria (21). In fact, there is a small increase of the MOM permeability, which is understandable, considering that this highly charged molecule can decrease the voltage difference across the VDAC channels in the MOM (9) and thus could favor the open state.

To evaluate the importance of the G3139 sequence in its interaction with VDAC, phosphorothioate oligonucleotides with a completely randomized sequence were used. These so-called N-mers ($n = 12, 14, 16, 18$), which contain an equal mixture of each nitrogenous base at each position in the oligonucleotide chain, were added to isolated mitochondria, and the MOM permeability was measured. All tested N-mers inhibited this permeability to some extent (Table 2). This is consistent with their abilities to induce cytochrome *c* release from isolated mitochondria of melanoma cell lines 518A2 (Fig. 8). G3139 and N-mers, except 10-mers, all induce significant release of cytochrome *c* from isolated mitochondria after 2 h treatment in a concentration-dependent manner (Fig. 8), as demonstrated by Western blot analysis. Those results indicate that the specific G3139 sequence is not critical for the interaction with VDAC and induction of cytochrome *c* release from isolated mitochondria. In addition, the interaction is probably not limited to just a small number of sequences because the concentration of any one specific sequence in an N-mer is less than femtomolar. However, as the length increases, the ability of the N-mer to reduce the MOM permeability and induce cytochrome *c* release also increases (Table 2, Fig. 8). These data indicate that longer oligonucleotides in the range we tested have a stronger interaction with VDAC channels on the MOM and subsequently a greater ability to induce cytochrome *c* release from isolated mitochondria. This is also consistent with the length dependence reported for closure of pure VDAC reconstituted into phospholipid membranes (21).

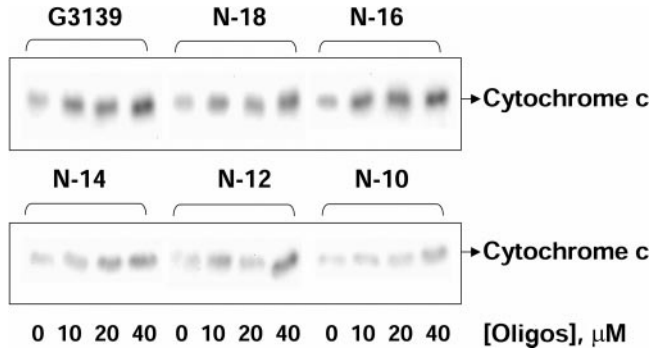

Fig. 8. G3139 and equivalent random sequences release cytochrome *c* from mitochondria in a concentration-dependent manner. Isolated mitochondria from 518A2 melanoma cells were treated with 10–40 μM of indicated oligonucleotides for 2 h at 10°C in *buffer B* and the supernatants were collected. Cytochrome *c* released into the supernatant from the mitochondria was analyzed by Western blotting.

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DISCUSSION

G3139 targets the initiation codon region of the Bcl-2 mRNA. Initially, it was believed that its ability to induce apoptosis in cancer cells relied on the downregulation of Bcl-2 protein, at least in vitro. However, recent work casts doubt on this hypothesis and implicates VDAC as a major target of G3139 (21). In this study, we provide direct evidence that G3139 reduces the MOM permeability, building a bridge between G3139 binding to mitochondria and the subsequent release of cytochrome *c*.

The fact that VDAC is the major pathway for metabolites indicates that G3139 reduces the permeability of the MOM by closing VDAC. This is supported by the fact that phosphorothioate oligodeoxynucleotides induce closure of VDAC reconstituted into planar phospholipid membranes.

The major effect of G3139 is on the MOM. We have demonstrated this in a variety of ways: *1*) The G3139-induced reduction of permeability to ADP is not inhibited by cyclosporin A (Fig. 4*B*), indicating that it is not due to a permeability transition. *2*) At concentrations less than or equal to 1 μM, the effect of G3139 on the MIM is insignificant (Figs. 5 and 6*B*), and even at higher concentrations, the effect on MIM cannot fully explain the permeability reduction. (Figs. 5, 6*B*). *3*) Damaging the MOM actually restores state III respiration that was inhibited by 0.5 and 1 μM G3139, indicating that the inhibition is on the outer membrane, not on the ANT (Fig. 6*A*). *4*) The constant ratio of state III to CCCP uncoupled respiration rate confirms that ANT was not inhibited by G3139 (Fig. 5*B*).

Additionally, the mitochondrial permeability reductions induced by G3139 were essentially the same when 5 mM malate and glutamate were used as the substrate (Fig. 9), showing that pyridine nucleotides were not depleted from the matrix during G3139 treatment.

Thus, the main effect is that G3139 is able to reduce MOM permeability to ADP by closing VDAC channels in the outer membrane. From Fig. 2, closure of VDAC by G3139 results in a total loss of conductance, and thus both ATP and ADP are not permeable through the channels. Thus the remaining permeability of the outer membrane is through VDAC channels that have not been closed by G3139. What we report is an average drop of the mitochondrial outer membrane permeability to ADP. This drop slows down both the steady-state ADP influx and ATP efflux, resulting in an inhibition of respiration at physiological ADP level (Fig. 1*B*).

Table 2. *Permeability of mitochondria to ADP in the presence or absence of 0.5 μM G3139 or N-mers (n = 12, 14, 16, 18)*

	Permeability $cm^3 \cdot s^{-1} \cdot g\ protein^{-1}$	Percent Inhibition
Control	244 (23)	
G3139	135 (16)†	45
12mers	168 (13)*	31
14mers	135 (7)†	45
16mers	132 (7)†	46
18mers	116 (20)†	52

Values are presented as average (SD) of three experiments. Statistical tests indicate significance in the reduction of permeability (*$P < 0.01$, †$P < 0.005$). All the results of oligomers except 12mers are not significantly different from that of G3139.

The mechanism and specificity of the oligonucleotide-induced permeability decrease. Our study has shown that the length of the oligonucleotide plays a role in its ability to affect the MOM permeability and cytochrome *c* release from mitochondria, while the sequence may be less important (Table 2). The presence of phosphorothioate linkages in the oligomer is very important for its ability to reduce the MOM permeability (Fig. 7) and subsequently induce cytochrome *c* release and cell apoptosis (21).

The importance of the phosphorothioate linkage has also been observed in other oligonucleotide-protein interactions. The binding of a phosphorothioate oligodeoxyribonucleotide to albumin increased with the number of phosphorothioate linkages (16). The binding of dA_{36} to the g5p protein increases more than 300-fold after substitution of sulfur for oxygen in an oligonucleotide (29).

The differences between sulfur and oxygen atoms may provide insight into the requirement for the phosphorothioate

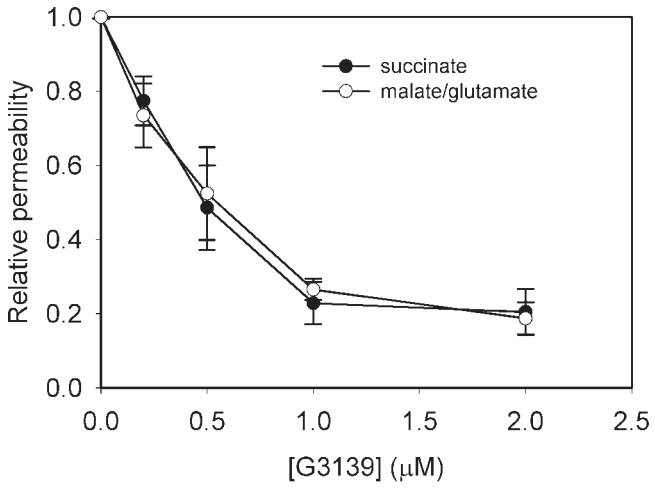

Fig. 9. Comparison of the concentration dependence of the reduction in MOM permeability induced by G3139 in the presence of 5 mM of either succinate or malate/glutamate as the substrate. The mitochondrial protein concentration was 320 μg/ml. The results shown here were from one experiment performed in triplicate. This is typical of three independent experiments.

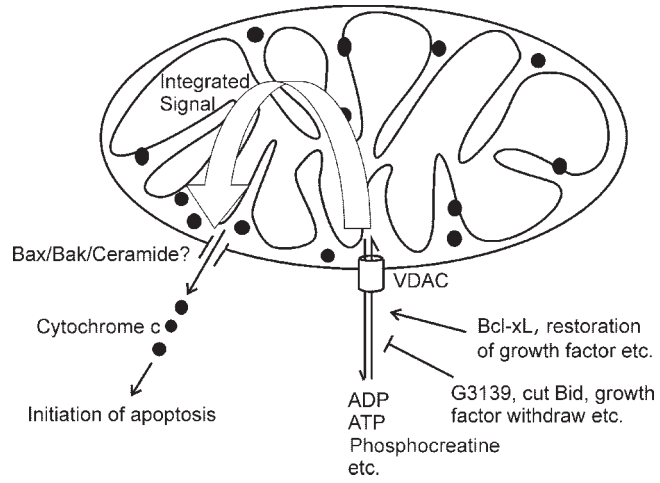

Fig. 10. Hypothetical model of the role of VDAC in apoptosis. VDAC integrates apoptotic signals changing the propensity for release of intermembrane space proteins. VDAC closure favors cytochrome *c* release through some not clearly identified mechanism.

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linkage. Compared with oxygen, the sulfur atom has less electronegativity, and its larger van der Waal's radius decreases its surface charge density, which may allow it to carry a full negative charge in solution. (12, 14). The sulfur also lacks the ability to hydrogen bond with water, and so water dissociates more readily from the oligonucleotide. This is critical because the binding energy is the difference between the energy of interaction between the protein and the oligonucleotide and the energies of dehydration of the interacting surfaces. The low-charge density of the sulfur also reduces the enthalpy needed to strip small ions before binding to VDAC (6). This property also increases the polarizability (37) of the sulfur atoms, strengthening the interaction with lower charge density groups found in proteins.

A simple explanation for the length dependence of the permeability decrease induced by the phosphorothioate oligonucleotides with a randomized sequence is the importance of sheer size. A larger inhibitor would generate a more effective steric block or electrostatic block of the channel. However, the reality may be more complex. For example, multiple interactions may be needed to stabilize the complex.

The results are consistent with a one-to-one interaction between G3139 and VDAC. This might indicate a single binding site on VDAC or that electrostatic repulsion precludes the binding of two oligonucleotides. The analysis is complicated by an incomplete reduction in permeability. The latter finding is in harmony with the finding that some VDAC channels are resistant to closure by G3139 (21).

The physiological conditions may modify the interaction. The ability of G3139 to influence mitochondrial respiration rates depends on the free [ADP]. At high ADP levels the effects of G3139 are quite small because the lower MOM permeability is compensated by the higher [ADP]. Inside the cell, more than half the ADP is bound to proteins (3, 4, 28). The free [ADP] from various cell types is not directly known but has been estimated to be 6–90 μM (3, 36, 45). The failure to detect the ADP by ^{31}P-NMR argues for the real concentration to be in the low end of that range (3, 36). In any event, this range is within the concentration range at which G3139-induced MOM permeability reduction limits the rate of ADP phosphorylation.

G3139 induces reductions of MOM permeability at doses that are more than one order of magnitude lower than those needed to achieve comparable reductions in the conductance of a VDAC-containing membrane. These differences in sensitivity between experiments on isolated mitochondria and those performed on pure VDAC in phospholipid membranes indicate that physiological conditions may actually augment the interaction and/or magnify the effect (Fig. 3, *A* and *B*). The presence of regulatory proteins and other local factors that favor VDAC closure (9) may act synergistically with G3139. The voltage difference across MOM may also contribute to the stronger in vivo effect.

Differences in sensitivity also appear among different mitochondria isolated on different days. The K_i values vary between 0.2 to 0.5 μM. These differences could not be attributed to differences in weight/age of the animal. Also all animals were male and were on the same diet. Hence, the difference likely arises from differences in the physiological state of the animal: different levels of VDAC regulatory proteins in the intermembrane space, different levels of electrical potential across the outer membrane, or different amounts of Bcl-2 family proteins in the MOM. For example, Bcl-xL is antiapoptotic and has been reported to open VDAC channels and to increase the MOM permeability (44). There may be competition between G3139 and Bcl-2 family proteins.

The apoptotic implication of the interaction. VDAC's role in early apoptotic events is still controversial. There is evidence that VDAC is not involved in the apoptosis of *Saccharomyces cerevisiae* (17), but apoptosis in yeast is very different from apoptosis in multicellular organisms. Indeed, it is generally believed that VDAC is important in the initiation of apoptosis, but different and sometimes conflicting mechanisms have been proposed. Many reports claim that VDAC is a part of the permeability transition pore (PTP) (10, 26, 41, 42). The opening of PTP causes dissipation of the mitochondrial membrane potential and matrix swelling, which leads to cytochrome *c* release and cell apoptosis. Some claim that VDAC can oligomerize (46) or associate with Bax (39) to form a pathway for the release of proteins from mitochondria. We favor a third mechanism, which is that VDAC closure leads to the initiation of apoptosis (43). A drastic reduction in the rate of exchange of metabolites between the mitochondria and the cytosol leads to unidentified changes that result in the release of proteins from mitochondria. This hypothesis is supported by a variety of observations (34, 44) and is consistent with the present findings. G3139 closes VDAC and induces cytochrome *c* release and apoptosis in cells. The perfect match of the abilities of N-mers to decrease MOM permeability and to induce cytochrome *c* release from isolated mitochondria (Table 2, Fig. 8) further implicate VDAC closure as the initial step leading to cytochrome *c* release from mitochondria.

In conclusion, phosphorothioate oligonucleotides ($n > 12$) are able to reduce MOM permeability through closing VDAC channels. Our findings support the hypothesis that VDAC closure leads to apoptosis and that G3139 closes VDAC channels in the MOM. In the cell the situation is more complex. G3139 is more potent than a random sequence. Among the possible factors is the presence of CpG motifs (15). Therefore, the action of G3139 is multifaceted with VDAC closure being only one of these facets.

VDAC can be viewed as an antiapoptotic protein, whose open state guarantees the exchange of metabolites through the MOM and inhibits cell apoptosis. It is also a transducer of apoptotic signals, integrating the information and communicating it in the form of a change in MOM permeability (Fig. 10).

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