# Virus-induced permeability transition in mitochondria

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**Abstract Isolated rat liver mitochondria undergo permeability transition after supplementation with a suspension of tobacco mosaic virus. Four mitochondrial parameters proved the opening of the permeability transition pore in the inner mitochondrial membrane: increased oxygen consumption, collapse of the membrane potential, release of calcium ions from mitochondria, and high amplitude mitochondrial swelling. All virus-induced changes in mitochondria were prevented by cyclosporin A. These effects were not observed if the virus was treated with EGTA or disrupted by heating. Protein component of the virus particle in the form of 20S aggregate A-protein, or helical polymer, as well as supernatant of the heat-disrupted virus sample, had no effect on mitochondrial functioning. Electron microscopy revealed the direct interaction of the virus particles with isolated mitochondria. The possible role of the mitochondrial permeability transition pore in virus-induced apoptosis is discussed.**



*Key words:* Tobacco mosaic virus; Mitochondrion; Permeability transition pore; $Ca^{2+}$

## 1. Introduction

The list of described inducers of the $Ca^{2+}$-dependent mitochondrial megachannel (permeability transition pore, PTP) is long and includes many different chemical and physical factors all synergistic to $Ca^{2+}$ [1,2]. As a result of PTP opening intramitochondrial solutes of molecular mass lower than 1.5 kDa equilibrate with those in cytosol (reviewed in [2]). This is accompanied by the activation of mitochondrial respiration, the loss of ions accumulated in the matrix and high amplitude swelling of mitochondria.

Although PTP has been studied for several decades, its physiological relevance is not clear yet. Recent findings suggest PTP involvement in either programmed mitochondrial destruction [3] and, hence, in mitochondrial selection [4] or in programmed cell death [5–7]. The mitochondrial contribution to apoptosis opens a vast field for investigating a new mitochondrial function.

Viral-induced apoptosis has been described for several viruses. Viruses like human immunodeficiency virus (HIV) [8], influenza A and B [9], Sendai virus, herpes virus type 1 [10] and others were found to be apoptogenic. In circulating T-lymphocytes from HIV carriers, mitochondria exhibit a reduction of the membrane potential [11] which supports mitochondrial dysfunctioning in the early stages of the apoptotic process. The HIV RNA accumulates in mitochondria apparently using the transport system for importing RNA from the MRP-RNase complex [12].

It may be suggested that mitochondrial depolarization, observed when HIV interacts with mitochondria, is a result of the activation of PTP. In order to test the suggestion of virus-induced PTP in mitochondria, we studied the effect of tobacco mosaic virus (TMV) on rat liver mitochondria. Models of this kind have already been used. Dimitriadis and Georgatsos [13] demonstrated synthesis of the TMV coat protein after penetration of viral RNA into isolated mouse liver mitochondria. Zhdanov et al. [14] reported the reproduction of TMV in isolated rat liver mitochondria.

## 2. Materials and methods

Rat liver mitochondria were obtained by a conventional method described elsewhere [15]. The isolation medium contained 0.3 M sucrose, 5 mM HEPES, 500 μM EDTA, pH 7.4 (adjusted with Tris). After spinning at $8000\times g$, the mitochondrial pellet was washed in an EDTA-free medium. The incubation medium contained 0.3 M sucrose, 5 mM HEPES, pH 7.4 (adjusted with Tris).

TMV was propagated on *Nicotiana tabacum* var. *Samsun* plants and purified from the plant sap by extraction with 5 mM EDTA-containing buffer, chloroform–butanol treatment, polyethylene glycol precipitation and differential centrifugation [16]. TMV coat protein was prepared by the acetic acid method [17,28]. The intact virus and small protein aggregates (A-protein) of TMV coat protein were stored in 5–10 mM borate buffer, pH 7.0 and 8.0 respectively.

The preparations of the virus-like helical TMV coat protein were obtained by the addition of 0.5 M phosphate buffer, pH 5.6 to a cold A-protein solution (1:9 by volume) at 4°C. The mixture was incubated for 2 h at 20°C and repolymerized protein was pelleted by centrifugation at $105\,000\times g$ for 90 min. The protein was resuspended in 50 mM citrate buffer, pH 5.5 and clarified by centrifugation at $15\,000\times g$ for 20 min. The virus and coat protein concentrations were measured spectrophotometrically [15].

To remove bound $Ca^{2+}$ from the virus preparation, EGTA was added to TMV suspension (~15 mg/ml) in 10 mM borate buffer, pH 7.0 to a final concentration of 0.5 mM. The virus was pelleted twice by high speed centrifugation, resuspended in 20 mM borate buffer, pH 7.0 and clarified by low speed centrifugation.

For electron microscopic study mitochondria were fixed by 2.5% glutaraldehyde prepared on phosphate buffer (pH 7.2) with postfixation by 1% osmium tetroxide, dehydration in ethanol, staining with uranyl acetate and embedding in Epon 812. Ultrathin sections were prepared on an LKB-3 ultramicrotome and examined at 75 kV in a Hitachi HU-11 electron microscope.

$Ca^{2+}$ content of the virus preparations was measured by the ICP-AE (atomic emission with inductively coupled plasma) method [18].

Four mitochondrial parameters were recorded simultaneously in the same 1.5 ml chamber: oxygen consumption with a platinum Clark-type closed electrode, calcium ion concentration with a $Ca^{2+}$-selective

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*Abbreviations:* TMV, tobacco mosaic virus; HIV, human immunodeficiency virus; PTP, permeability transition pore


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electrode, mitochondrial membrane potential with a tetraphenylphosphonium$^+$-selective electrode and mitochondrial swelling with the light scattering at 660 nm.

## 3. Results and discussion

Rat liver mitochondria supplemented with 200 μg/ml of TMV (corresponding to $5\times10^{12}$ virions per mg mitochondrial protein) undergo characteristic changes typical for opening of PTP in the inner mitochondrial membrane. Fig. 1 (curves c) demonstrates the TMV-induced activation of the mitochondrial respiration, the decline of the membrane potential, the loss of mitochondrial $Ca^{2+}$ and the high amplitude swelling. A similar effect could be achieved by the addition of 2 μM $Ca^{2+}$ (Fig. 1, curves b). TMV-induced mitochondrial changes were completely blocked by cyclosporin A (CSA) (Fig. 1, curves d). All these data testify to the virus-induced activation of PTP in mitochondria.

Considering the architecture of the TMV particle, which is composed of a coat protein molecule helically wrapped around an RNA molecule, the effect of TMV constituents on the mitochondrial functioning was also studied. Depending on pH, ionic strength and temperature, TMV coat protein can in solution form a large number of aggregates of different structure. At low ionic strength and pH≥8.0, the protein exists as small 4S aggregates (A-protein), consisting of 3–5 subunits. At pH around 7.0 and mild ionic strength, TMV exist mainly in the form of 20S aggregates formed by ~34 identical protein subunits [17,18] and at pH < 6.0 in the form of RNA-free virus-like helical protein assemblies (repolymerized protein). All these forms of TMV coat protein were tested for the ability to induce PTP in rat liver mitochondria. Although A-protein (Fig. 1, curves a), repolymerized protein (Fig. 2A, curves b) and 20S aggregates (Fig. 2B, curves d) did not show apparent features of PTP inducers, all these protein aggregates sensitized mitochondria to added $Ca^{2+}$ (2 μM $Ca^{2+}$ when added after these components, activated PTP-related phenomena without a visible lag period). It should be noted that all three forms of TMV coat protein were able to induce PTP but 30–50 times more slowly than the intact virus (data not shown).

Only intact virus particles retained the ability to induce PTP in mitochondria. TMV virions disrupted by 15 min heating at 70°C lost the ability to induce PTP (Fig. 3, curves a) within the time period observed for native particles.

Virus-induced mitochondrial permeability transition required $Ca^{2+}$ to develop the process, the latter is known to be an attribute of PTP and to our knowledge the removal of $Ca^{2+}$ from the medium abolishes the ability to activate PTP for any described inducers. When $Ca^{2+}$ was removed from TMV by EGTA treatment (see Section 2), TMV did not induce PTP, although mitochondria were sensitized to added $Ca^{2+}$ as had been observed with TMV components (Fig. 3). Based on this aspect, TMV can be placed in the group of conventional PTP inducers. Apparently, TMV-induced PTP was due to the bound calcium ions. These ions seem to be tightly bound to the virus particle structure since the supernatant obtained by spinning down the viral suspension did not show any effect on the mitochondrial permeability. We measured the total amount of $Ca^{2+}$ bound to TMV by ICP-AE. This method was chosen because of the lack of the errors common to other methods when used in mixtures containing proteins and phosphate. This method gave us values in a range of ~1 μg $Ca^{2+}$ per mg of TMV protein. It means that 200 μg TMV induced PTP in rat liver mitochondria contained 0.2 μg or 5 nmol of bound $Ca^{2+}$. In the experiments presented above, 3–4.5 nmol of added $Ca^{2+}$ induced PTP with kinetics similar to those induced by 200 μg TMV. It becomes clear that TMV may induce mitochondrial PTP due to the calcium ions that are tightly bound to TMV. There are many conflicting data concerning the evaluation of $Ca^{2+}$ binding sites in plant viruses [19–23]. The suggestion that calcium ions stabilize the viral structure and are responsible for assembly/disassembly of the virus [24] seems to be more likely. Based on these data, it becomes clear that intact VTM keeps its tubular protein-RNA architecture due to $Ca^{2+}$ bonds. $Ca^{2+}$ stabilizes the structure and the loss of these tightly bound ions results in the disaggregation of the virus particle. In his letter to *Nature* entitled 'Do viruses use calcium ions to shut off host cell functions?' Durham [25] suggested that in some cases structural components of both plant and animal viruses act as calcium ionophores. This conclusion fits very well with the data obtained in the present study, showing the synergism of viral and $Ca^{2+}$ effects. We develop a model of the $Ca^{2+}$-

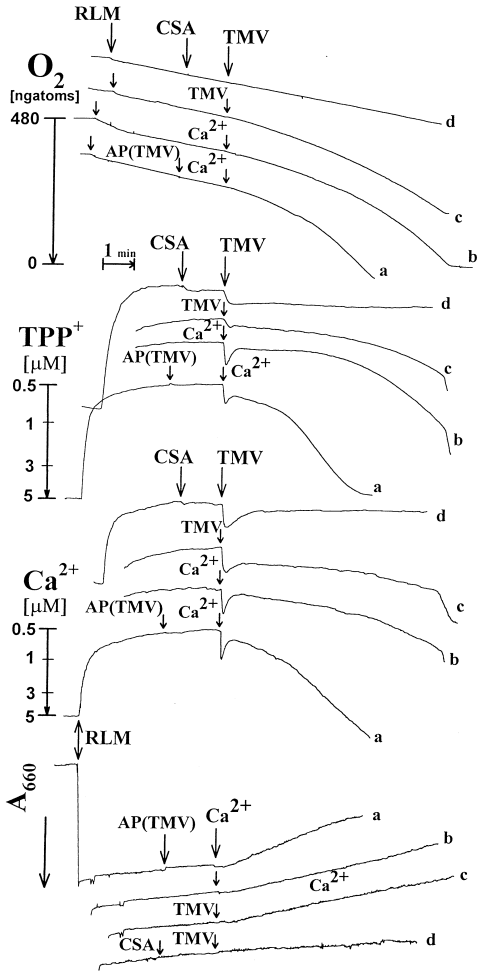


Fig. 1. Induction of PTP in rat liver mitochondria by TMV and the inability to induce it by its coat protein. The effects of TMV and its coat protein, AP(TMV) (200 μg viral protein per mg mitochondrial protein) are shown. RLM, rat liver mitochondria (1 mg/ml); CSA, cyclosporin A ($10^{-7}$ M); TPP, tetraphenylphosphonium chloride; $Ca^{2+}$, calcium chloride, 3 nmol/mg mitochondrial protein.

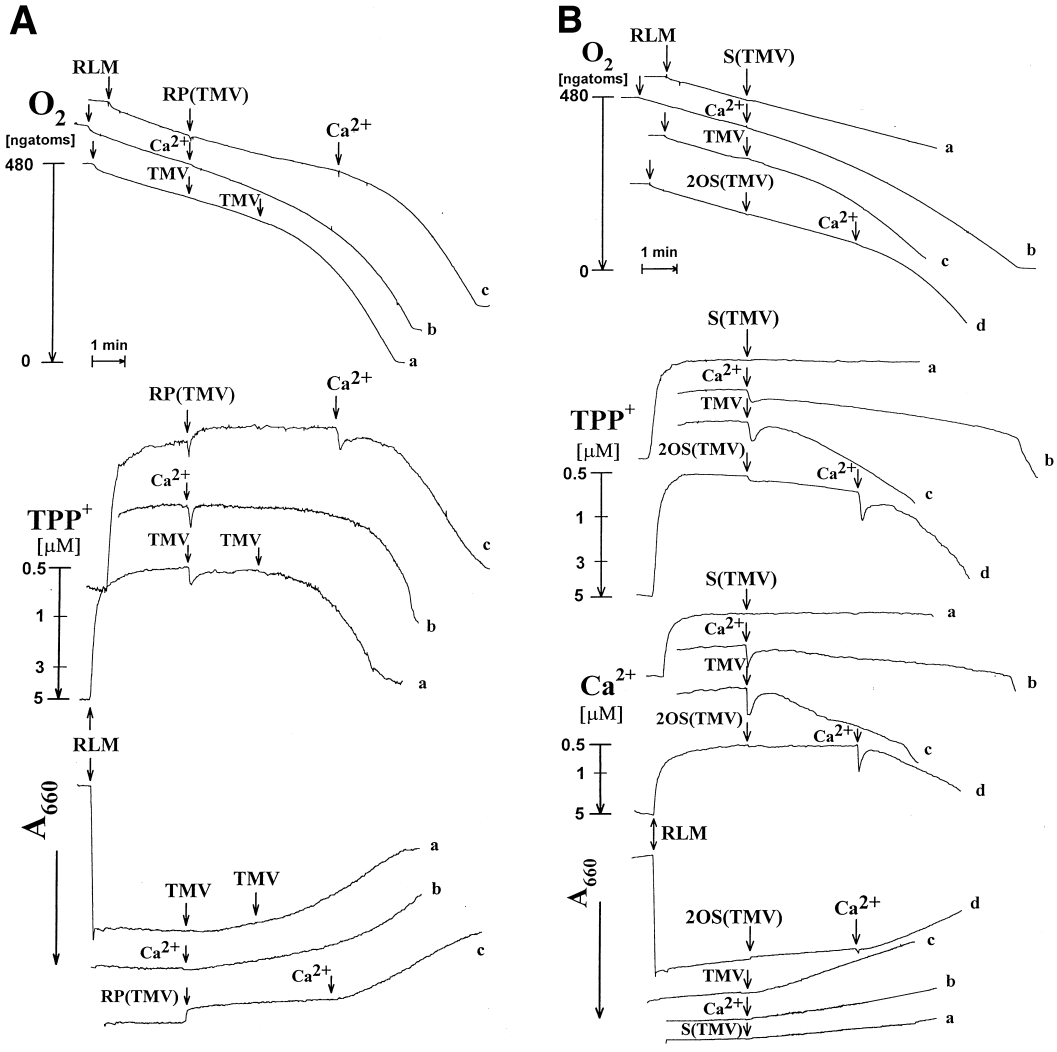


Fig. 2. Induction of PTP in rat liver mitochondria by TMV and the inability to induce it by its repolymer. A: The effect of TMV (two successive additions, 100 μg viral protein per mg mitochondrial protein each) and its repolymer, RP(TMV), 200 μg protein per mg mitochondrial protein. B: The effect of viral protein disks, 20S(TMV) (200 μg of viral protein per mg of mitochondrial protein) and supernatant obtained after high speed centrifugation, S(TMV). The volume of supernatant added was the same as the volume of virus suspension. Other notes as in Fig. 1.

mediated mechanism of TMV-induced permeability transition in mitochondria. According to the model, the specific interaction of TMV with the outer mitochondrial membrane results in $Ca^{2+}$ being bound to TMV to be taken by mitochondria without release into the medium. The transfer of $Ca^{2+}$ into the matrix results in a conformational change of the adenine nucleotide translocator with the following opening of the permeability transition pore.

Two very important points should fit this model. First, $Ca^{2+}$ bound to TMV should be in close proximity to the mitochondrial membrane or, in other words, viral particles should have rather specific binding sites on the mitochondrial surface. The impressive electron microscopic pictures of the association of tobacco rattle virus with cellular mitochondria made by Harrison and Roberts [26] showing apparent regularity of this association support the specific interaction of mitochondria with viruses. Our electron micrographs presented as a few examples in Fig. 4 also confirm the physical interaction of TMV particles with isolated rat liver mitochondria. The interaction seems to be independent of the presence of CSA. Note that the visual crossing of the outer mitochondrial membrane by two virus particles presented in Fig. 4B might be mistaken due to possible tangential sectioning to the plane of mitochondria with attached virus particles. The second important point is that TMV in the suspension with rat liver mitochondria does not release its bound $Ca^{2+}$ into the incubation medium since electron microscopic pictures demonstrate a filamentous rather than disaggregated virus appearance. The preservation of the virus structure when it interacts with the mitochondrion supports the point that the virion does not release $Ca^{2+}$ into the medium which itself can activate PTP. The experiment presented in Fig. 2B, curve a, apparently shows the absence of the release of PTP-inducible amounts of $Ca^{2+}$ from TMV into the mitochondrial incubation medium, since the supernatant after high speed centrifugation of TMV, added in an equal volume as TMV alone, did not activate PTP. This functional experiment together with morphological study give a strong line of evidence that TMV may activate PTP in rat liver mitochondria. This particular induction like other described ways of PTP induction

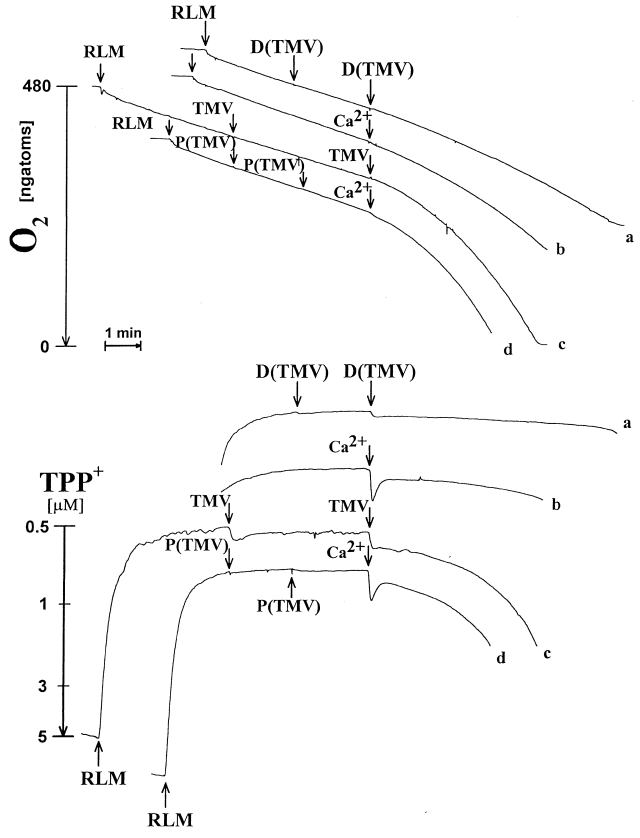


Fig. 3. Induction of PTP in rat liver mitochondria by intact TMV and the inability to induce it by inactivated virus, D(TMV), or by virus stripped of $Ca^{2+}$, P(TMV). Two successive additions, 100 μg protein per mg mitochondrial protein each, were done. $Ca^{2+}$, calcium chloride, 4.5 nmol/mg mitochondrial protein. Other notes as in Figs. 1 and 2.
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goes synergistically with $Ca^{2+}$ but is not mediated by solute $Ca^{2+}$ as a result of its dissociation from the virion structure. The only tentative explanation is in the direct transfer of bound to virion $Ca^{2+}$ into mitochondria through tight interaction of these structures, thus resulting in the formation of a mitochondrial megachannel. Alternatively, the PTP opening may be caused by TMV RNA released from the virion during the contact of the organelle with the virus particle [27].

The observed viral-induced activation of the permeability transition in mitochondria may be relevant to viral-induced apoptosis because the suggestion that PTP participates in apoptosis has been put forth [3–5]. Human immunodeficiency virus is one of the most interesting apoptogenic viruses and is

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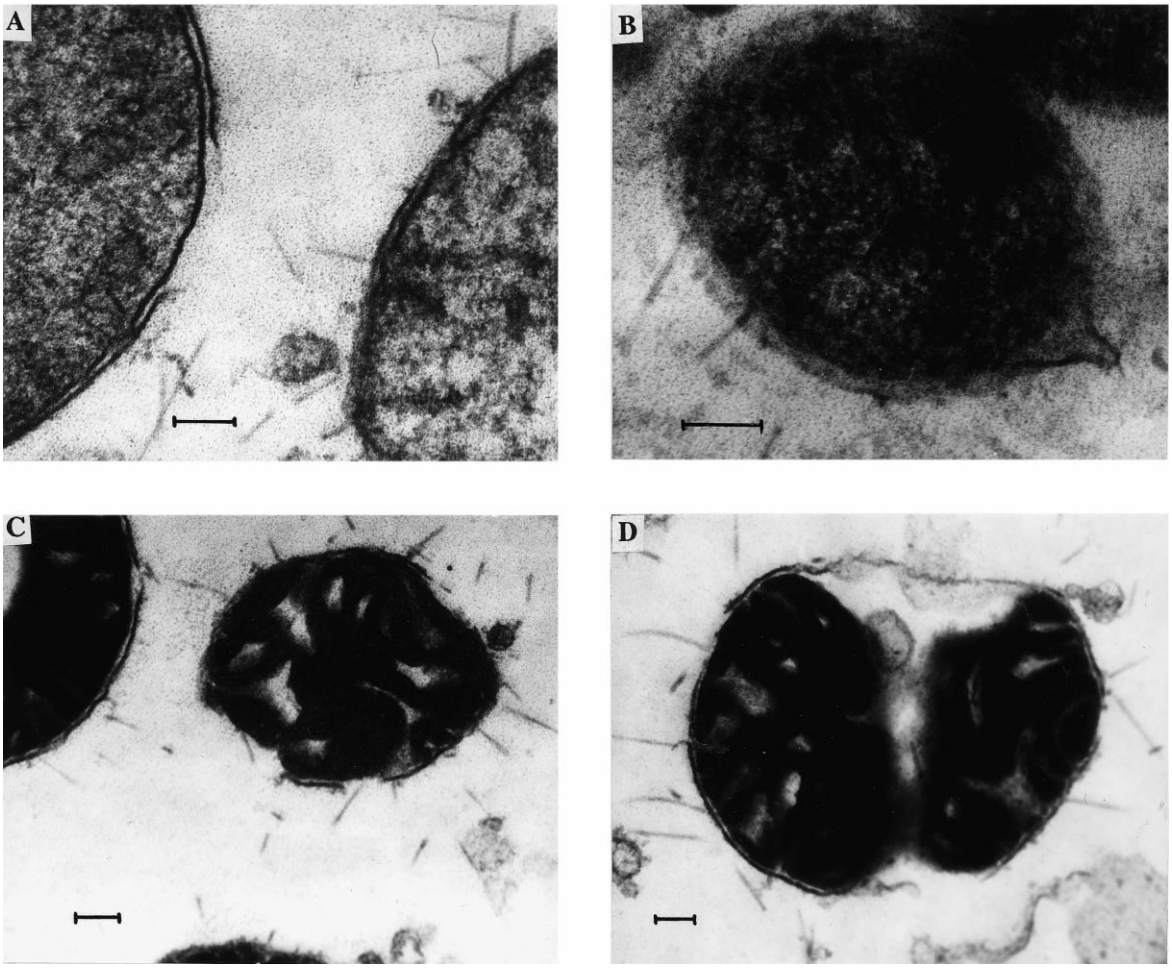
Fig. 4. Electron micrographs of the fraction of isolated rat liver mitochondria incubated with TMV. A, B: Incubation with TMV (200 μg viral protein per mg mitochondrial protein) alone; B: tangential section through the mitochondrion). C, D: Two examples of TMV interaction with isolated rat liver mitochondria incubated with $10^{-7}$ M cyclosporin A. Note an apparent mitochondrial swelling in A and B. Bars, 0.05 μm.



of prime interest, especially if one can take into account its specific interaction with mitochondria [9,10,12].

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