



TRIANA, URIBE & MICHELSEN

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SEÑOR,
SUPERINTENDENTE DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES
E. S. D.

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REFERENCIA : SOLICITUD DE PATENTE DE INVENCION TITULADA:
"COMPOSICIÓN FARMACÉUTICA QUE COMPRENDE
LA COMBINACIÓN DE PIRACETAM Y L-CARNITINA"
EXPEDIENTE No. 10-110534
SOLICITANTE : BIOLAB SANUS FARMACEUTICA LTDA.

JUAN CARLOS URIBE, abogado, en mi condición de apoderado de la sociedad **BIOLAB SANUS FARMACEUTICA LTDA.**, atentamente manifiesto que estando dentro del término de ley y por orden expresa de mi representada, respetuosamente me dirijo a usted con el fin de interponer Recurso de Reposición, en contra de la Resolución No. 73062 del 29 de Noviembre de 2013, emitida por su Despacho.

1. PRETENSIONES.

- 1.1. Que se revoque la Resolución No. **73062**, proferida por el Señor Superintendente de Industria y Comercio el día 29 de Noviembre de 2013, la cual denegó la patente de invención titulada: "**COMPOSICIÓN FARMACÉUTICA QUE COMPRENDE LA COMBINACIÓN DE PIRACETAM Y L-CARNITINA**".

2. HECHOS.

- 2.1. El 7 de septiembre de 2010, la solicitante radicó la solicitud de patente de invención titulada: **COMPOSICIÓN FARMACÉUTICA QUE COMPRENDE LA COMBINACIÓN DE PIRACETAM Y L-CARNITINA**, a la cual se le asignó el expediente No. 10-110534. Esta solicitud corresponde a la publicación internacional PCT número WO2009/105853 del 3 de septiembre de 2009, cuyo número de solicitud internacional corresponde a PCT/BR2009/000053.
- 2.2. Una vez publicada dicha solicitud en la gaceta de propiedad industrial número 640 del 15 de marzo de 2012, la Dirección de Nuevas Creaciones procedió a realizar el examen de fondo con base en el artículo 45 de la Decisión 486 de la CAN, sobre el cual emitió el Oficio No. 13104 de fecha 26 de julio de 2012.

- 2.3. El Oficio No. 13104, solicitó claridad al título, descripción y reivindicaciones respecto de la materia no patentable y sobre algunos términos que no estaban debidamente soportados. En materia de los requerimientos, se contestaron uno a uno, anexando un nuevo capítulo reivindicatorio y las páginas 17 y 19 de la descripción, además del debido ajuste en el título.
- 2.4. En materia de las anterioridades expuestas en este oficio 13104, se advierte que carece de novedad y nivel inventivo respecto a los documentos del estado de la técnica referenciados como:
- D1** - US 5,977,162;
D2 - US 6.245.800;
- 2.5. En respuesta a la mención de carencia de altura inventiva, se presentó un nuevo capítulo reivindicatorio, en el que se definen una a una las diferencias de dicho arte anterior y la presente solicitud.
- 2.6. Finalmente, su Despacho expide la Resolución No. **73062** por medio de la cual niega la patente de invención titulada: **COMPOSICIÓN FARMACÉUTICA QUE COMPRENDE LA COMBINACIÓN DE PIRACETAM Y L-CARNITINA.**

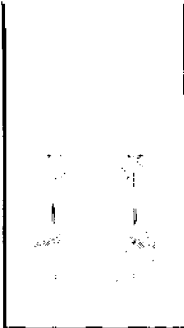
3. CONSIDERACIONES.

- 3.1. El examinador, en el escrito de la Resolución, se refiere a los documentos: US5977162 del 02 de noviembre de 1999 referenciando como D1; y US6245800 del 12 de junio 2001 referenciado como D2.
- 3.2. Por su parte, el documento D1 se refiere al tratamiento terapéutico de la función auditiva. Según el examinador, este documento D1 da a conocer un líquido o forma de tableta que comprende el piracetam en el rango de 100-800 mg, preferiblemente 300 mg de la composición y al menos dos componentes seleccionados en cantidades con eficacia sinérgica de ácido lipóico, acetil-L-carnitina, coenzima Q-10 y glutatión, en el que la acetil-L-carnitina se encuentra en un rango entre 10 y 1000 mg (Col. 6 , líneas 51-67 y columna 7, líneas 1 a 10). Según D1, dicho suplemento dietético mejora la función mitocondrial mediante el aumento de la producción de energía celular en atletas, personas sedentarias y pacientes con trastorno cognitivo tal como la demencia y la enfermedad de Alzheimer (Col. 4, líneas 52-67).
- 3.3. El documento D2 se refiere a un método para prevenir o tratar los efectos tóxicos inducidos por las estatinas usando L-carnitina o un alkanoil L-carnitine. Según el examinador, el documento D2 describe un método para prevenir los efectos adversos o

la toxicidad inducida por el tratamiento que comprende la administración de una estatina que comprende la administración coordinada (coadministración o administración de una composición aislada, Col.3 , líneas 5 a 13) de una cantidad detoxificante de L-carnitina o un derivado alcanilo lineal o ramificada que tiene de 2 a 6 átomos de carbono o una sal farmacéuticamente aceptable del mismo y una estatina seleccionada de entre el grupo constituido por: lovastatina, simvastatina, pravastatina y fluvastatina en una cantidad mayor que 20 mg/día (Col. 6, líneas 16 a 36).

- 3.4. Ahora bien, respecto a la actividad inventiva, el señor examinador presenta un cuadro comparativo entre las principales características técnicas de la solicitud estudiada y se menciona en la reivindicación 1 en comparación con lo que revelan los documentos D1 y D2 que representan el estado de la técnica, donde:

Características esenciales	D2	D1
Composición farmacéutica que comprende:	Composición (Col. 3, líneas 5 a 13) que comprende:	Composición líquida o en forma de tableta (Col. 6, líneas 51 a 67 y Col. 7, líneas 1 a 10) que comprende:
una cantidad terapéuticamente eficaz de piracetam o una sal farmacéuticamente aceptable	No menciona	Piracetam en el rango de 100 a 800 mg, preferiblemente 300 mg
una cantidad terapéuticamente eficaz de L-carnitina o una sal farmacéuticamente aceptable,	Una cantidad detoxificante de L-carnitina o un derivado alcanilo lineal o ramificado con 2 a 6 átomos de carbono o una sal farmacéuticamente aceptable	Acetil-L-carnitina en un rango entre 10 y 1000 mg
un vehículo farmacéuticamente aceptable	Un vehículo farmacéuticamente aceptable	Un vehículo farmacéuticamente aceptable
(IV) una cantidad terapéuticamente eficaz de por lo menos un agente terapéutico asociado con trastorno mitocondrial y/o metabólico seleccionado de simvastatina,	Una estatina seleccionada del grupo que comprende: lovastatina, simvastatina, pravastatina y fluvastatina en una cantidad mayor a 20 mg/día (Col. 6, líneas 16 a 36)	No menciona



pravastatina, lovastatina, atorvastatina, fluvastatina, dalvastatina, cerivastatina, mevastatina, pitavastatina y rosuvastatina		
		Ácido lipóico, coenzima Q-10 y glutatión

- 3.5. Según el examinador, los documentos D1 y D2 se consideran la técnica anterior más cercana a la invención definida en la reivindicación 1, debido a que el documento D2 enseña una composición para prevenir la toxicidad adversa inducida por el tratamiento con una estatina que comprenden los efectos de: una cantidad detoxificante L-carnitina o un derivado alcanofilo lineal o ramificado que tiene de 2 a 6 átomos de carbono, o una sal farmacéuticamente aceptable del mismo (Col. 3, líneas 5 a 13) y una estatina seleccionada de entre el grupo constituido por: lovastatina, simvastatina, pravastatina y la fluvastatina en una cantidad mayor a 20 mg/día (Col. 6, líneas 16 a 36). El documento D1 enseña una composición en líquido o forma de la tableta, que comprende piracetam en el intervalo de 100 a 800 mg, preferiblemente 300 mg, acetyl L-carnitina en una gama de 10 a 1000 mg , ácido lipoico, coenzima Q-10 y glutatión (Col. 6 , líneas 51-67 , y la Col. 7, líneas 1 a 10).
- 3.6. Respecto a tales argumentos, la Solicitante optó por mantener el mismo conjunto de reclamaciones presentadas en respuesta al primer oficio y presenta algunos argumentos a continuación, con base en que a su juicio, demuestran que la invención, como se afirma, presenta actividad inventiva contra los documentos identificados.
- 3.7. Teniendo en cuenta los argumentos presentados por el señor examinador, respetuosamente el solicitante no está de acuerdo con que los documentos D1 y D2 afectan la actividad inventiva respecto a la presente solicitud, toda vez que el documento D1 describe un "suplemento nutricional", que tiene en su composición al menos dos componentes y cantidades con eficacia sinérgica para promover la "sensibilidad auditiva", lo que sugiere que el efecto sinérgico de los componentes se selecciona de entre el grupo que consiste sólo de **ácido lipoico, acetyl-L-carnitina, glutatión y una coenzima Q10** (D1, Col. 6, líneas 51 a 57). Además, este "suplemento alimenticio", contiene una combinación sinérgica que puede contener además piracetam en el rango de 100 a 800 mg. Sin embargo, en ningún momento, el documento D1 menciona que existe un efecto sinérgico entre el piracetam y el ácido lipoico, acetyl-L-carnitina, la coenzima Q10 y los componentes de glutatión. Más bien, el efecto sinérgico que se sugiere en este documento, sólo se produce entre la combinación de los componentes ácido lipoico, acetyl-L-carnitina, la coenzima Q10 y el glutatión. Esta comprensión puede ser confirmada por la reivindicación 12 del

documento D1 (Col. 7, líneas 11 a 14 y Col. 8, líneas 1-2), que afirma específicamente para el producto de interés, que es un "suplemento alimenticio" a "promover la sensibilidad auditiva" que comprende sólo la combinación de 150 mg de **ácido alfa-lipóico**, 150mg de **acetil-L-carnitina**, 60 mg de **coenzima Q-10** y 30 mg **glutathion**. En esta realización, afirman que el producto particular de interés no reclama la adición opcional de piracetam. Por lo tanto, un experto en la técnica, basado en las enseñanzas de D1, no tendría ninguna motivación para probar la combinación de piracetam y L-carnitina, para llegar al objeto de la presente solicitud de patente.

El solicitante declara respetuosamente que el documento D2 sólo describe el uso de L-carnitina en la prevención y el tratamiento de los efectos tóxicos o secundarios inducidos por las estatinas. Por lo tanto, con el conocimiento de los documentos D1 y D2, un experto en la técnica no estaría motivado para reemplazar **acetil-L-carnitina** por **L-carnitina** y de combinar **L-carnitina** con **ácido lipoico**, una coenzima Q10 o glutathion, toda vez que el documento D1 sugiere el efecto sinérgico entre estos 4 componentes y no tendría ninguna motivación para probar la combinación de **piracetam** y **L-carnitina**, como si lo hace el objeto de esta solicitud de patente. Por lo tanto, el solicitante cree que se ha demostrado que sobre la base de las enseñanzas de D1 y D2, no sería obvio para un experto en la técnica usando los activos combinados, y mucho menos suponer que la combinación de **piracetam** y **L-carnitina** (objeto de la presente invención) podría ser presentado en un **efecto sinérgico positivo**.

- 3.8. Para una mejor comprensión de la presente invención, el solicitante trae a colación el artículo titulado: "*Protective effects of L-carnitine and piracetam against mitochondrial permeability transition and PC3 cell necrosis induced by simvastatin; Costa RA, Fernandes MP, de Souza-Pinto NC, Vercesi AE. Eur J Pharmacol. 2013 Feb 15;701(1-3):82-6*", que es un estudio requerido por el solicitante a las instituciones de investigación de renombre, que explica el efecto sinérgico de la combinación identificada Piracetam y L-carnitina, objeto de la presente invención. El solicitante presenta a continuación en la Tabla 1A y Tabla 1B, que son las cifras relativas a las figuras 1A y 1B de este artículo, donde:

Table 1A

Concentration μM (L-carnitine)	PC3 cell death (%)
Control	1,89
0	46,51
1	50,68
2	41,70
4	25,61
5	10,44
6	1,35
12	1,23

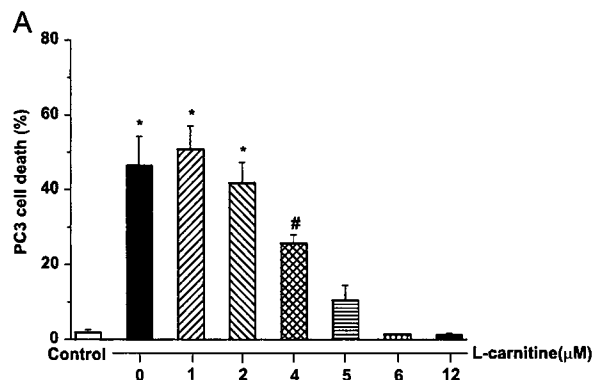
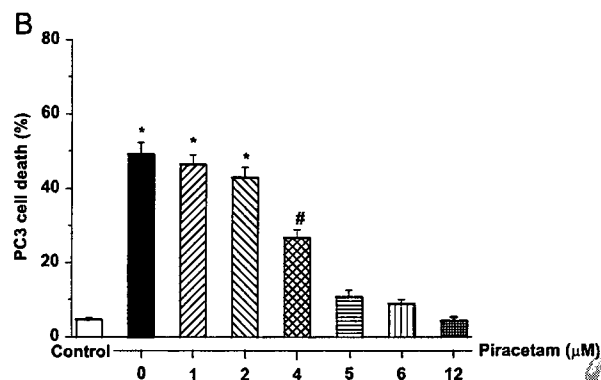


Table 1B

Concentration μM (Piracetam)	PC3 cell death (%)
Control	4,61
0	49,17
1	46,48
3	43,00
4	26,68
5	10,67
6	8,84
12	4,32



- 3.9. En ese artículo, las figuras 1A y 1B muestran la protección otorgada por la L- carnitina o piracetam contra la necrosis celular inducida por la simvastatina en células PC3. Como se puede ver en los resultados, la adición de simvastatina causó la muerte celular de aproximadamente 50% (columna 0), y cuando se adicionaron 4 μM de L-carnitina se redujo la muerte a 25,61% (figura y la tabla 1A) y añadiendo Piracetam en 4 μM , la muerte celular se redujo a 26,68% (figura y tabla 1B). Sin embargo, para reducir la muerte celular en los mismos niveles del grupo de control se les exigía 6 μM de L-carnitina y 12 μM de piracetam. Además, el solicitante presenta bajo la tabla 4B y la figura 4B, que son complementarios a la figura 4, los datos de dicho artículo para facilitar la visualización del efecto sinérgico inesperado, donde:

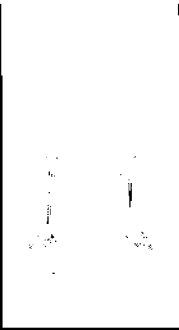
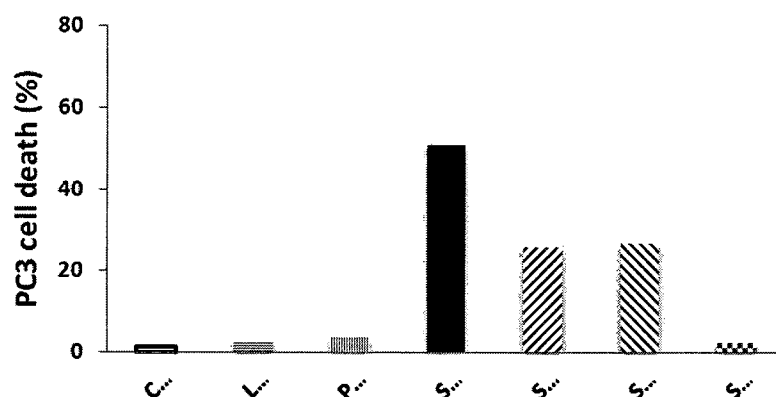


Table 4B

Concentration μM	PC3 cell death (%)
Control	1,37
L-carnitine (4 μM)	2,55
Piracetam (4 μM)	3,54
Sinvastatin (60 μM)	50,96
Sinvastatin (60 μM) + L-carnitine (4 μM)	25,61
Sinvastatin (60 μM) + Piracetam (4 μM)	26,68
Sinvastatin (60 μM) + L-carnitine (4 μM) + Piracetam (4 μM)	2,21



- 3.10. Los resultados obtenidos *in vitro* (Figura 4B arriba y las Figuras 1A y 1B del artículo) muestran que para reducir la muerte celular en el mismo nivel en el grupo de control, se obtuvo: (a) requiere una alta dosis de piracetam (12 μM), fig. 1B; (b) requiere una dosis de 6 μM de L-carnitina (fig. 1A); y, (c) posibilidad para reducir las concentraciones del activo cuando se administra en combinación, ya que el mismo efecto protector se obtuvo con 4 μM de piracetam asociado a 4 μM de L-carnitina.
- 3.11. Estos resultados demuestran la necesidad de **dosis 150% y 300% mayores de L-carnitina o piracetam aislados**, respectivamente, para una buena reducción de la muerte celular inducida por las estatinas, en comparación con la dosis utilizada en la combinación de la invención para efectos similares. Por lo tanto, el efecto obtenido con la **combinación de los activos excede lo que se esperaría de la asociación**, y permite una reducción de la dosis de los activos, que desde el punto de vista

terapéutico, es una contribución importante en términos de eficacia y de relación de los riesgos y beneficios.

- 3.12. Por último, el solicitante respetuosamente explica que un experto en la técnica basándose en las enseñanzas de D1 y D2 no podría predecir que la combinación de L-carnitina y Piracetam, mostraría un efecto sinérgico en la prevención de daño mitocondrial inducido por las estatinas. Por lo tanto, de acuerdo con lo anterior, el solicitante ha demostrado que un experto en la técnica basándose en las enseñanzas con D1 y D2 no tendría ninguna motivación para probar la combinación de L-carnitina y Piracetam, para el propósito de prevenir mitocondrial, ni se deduce que ésta combinación podría tener un **efecto sinérgico positivo**. Es decir, un experto en el arte no sería capaz de asumir una **interacción positiva entre activo (piracetam y L - carnitina)**, lo que reduciría el nivel de activos, como se identifica en la combinación de la presente invención. Por lo tanto, la presente solicitud supera una pre-prueba técnica, contemplando actividad Inventiva manifiesta contra D1 y D2.
- 3.13. Además, el solicitante resalta que dicha combinación y su sinergia en la protección de las mitocondrias no se describen o dan a conocer en los documentos de la técnica anterior identificada. Adicionalmente, el solicitante cree que es útil destacar que el efecto sinérgico que se describe en el presente documento es de gran interés, ya que dosis inferiores pueden ser utilizadas para obtener un efecto deseado, permitiendo de este modo una reducción de los efectos secundarios.
- 3.14. A la luz de lo anterior, el solicitante ha demostrado que los documentos identificados por el examinador no afectan la novedad y la actividad inventiva de la invención. Frente a lo anterior, el solicitante pide la revisión de la solicitud y espera su respectiva **CONCESIÓN**, quedando a su disposición para hacer las aclaraciones que se consideren necesarias.
- 3.15. En vista de lo anterior, ya que los documentos citados por este Despacho no afectan la **NOVEDAD** ni la **ACTIVIDAD INVENTIVA** de la invención, por los motivos antes expuestos, ni contradice disposición legal o reglamentaria alguna, la sociedad solicitante espera que ésta sea concedida dentro del derecho que sigue la norma.
- 3.16. Por todo lo anterior, solicito respetuosamente que los anteriores argumentos sean evaluados frente a lo reiterado a lo largo del expediente y en consecuencia, se **revoque** la Resolución No. **73062**, proferida por el Señor Superintendente de Industria y Comercio el día 29 de Noviembre de 2013, la cual denegó la patente de invención denominada: **"COMPOSICIÓN FARMACÉUTICA QUE COMPRENDE LA COMBINACIÓN DE PIRACETAM Y L-CARNITINA"**, y en consecuencia la **CONCEDA** bajo el tenor de sus reivindicaciones.

4. FUNDAMENTO DE DERECHO.

Fundamento esta remisión en las normas legales siguientes:

- 4.1 Decisión 486 de la Comisión del Acuerdo de Cartagena.
- 4.2 En las demás normas concordantes.

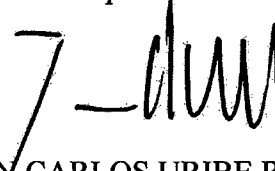
5. ANEXOS.

- 5.1 Artículo: "*Protective effects of L-carnitine and piracetam against mitochondrial permeability transition and PC3 cell necrosis induced by simvastatin; Costa RA, Fernandes MP, de Souza-Pinto NC, Vercesi AE. Eur J Pharmacol. 2013 Feb 15;701(1-3):82-6*".

6. NOTIFICACIONES.

El suscrito recibirá notificaciones en la Calle 93B No. 12-48, P.4 de la ciudad de Bogotá, D.C., o en la Secretaría de su Despacho.

Del Señor Superintendente,



JUAN CARLOS URIBE RESTREPO
C.C. No. 79.159.313 de Usaquén
T.P.A. No. 54.493 del C.S de la J.



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Molecular and cellular pharmacology

Protective effects of L-carnitine and piracetam against mitochondrial permeability transition and PC3 cell necrosis induced by simvastatin

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ABSTRACT

Mitochondrial oxidative stress followed by membrane permeability transition (MPT) has been considered as a possible mechanism for statins cytotoxicity. Statins use has been associated with reduced risk of cancer incidence, especially prostate cancer. Here we investigated the pathways leading to simvastatin-induced prostate cancer cell death as well as the mechanisms of cell death protection by L-carnitine or piracetam. These compounds are known to prevent and/or protect against cell death mediated by oxidative mitochondrial damage induced by a variety of conditions, either in vivo or in vitro. The results provide evidence that simvastatin induced MPT and cell necrosis were sensitive to either L-carnitine or piracetam in a dose-dependent fashion and mediated by additive mechanisms. When combined, L-carnitine and piracetam acted at concentrations significantly lower than they act individually. These results shed new light into both the cytotoxic mechanisms of statins and the mechanisms underlying the protection against MPT and cell death by the compounds L-carnitine and piracetam.

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1. Introduction

Statins are natural or synthetic compounds which inhibit the conversion of 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) into mevalonic acid, the rate-limiting step in cholesterol synthesis (Endo, 1992). These compounds are widely used in the clinic for the treatment of hypercholesterolemia (Shepherd et al., 1995; Collins et al., 2003) and many studies suggest an antioxidant effect of these compounds when used in therapeutic doses (Cook et al., 1988; Guzman et al., 1993; Bhuiyan and Secombe, 1996; Hsu et al., 2006). In addition, literature data indicate that statins cause a series of adverse (Sirvent et al., 2005; Kwak et al., 2012) or beneficial (Manfredini et al., 2010; Li et al., 2010) effects that include protection against prostate cancer (Oliveira et al., 2008; Marcella et al., 2011; Mondul et al., 2011).

We have previously reported that, at 10 μM, simvastatin induced mainly apoptosis in PC3 cells, which was prevented by mevalonic acid but not by Cyclosporine A, an inhibitor of the mitochondrial membrane permeability transition (MPT) (Oliveira et al., 2008). At higher concentrations (60 μM), simvastatin induced necrosis, which was prevented by Cyclosporine A but not by mevalonic acid. Cell necrosis was preceded by a threefold increase in cytosolic free Ca²⁺ and a significant decrease in both respiratory rate and mitochondrial membrane potential. Both mitochondrial dysfunction and necrosis

were prevented by Cyclosporine A, indicating that necrosis, in this model, is dependent on MPT (Oliveira et al., 2008).

MPT is a nonselective permeabilization of the inner mitochondrial membrane, promoted by mitochondrial Ca²⁺ loading (Hunter et al., 1976), changes in membrane structure (Ricchelli et al., 1999; Grijalba et al., 1999) and oxidative stress (Vercesi, 1984; Fagian et al., 1990; Kowaltowski et al., 2001; Figueira et al., 2012a). A recent study (Vaseva et al., 2012) demonstrated that inhibition of MPT by Cyclosporine A is mediated by the blockade of a p53-Cyclophilin D (CypD) complex formation under oxidative stress-induced necrosis in brain ischemia/reperfusion injury. The authors provided evidence that p53 accumulates in the mitochondrial matrix in response to oxidative stress and triggers permeability transition pore (PTP) opening and necrosis by physical interaction with the PTP regulator CypD. This study confirms the participation of MPT in oxidative stress-induced necrosis in cerebral stroke and other vascular disorders that are leading causes of death (Vaseva et al., 2012). Therefore, targeting PTP may provide avenues for therapeutic protection and/or prevention against cell death that occurs in these pathologies.

A large body of evidences indicate that either L-carnitine or piracetam prevent and/or ameliorate mitochondrial dysfunction caused by a series of conditions either in vivo or in vitro (Moretti et al., 2002; Virmani et al., 2005; Silva-Adaya et al., 2008; Elinos-Calderon et al., 2009; Zhang et al., 2010). Therefore, the aims of the present paper were two: first, to dissect the events leading to simvastatin induced MPT and cell death and second, to better understanding the mechanisms underlie the protection by L-carnitine and piracetam. The results indicate that L-carnitine

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or piracetam, either alone or in combination, prevents simvastatin induced superoxide generation thus protecting against MPT and cell necrosis.

2. Material and methods

2.1. Chemicals

Simvastatin (99.5% purity) was purchased from Galena Química e Farmacêutica Ltda (Campinas, SP, Brazil). Piracetam was obtained from BioLab Farmacêutica (São Paulo, SP, Brazil). RPMI 1640 medium and fetal bovine serum (FBS) were obtained from Cultilab (Campinas, SP, Brazil). L-carnitine, Cyclosporin A, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (CCCP), adenosine 5-diphosphate (ADP) dimethyl sulfoxide (DMSO), propidium iodide and digitonin were obtained from Sigma (St. Louis, MO, USA). MitoSox was obtained from Life Technologies. All other chemicals were standard commercial products of reagent-grade quality.

2.2. Cell culture and simvastatin treatment

The PC3 human prostate cancer cell line was obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in RPMI 1640 medium supplemented with 10% FBS and 10 mM (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; HEPES). The cells were maintained at 37 °C in air atmosphere with 5% CO₂. The cultures were maintained below 80% confluence. To evaluate the effects of simvastatin the cells were plated in growth medium containing either 0.1% DMSO or simvastatin dissolved in DMSO (stock solution of 100 mM) and diluted in the culture medium immediately prior to use. When indicated, cyclosporin A, L-carnitine and piracetam were also added during the treatments.

2.3. Flow cytometry analysis

Cell death was analyzed by flow cytometry in a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with an argon laser and CellQuest software (version 4.1). Ten thousand events were acquired for each sample. The PC3 population was identified by their light-scattering characteristics, enclosed in electronic gates, and analyzed for the intensity of the fluorescent probe signal, as described below.

2.4. Analysis of cell viability by propidium iodide staining

PC3 were incubated with propidium iodide (PI, 1:50) immediately before analyzing the samples in FACS. Necrosis was quantified as the number of PI positive cells divided by the total number of cells.

2.5. Mitochondrial superoxide generation

For the quantification of mitochondrial superoxide generation, PC3 cells were treated with 6 μM L-carnitine, 6 μM piracetam or 4 μM L-carnitine+4 μM piracetam in the presence or absence of 60 μM simvastatin for 2 h. Then they were harvested, washed with cold PBS and trypsinized, followed by staining with 3 μM MitoSox at 37 °C for 10 min. The samples were analyzed in the FL-2 channel of the flow cytometer (Payne et al., 2007).

2.6. Determination of mitochondrial membrane potential ($\Delta\Psi_m$) in digitonin-permeabilized PC3 Cells

Mitochondrial membrane potential ($\Delta\Psi_m$) in digitonin-permeabilized cells was estimated as changes in the fluorescence of safranin O (Figueira et al., 2012b), recorded using a spectrofluorometer (Hitachi, model F4500, Tokyo, Japan), operating at excitation and emission wavelengths of 495 and 586 nm, respectively, with slit width of 5 nm. After treatment with simvastatin, PC3 cells (10⁶ cells) were permeabilized with 20 μM digitonin (Campos et al., 2004) in 0.5 ml of standard reaction medium (125 mM sucrose, 65 mM KCl, 10 mM Tris-HCl (pH 7.2), 2.5 mM Na₂HPO₄, 0.33 mM EGTA, 5 mM succinate, and 5 μM safranin O) under constant stirring at 37 °C, in a 0.5 ml thermostatically sealed glass cuvette equipped with a magnetic stirrer.

2.7. Statistical analysis

The results from six independent experiments, except for mitochondrial membrane potential (n=4) and reactive oxygen species (n=3) measurements, are presented as means ± SEM. Comparisons between groups were performed using a one-way Analysis of Variance (ANOVA) with Kruskal-Wallis' post-hoc analysis. The level of significance was set at P < 0.05.

3. Results

3.1. Either L-carnitine or piracetam protect against PC3 cell death induced by simvastatin

The effects of L-carnitine or piracetam on simvastatin-induced cell death were investigated in PC3 cells treated during 2 h with 60 μM simvastatin (Oliveira et al., 2008). As analyzed by flow cytometry (Fig. 1 A and B), this treatment induced significant necrotic cell death. Either L-carnitine (A) or piracetam (B) caused a dose-dependent protection; being 50% inhibition obtained at concentrations in the range of 4 μM of either compound. An almost complete protection was obtained at the concentration of 6 μM of either L-carnitine (A) or piracetam (B).

3.2. L-Carnitine or piracetam decreased the rate of mitochondrial superoxide generation induced by simvastatin in PC3 cells

The effects of L-carnitine or piracetam on the rates of reactive oxygen production by mitochondria were estimated by the MitoSox method in PC3 cells treated or not with simvastatin for 2 h (Fig. 2). Simvastatin increased the rate of superoxide generation by 73% (black bar). Either L-carnitine or piracetam, at 6 μM, significantly decreased the rates of superoxide generation both in the presence or absence of simvastatin. In the presence of simvastatin the inhibition by 6 μM of either L-carnitine or piracetam shifted the rates of superoxide production to the control levels. In addition, Fig. 2 shows that the combination of L-carnitine and piracetam, at the concentrations of 4 μM, also shifted the rates of superoxide production to the levels induced by the individual compounds, at the concentration of 6 μM.

3.3. Cyclosporine A, L-carnitine or piracetam protects PC3 cells against MPT caused by simvastatin

Simvastatin-induced cell death had been associated with MPT opening in PC3 cells (Oliveira et al., 2008). Thus, we investigated whether L-carnitine or piracetam protect against $\Delta\Psi_m$ decrease that occur prior to necrosis mediated by MPT. PC3 cells were treated with 60 μM simvastatin (Fig. 3) in the presence or absence

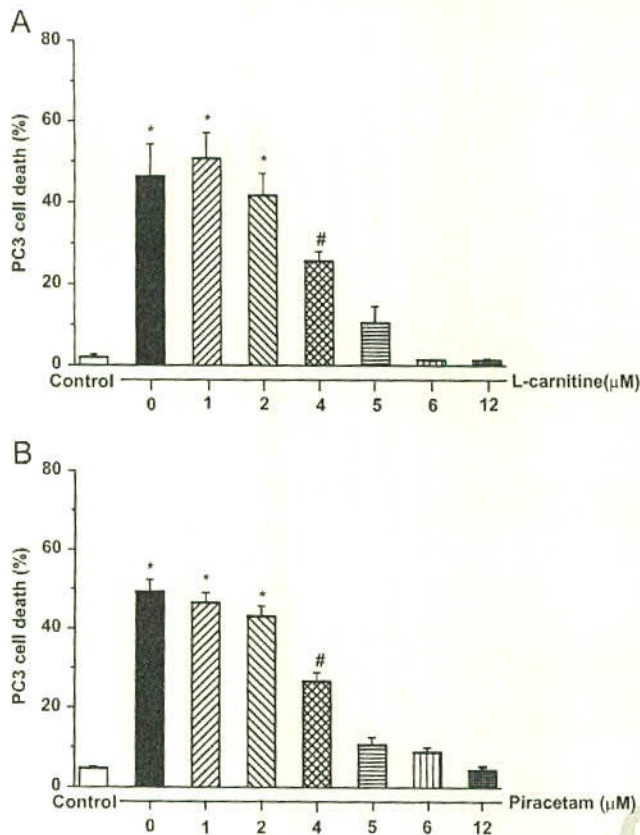


Fig. 1. L-Carnitine or piracetam protect against PC3 cell necrosis induced by simvastatin. PC3 cells were treated with 0.1% DMSO (control) or with 60 μM simvastatin+L-carnitine (A) or 60 μM simvastatin+piracetam (B) for 2 h. After treatment, cells were stained with propidium iodide to estimate dead cells. Values represent the mean $\pm$ SEM ($n=6$). * $P < 0.05$ control versus L-carnitine 0–2 μM, # $P < 0.05$ control versus L-carnitine 4 μM.

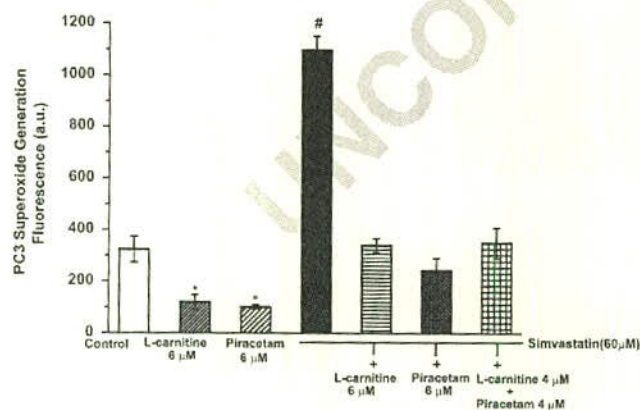


Fig. 2. Protection by L-carnitine or piracetam against superoxide generation induced by simvastatin in PC3 cells. Cells were treated with 6 μM L-carnitine or 6 μM piracetam or 4 μM L-carnitine+4 μM piracetam in the presence (or absence) of 60 μM simvastatin for 2 h. After this treatment they were stained with MitoSox as described in Material and methods. Values represent the mean $\pm$ SEM ($n=3$). * $P < 0.05$ control versus L-carnitine 6 μM and Piracetam 6 μM, # $P < 0.05$ control versus simvastatin 60 μM.

of Cyclosporine A, L-carnitine or piracetam. After these treatments cells were washed and placed in the standard reaction medium in the presence of the fluorescent dye safranin O. The permeabilization of the plasma membrane by digitonin allows the entry of safranin O in the cells, which binds to the inner mitochondrial membrane in response to the proton gradient. This decreases the

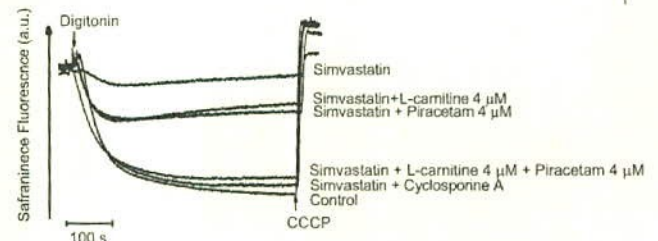


Fig. 3. Protective effects of Cyclosporine A, L-carnitine or piracetam on $\Delta\Psi_m$ decrease of PC3 cells caused by simvastatin. PC3 cells (1×10^6 cells) were treated with 0.1% DMSO (control) or with 60 μM simvastatin in the presence (or absence) of 0.5 μM Cyclosporine A, 4 μM L-carnitine, 4 μM piracetam or 4 μM L-carnitine+4 μM piracetam, as indicated in the figure. After these treatments the cells were washed and resuspended in 0.5 ml of standard reaction medium (125 mM sucrose, 65 mM KCl (pH 7.2), 2.5 mM Na_2HPO_4 , 0.33 mM EGTA, 5 μM safranin O and 5 mM succinate) for $\Delta\Psi_m$ measurements. The arrows indicate additions of 20 μM digitonin and 1 μM CCCP. The figure is representative of four independent experiments.

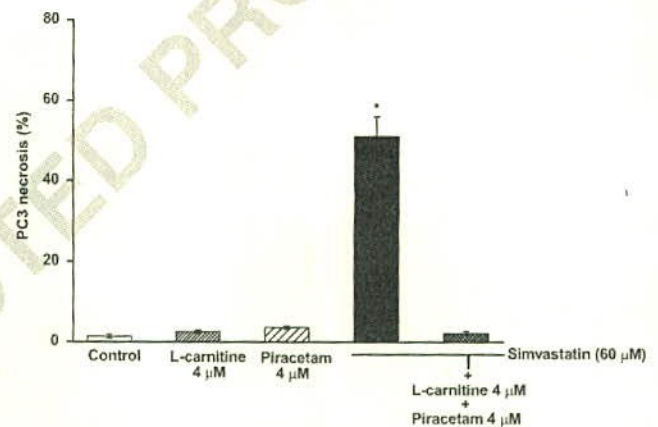


Fig. 4. Protection by the association of L-carnitine plus piracetam against PC3 cell death. PC3 cells were treated with 4 μM L-carnitine, 4 μM piracetam or 4 μM L-carnitine+4 μM piracetam in the presence (or absence) of 60 μM simvastatin. Values represent the mean $\pm$ SEM ($n=6$). * $P < 0.05$ control versus simvastatin 60 μM.

intensity of the fluorescence emitted (Figueira et al., 2012b). The addition of CCCP leads to $\Delta\Psi_m$ elimination and release of safranin O from the membrane, thus allowing for $\Delta\Psi_m$ estimation. Simvastatin caused a large decrease in $\Delta\Psi_m$ sensitive to the MPT inhibitor Cyclosporine A (Fig. 3, compare lines *simvastatin* and *simvastatin+Cyclosporine A*). Similar to the effects observed with Cyclosporine A, the $\Delta\Psi_m$ decrease induced by simvastatin was inhibited either by L-carnitine or piracetam alone, or additively by the combination of these compounds (Fig. 3).

3.4. The combination of L-carnitine and piracetam has additive effects on the protection against necrosis induced by simvastatin

The dose–response experiments shown in Fig. 1 indicated that, at 4 μM, either L-carnitine or piracetam protected the PC3 cells against necrosis by 50%. In addition the experiments of Fig. 3 indicated that the combination of these compounds have additive effects on the protection against simvastatin induced $\Delta\Psi_m$ decrease. In order to ascertain the different molecular mechanisms of these compounds on the protection against cell necrosis we used a combination of both, at the concentrations of 4 μM. Fig. 4 shows that the association of these compounds increased their protective effects against cell necrosis from 50% to about 100%.

4. Discussion

The results described above demonstrate that either L-carnitine or piracetam protect against simvastatin-induced PC3 cell death mediated by increased cytosolic Ca^{2+} concentrations. Considering that calcium is a known signaling agent for mitochondrial reactive oxygen generation, in the present study we investigated whether preventing and/or ameliorating oxidative mitochondrial dysfunction with L-carnitine (Binienda, 2003; Sener et al., 2004; Yapar et al., 2007; Nishimura et al., 2008; Shen et al., 2008; Elinos-Calderon et al., 2009; Ye et al., 2010; Ghavami et al., 2012) or protecting against changes in mitochondrial membrane fluidity by piracetam (Keil et al., 2006) would prevent necrotic cell death induced by toxic concentrations of simvastatin (Oliveira et al., 2008). The results show that the necrotic death of PC3 cells was inhibited in a dose-dependent manner by either L-carnitine or piracetam (Fig. 1). The additive effects of these compounds confirmed their concerted actions through different mechanisms leading to the same final effect, that is, a decreased mitochondrial overproduction of the superoxide anion induced by simvastatin (Fig. 2). Similar results were also obtained with the primary fibroblasts cell line GN16 obtained from normal gingival biopsy and the non-tumorigenic immortalized cell line HaCaT, derived from human keratinocytes (Costa, R.A.P., Fernandes, M.P. and Vercesi, A.E., unpublished results).

Literature data indicate that, at the concentrations used here, statins promote cell death mediated by mitochondrial dysfunction associated with alterations in calcium homeostasis (Sirvent et al., 2005), inhibition of beta-oxidation (Kaufmann et al., 2006) and partial inhibition of respiration (Kwak et al., 2012). Interestingly, all these alterations stimulate reactive oxygen generation by mitochondria supporting the notion that oxidative stress is a common denominator in statins toxicity. Therefore, we next discuss the possible mechanisms underlying the protective effects of L-carnitine and piracetam on the simvastatin effects described above such as cytosolic Ca^{2+} increase, MPT and reactive oxygen generation.

As it might occur in some degree in our experiments, L-carnitine has been shown to stimulate beta-oxidation by increasing the mRNA expression of carnitine palmitoyltransferase 1A. This prevented free fatty acid induced oxidative stress and reduced hepatotoxicity either in vitro or in vivo by enhancing mitochondrial function (Jun et al., 2011). Indeed, the property of free fatty acids as mitochondrial oxidative damaging agents are very well documented either in vivo (Madesh and Balasubramanian, 1997; Ghosh et al., 2006; Tonin et al., 2010; Ruiz-Ramirez et al., 2011) or in vitro (Yao et al., 2005; Zhou et al., 2009; Barazzoni et al., 2012).

With respect to the effect of statins on the mitochondrial electron transport chain, current literature data indicate that like simvastatin (Sirvent et al., 2005), the metabolic inhibitors 3-nitropropionic acid (3-NTA) (Solesio et al., 2012), rotenone (Panov et al., 2005) and 1-methyl-4-phenylpyridinium (MPP+) (Brill and Bennett, 2003) inhibit electrons transport, at the levels of Complexes I or II (Virmani et al., 2004, 2005; Binienda et al., 2005; Panov et al., 2005; Vamos et al., 2010). This causes damage to Complex I and II, presumably at the level of 4Fe–4S clusters (Panov et al., 2005). These alterations diminish resistance to Ca^{2+} thus increasing probability of MPT and necrotic cell death (Panov et al., 2005; Vaseva et al., 2012). Since L-carnitine plays significant neuroprotective effects against all these conditions (Virmani et al., 2002, 2003; Binienda et al., 2003) we propose that L-carnitine, in addition to its metabolic role, may directly interact with 4Fe–4S clusters decreasing the superoxide radical generation. In this respect, it is worthy to mention that L-carnitine has also the ability to bind Fe^{2+} (Gulcin, 2006) a transition metal, supposed to participate in the mitochondrial oxidative stress that leads to MPT (Castilho et al., 1995).

Concerning the molecular mechanisms of piracetam protection against simvastatin toxicity (Fig. 1B), we may consider that

piracetam protects against changes in membrane fluidity via unspecific interactions with the polar head groups of the cellular membranes (Keil et al., 2006). In this regard, we have previously demonstrated that Ca^{2+} binding to inner membrane cardiolipins causes alterations in membrane lipid organization that result in a burst of mitochondrial superoxide generation that triggers MPT (Grijalba et al., 1999). Therefore, it can be proposed that inhibition of simvastatin-induced MPT and necrosis by piracetam is mediated by membrane stabilization that minimizes Ca^{2+} stimulated superoxide production (Fig. 2).

Having investigated PC3 toxicity of statins in vitro and the protection by L-carnitine and piracetam, the question arises to what extent our findings are relevant for the in vivo situation. The plasma concentrations of statins after oral doses of 20–40 mg are around 0.1 $\mu\text{mol/L}$ (Kaufmann et al., 2006) much lower than the toxic concentrations used in our assays. However, the lipophilic nature of simvastatin, presumably, favors its accumulation into the cells, as a function of time, rendering it difficult to estimate its effective concentrations in the mitochondrial environment.

Taken together, our investigations do not offer an explanation for in vivo toxicity of simvastatin. Rather, the findings are more relevant with respect to the understanding of MPT mechanisms and the effects of L-carnitine, piracetam and their combination on the protection against necrosis, either in tumor or non-tumorigenic cells. According mounting evidences accumulated during recent years (Lemasters et al., 2009; Rasola et al., 2010; Toman and Fiskum, 2011; Figueira et al., 2012a; Kim et al., 2012) that MPT-induced necrosis underlies the pathogenesis of leading causes of human death such as cerebral stroke and other vascular diseases (Vaseva et al., 2012).

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