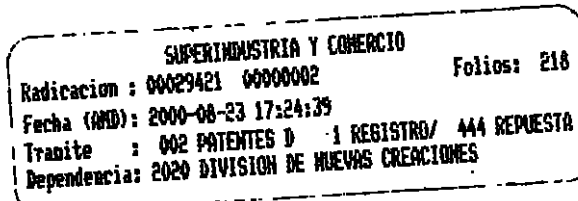


RAISBECK, LARA, RODRIGUEZ & RUEDA

MIEMBROS DE LA FIRMA

BAKER & McKENZIE

CALLE 35 No. 7-25, Piso 4
TELÉFONO: (57-1) 285-1400 / 570-0010
SANTA FE DE BOGOTÁ, D.C.
COLOMBIA



Señores

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

DIVISION DE NUEVAS CREACIONES

E. _____ S. _____ D. _____

Re: **Expediente No. 00.029.421**

Solicitud de Patente de Invención titulada :

“EFECTOS SINERGICOS DE LA
AMLODIPINA Y ATORVASTATINA O
METABOLITO DE LA ATORVASTATINA
COMO BASE PARA UNA TERAPIA
COMBINADA”.

Solicitante : DR. R. PRESTON MASON.

**RESPUESTA AL AUTO NOTIFICADO POR ESTADO No. 073 DE FECHA
25 DE MAYO DE 2000, CONCEPTO TECNICO No. 746**

CARLOS R. OLARTE, mayor de edad y vecino de Santa fe de Bogotá, identificado tal como aparece al pie de mi firma en mi carácter de apoderado de la sociedad solicitante, respetuosamente me permito manifestar lo siguiente ante el auto emanado por ese Despacho, dentro del término termino de prorroga solicitado en mi memorial de fecha 11 de Julio de 2000:

RAISBECK, LARA, RODRIGUEZ & RUEDA

MIEMBROS DE LA FIRMA

BAKER & McKENZIE

CALLE 35 No. 7-25, PISO 4
TELÉFONO: (57-1) 285-1400
SANTA FE DE BOGOTÁ, D.C.
COLOMBIA

- 2 -

1. Sin tomar una posición respecto a las observaciones del Examinador Técnico, **DESISTO** de las reivindicaciones 6 a 182. Para tal efecto, adjunto un Capítulo Reivindicatorio Modificado.

2. En atención a las observaciones del Examinador Jurídico, adjunto las copias certificadas de los Documentos de Prioridad junto con la traducción de sus Capítulos Reivindicatorios. Igualmente adjunto el documento de poder, debidamente legalizado y oficialmente traducido.

Atentamente,



CARLOS R. OLARTE
C.C. No. 79.782.747 de Bogotá
T.P. 74.295

Anexos: Poder.
Capítulo Reivindicatorio Modificado.
Copias Certificadas de los Documentos de Prioridad junto con las Traducciones de sus Capítulos Reivindicatorios.

Traducción Oficial No. 00-307

Efectuada el día 13 de julio del 2000, de un documento escrito en Inglés, al cual para su identificación se le estampa el sello de María del Pilar Iglesias, Traductora e Intérprete Oficial por Resolución No. 819 del 15 de abril de 1971 expedida por el Ministerio de Justicia de la República de Colombia.

Autenticaciones notariales y subsiguientes correspondientes a un Poder otorgado el 9 de junio del 2000 por R. Preston Mason a Alvaro Correa Ordoñez y/o Carlos Reinaldo Olarte y/o Luz Helena Villamil, el cual viene en Español y se explica por sí solo.

Estado de Massachusetts - Condado de Suffolk

Firmado ante mí por R. Preston Mason, a los 9 días del mes de junio del 2000.

(Fdo.) Jerry Cohen - Notario Público.

Estado de Massachusetts

William Francis Galvin

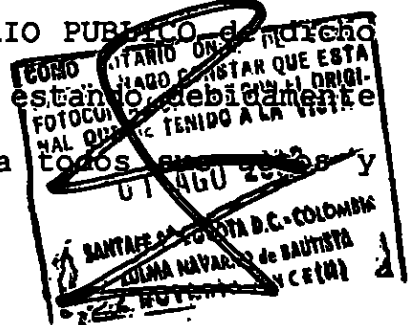
Secretario del Estado

C 235355

Boston, 23 de junio del 2000.

Por el presente certifico que Jerry Cohen, cuyo firma aparece en el certificado de reconocimiento, prueba o declaración jurada adjunta, se desempeñaba como NOTARIO PÚBLICO de dicho

Estado, en el momento de tomar el mismo, estando debidamente comisionado y constituido como tal; que a todos los efectos y



María del Pilar Iglesias
Traductora e
Intérprete Oficial
Resolución No. 819 del 15 de abril de 1971

M. Iglesias

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atestiguaciones debe dárseles toda fe y pleno crédito judicial y extrajudicialmente; que como Notario Público se encontraba legalmente autorizado para tomar declaraciones, administrar juramentos y tomar reconocimientos de escrituras o trasposos de tierras, herencias y demás instrumentos que deban registrarse en dicho Estado de acuerdo con la ley.

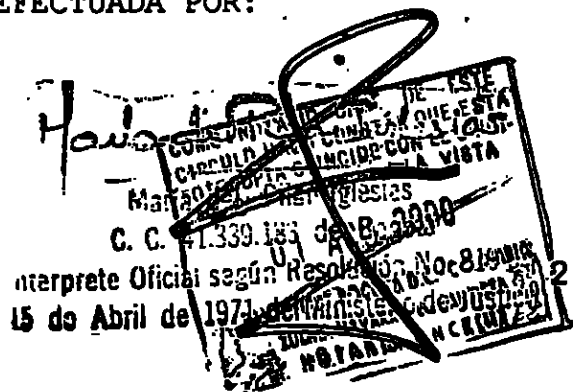
En fe de lo cual en el día de hoy, estampo el Gran Sello del Estado de Massachusetts.

(Fdo.) William Francis Galvin - Secretario de Estado de Massachusetts. (Sello del Estado de Massachusetts).

En Español, viene certificación No. 531 del 27 de junio del 2000, expedida por el Consulado de Colombia en Boston, Estados Unidos de América, certificando la firma y cargo precedentes. Dicha certificación está firmada por Isabel Pardo Uribe, Vicecónsul de Colombia, cuya firma fue finalmente reconocida por el Ministerio de Relaciones Exteriores de Colombia bajo el sello No. 094731 del 13 de julio del 2000.

ES TRADUCCION FIEL Y COMPLETA EFECTUADA POR:

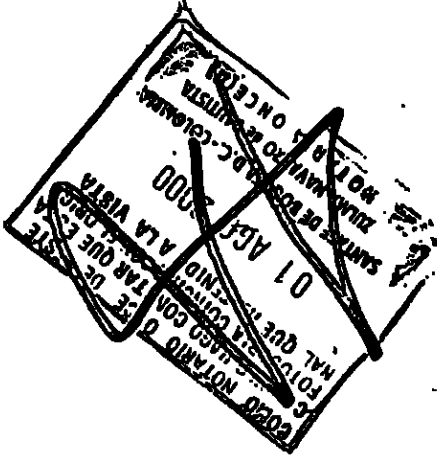
Maria del Pilar Iglesias
Maria del Pilar Iglesias
C.C. 41.339.183 de Bogotá.



MINISTERIO DE RELACIONES
EXTERIORES

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JURA FURTO ARREBES CENUBA
DOCUMENTO DESEMPENA
LAS FERTILES INDIADAS



NO SE ABOLME
RESPONSABILIDAD

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JEFE DE LEGALIZACIONES
SANTAFE DE BOGOTA D.C.



Consulado de Colombia
535 Boylston Street, 11th Floor Boston, MA 02116
Tel: (617) 536-6222 Fax: (617) 536-9372

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7:00-10:00
Marta del Pilar Iglesias
Traductora
Interpret Local
Boschella P.L. 270 Huntington
13 JULIO, 2000

No 531

FECHA: JUN 27 2000

El suscrito Cónsul de Colombia en Boston, MA Estados Unidos vistos los documentos presentados.

CERTIFICA:

Que el señor WILLIAM FRANCIS GALVIN, quien firma el documento adjunto, ejercía legalmente en la fecha allí indicada las funciones de SECRETARIO DE ESTADO DEL ESTADO DE MASSACHUSETTS, ESTADOS UNIDOS DE AMERICA y su firma y sello que aparecen en el documento son los que acostumbra a usar en sus actos oficiales.
El Cónsul no asume responsabilidad por el contenido del documento.

Pagado Impuesto de Timbre US\$22.00

NO SE ASUME
RESPONSABILIDAD DEL TEXTO

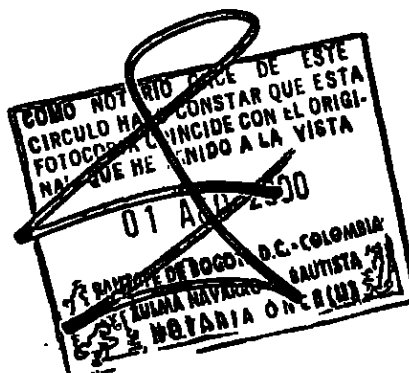
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ISABEL PARDO
JEFE DE LEGALIZACIONES
CONSULADO DE COLOMBIA



MINISTERIO DE RELACIONES
EXTERIORES

094731
CUYA FIRMA APOYO EN EL
DOCUMENTO DESEMPEÑA
LAS FUNCIONES INDICADAS





The Commonwealth of Massachusetts

William Francis Galvin
Secretary of the Commonwealth

Boston, June 23, 2000

I hereby certify that at the date of the attestation hereunto annexed,

Jerry Cohen

whose name is signed to the attached certificate of acknowledgment, proof or affidavit, was at the time of taking the same, a NOTARY PUBLIC for the said Commonwealth duly commissioned and constituted; that to his/her acts and attestations, as such, full faith and credit are and ought to be given in and out of Court; that as such Notary Public, he/she was by law authorized to take depositions, to administer oaths and take acknowledgments or deeds or conveyances of lands, tenements or hereditaments and other instruments throughout the Commonwealth to be recorded according to law.

In testimony of which, I have hereunto affixed the

Great Seal of the Commonwealth

on the date above written.

William Francis Galvin

William Francis Galvin
Secretary of the Commonwealth



RAISBECK, LARA, RODRIGUEZ & RUEDA

Members of the Firm

BAKER & MCKENZIE

ATTORNEYS AT LAW

APARTADO AEREO 3746

TELEFONO: 285-1400

SANTAFE DE BOGOTA, D.C.

COLUMBIA

POWER OF ATTORNEY

The undersigned.

Dr. R. Preston Mason

located in
**Sewickley,
Pennsylvania
USA**

declares that it hereby grants to

**ALVARO CORREA ORDOÑEZ and/or
CARLOS REINALDO OLARTE
LUZ HELENA VILLAMIL and/or**

a full and sufficient power of attorney to apply to the proper national officers and authorities for the issue of:

Letters Patent, mergers, changes of name, assignments, filing and prosecution of oppositions, changes of address

to which end it empowers them to take all steps necessary before said authorities for the objects stated, to file petitions, prepare descriptions, make protests, declarations, appeals and objections, pay imposts, apply for certifications, receive documents and securities, abandon applications and collect refunds. To the said mandatories sufficient power is granted hereby to receive notifications for and on behalf of the Company, to confer powers on those who are to represent the company judicially and extrajudicially, to answer in court in the matter of all claims and demands that may be presented in connection with the patents, and to do everything that may be required, before administrative or judicial officers of any class giving them likewise power to substitute these presents and revoke such substitutions if necessary. Additionally, the Company hereby ratifies all acts taken to date on its behalf and in furtherance of this Power of Attorney.

Done and subscribed in

Boston, Massachusetts USA

On the 9th day of June

2000

IX

RPM

R. Preston Mason

Commonwealth of Massachusetts)
County of Suffolk) ss

Signed before me this 9th day of June 2000
by the said R. Preston Mason,

Terry Cohen, Notary Public

PODER

La abajo firmada.

Dr. R. Preston Mason

domiciliada en
**Sewickley,
Pensilvania
USA**

declara por el presente otorgar a

**ALVARO CORREA ORDOÑEZ y/o
CARLOS REINALDO OLARTE
LUZ HELENA VILLAMIL y/o**

poder especial amplio y bastante para recabar de las oficinas y autoridades nacionales que correspondan, la obtención de:

Patentes de Invención, fusiones, cambios de nombre, traspasos, presentación y tramitación de oposiciones, cambios de dirección

a cuyo efecto los faculta para dar ante dichas autoridades, todos los pasos necesarios al objeto indicado, elevar solicitudes, formular descripciones, protestas, declaraciones, apelaciones y reclamos, oblar impuestos, solicitar testimonios, recibir documentos y valores, desistir y percibir. Se concede a los expresados mandatarios poder bastante para recibir notificaciones a nombre de la compañía, otorgar poderes a quienes vayan a representar a la compañía judicial y extrajudicialmente, responder en juicio a todas las reclamaciones o demandas que por motivo de las patentes se presentaren y hacer cuanto fuere necesario, ante las autoridades administrativas o judiciales de cualquier orden, dándoles asimismo facultad para sustituir el presente y en caso necesario revocar dichas sustituciones. Adicionalmente, la compañía por medio de la presente ratifica toda actuación tomada en representación suya y en relación con este poder.

Dado y firmado en

Boston, Massachusetts USA

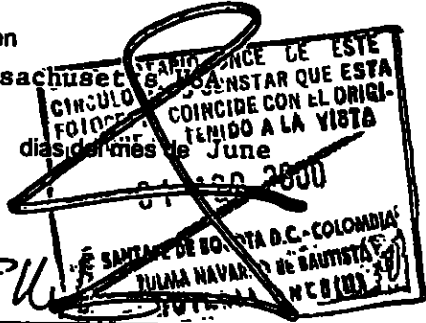
A los 9th

2000

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RPM

R. Preston Mason



REIVINDICACIONES:

Exp. 00-29421

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1. Una composición farmacéutica la cual comprende:

(a) una cantidad terapéuticamente efectiva de

5 amlodipina;

(b) una cantidad terapéuticamente efectiva de un compuesto de atorvastatina y metabolito de atorvastatina hidroxilado; y

(c) agentes de formulación farmacéuticamente

10 aceptables.

2. La composición farmacéutica de la reivindicación 1 en donde la amlodipina comprende un derivado terapéuticamente efectivo de la amlodipina.

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3. La composición farmacéutica de la reivindicación 2 en donde el derivado terapéuticamente aceptable de la amlodipina comprende besilato de amlodipina.

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4. La composición farmacéutica de la reivindicación 1 en donde el compuesto de atorvastatina comprende un derivado terapéuticamente efectivo del compuesto de atorvastatina.

5. La composición farmacéutica de la reivindicación 4 en donde el derivado terapéuticamente aceptable del compuesto de atorvastatina es una sal de hemicalcio.

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LOS ESTADOS UNIDOS DE AMERICA

A TODOS AQUELLOS A QUIEN SE LES PRESENTE ESTE DOCUMENTO

DEPARTAMENTO DE COMERCIO DE LOS ESTADOS UNIDOS

Oficina de Patentes y Marcas de los Estados Unidos

14 de julio de 2000

ESTO ES PARA CERTIFICAR QUE LO ANEXADO AQUI ES UNA COPIA VERDADERA DE LOS REGISTROS DE LA OFICINA DE PATENTES Y MARCAS DE LOS ESTADOS UNIDOS DE AQUELLOS PAPELES DE ESTA SOLICITUD IDENTIFICADA ENSEGUIDA QUE CUMPLIÓ CON LOS REQUISITOS PARA SER OTORGADA UNA FECHA DE PRESENTACIÓN BAJO 35 USC 111.

NUMERO DE SOLICITUD: 60/130.665

FECHA DE RADICACION: 23 de abril de 1999

Por autoridad de

El COMISIONADO DE PATENTES Y

MARCAS

Sello de la Oficina de	(Firmado N. Woodson)
Patentes y Marcas de	N. WOODSON
los Estados Unidos	Oficial de
de América	Certificación

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RESUMEN

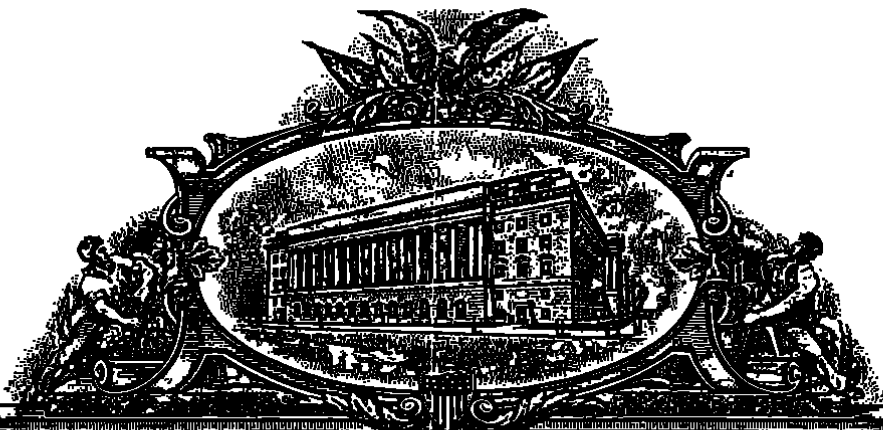
Por primera vez, los estudios demuestran que los medicamentos cardiovasculares ampliamente utilizados, amlodipina (Norvasc®) y la atorvastatina (Lipitor®), tienen efectos antioxidantes sinérgicos. Esta observación suministra una razón fundamental precisa para su uso combinado en un sistema de liberación única para pacientes con hipertensión y niveles elevados de colesterol en suero, puesto que el estrés oxidativo contribuye con la patofisiología de varios trastornos cardiovasculares, especialmente la enfermedad coronaria. Los efectos antioxidantes sinérgicos de estos compuestos fueron observados *in vitro* en preparaciones aisladas de membrana bajo condiciones experimentales bastante controladas. La base de este efecto antioxidante fue determinado mediante cálculos de mecánica cuántica y análisis de modelado molecular. Aunque los efectos antioxidantes de la amlodipina han sido reportados por separado por mi laboratorio¹, los efectos sinérgicos de estos agentes no han sido publicados hasta la fecha. Los métodos para medir las propiedades antioxidantes separadas y combinadas de la amlodipina y la atorvastatina han sido descritas detalladamente por mi laboratorio en una publicación reciente¹. Es igualmente importante anotar que los efectos antioxidantes para otros agentes antihipertensivos, que

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• tienen un mecanismo de acción similar a la amlodipina, no fueron observados en previos experimentos en mi laboratorio¹.

CONCLUSION

Los resultados de los análisis experimentales y de cómputo han demostrado, por primera vez, un efecto antioxidante sinérgico de la amlodipina y la atorvastatina bajo condiciones experimentales bastante controladas. Además de los mecanismos de acción establecidos para estos agentes, este beneficio adicional suministra razón fundamental para usar combinadamente estos agentes en un *sistema de liberación única* para el tratamiento de las enfermedades coronarias y la hipertensión.



THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

July 14, 2000

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: 60/130,665

FILING DATE: April 23, 1999



**By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS**


N. WOODSON

Certifying Officer

Please type a plus sign (+) inside this box → ☐

PTO/SB/16 (2-98)

Approved for use through 01/31/2001. OMB 0851-0037
Patent and Trademark Office, U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

INVENTOR(S)				
Given Name (first and middle (if any))	Family Name or Surname	Residence (City and either State or Foreign Country)		
R. Preston	Mason	905 Sewickley Heights Sewickley, PA 15143		
☐ Additional inventors are being named on the _____, separately numbered sheets attached hereto				
TITLE OF THE INVENTION (280 characters max)				
SYNERGISTIC ANTIOXIDANT EFFECTS OF AMLODIPINE (NOVASC) AND ATORVASTATIN (LIPITOR)				
Direct all correspondence to: CORRESPONDENCE ADDRESS				
☐ Customer Number				
OR		Type Customer Number here		
☒ Firm or Individual Name		Jerry Cohen, Esq.		
Address		Perkins, Smith & Cohen		
Address		One Beacon Street, 30th Floor		
City		Boston	State	MA
Country		USA	ZIP	02108
		Telephone	617-854-4000	Fax 617-854-4040
ENCLOSED APPLICATION PARTS (check all that apply)				
☒	Specification Number of Pages	11	☐	Small Entity Statement
☒	Drawing(s) Number of Sheets	8	☐	Other (specify)
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (check one)				
☒	A check or money order is enclosed to cover the filing fees			FILING FEE AMOUNT (\$)
☒	The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 03-2410			\$150
The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.				
☒ No.				
☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____				

Respectfully submitted,

SIGNATURE

Date 4/23/99

TYPED or PRINTED NAME

Jerry Cohen

REGISTRATION NO.

20,522

(if appropriate)

Docket Number:

2189 P02 PROV

TELEPHONE

617 854 4000

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.61. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C., 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C., 20231.

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SUMMARY

Studies demonstrate, for the first time, that the widely used cardiovascular medications, amlodipine (Norvasc[®]) and atorvastatin (Lipitor[®]) have synergistic *antioxidant* effects. This observation provides a compelling rationale for their combined use in a single delivery system for patients with hypertension and elevated levels of serum cholesterol, as oxidative stress contributes to the pathophysiology of various cardiovascular disorders, especially coronary artery disease. The synergistic antioxidant effects of these compounds were observed *in vitro* in isolated membrane preparations under well-controlled experimental conditions. The basis for this antioxidant effect was determined from quantum mechanics calculations and molecular modeling analyses. Although the antioxidant effects of amlodipine has been separately reported by my laboratory ¹, the synergistic effects of these agents has not been published to date. The methods for measuring the separate and combined antioxidant properties of amlodipine and atorvastatin have been described in detail by my laboratory in a recent publication ¹. It is also important to note that antioxidant effects for other antihypertensive agents that have a similar mechanism of action as amlodipine were not observed in previous experiments from my laboratory ¹.

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METHODS

Lipid peroxidation analysis: The separate and combined dose-dependent antioxidant activities of amlodipine, atorvastatin, and its metabolites statins were examined in membranes enriched with polyunsaturated fatty acids ¹. For these experiments, aliquots of 500 µl of membrane vesicles (5.0 mg/ml phospholipid) were added to separate test tubes. Membrane peroxidation was carried out at 37°C in a shaking water bath. At various time intervals, a 100 µl aliquot of sample was removed and 25 µl of 5.0 mM ethylenediaminetetracetic acid (EDTA) was immediately added to the sample to stop the peroxidation reaction.

The extent of membrane lipid peroxidation was measured by the CHOD-Iodide assay as previously described in detail ². The quantity of I₂⁻ was measured from the following reaction:



A 100 µl aliquot of the membrane sample was added to 20 µl of 35.0 mM butylated hydroxytoluene (BHT) (in ethanol) and 80 µl of 5.0 mM EDTA and 1.0 ml of CHOD color reagent (E.M. Science, Gibbstown, NJ). The sample was then covered with foil and allowed to incubate for 2 hours in the absence of light before measuring the absorbance of the sample at 365 nm ($\epsilon = 2.4 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$). The background sample was run along the test samples in triplicate and contains 76.7 µl of 0.652 mM HEPES, 20 µl of 5.0 mM EDTA and 3.3 µl of DDI water. The extent of lipid peroxidation was measured in triplicate for each drug concentration and compared to control samples that did not contain drug. The statistical significance of these experiments was assessed by the non-paired t-test. Significance was accepted at $p < 0.05$.

RESULTS AND DISCUSSION

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In Figure 1, the separate and combined (synergistic) antioxidant effects of amlodipine and atorvastatin are reviewed. At pharmacological levels (~100 nM), the combination of amlodipine and atorvastatin achieved a level of antioxidant activity that could not be achieved separately for these compounds, except at supra-pharmacologic levels ($\geq 1.0 \mu\text{M}$). In these experiments, the control level of lipid peroxide formation was typically 1.2 mM. Thus, an inhibition of lipid peroxide formation by over 35% indicates that 0.1 μM concentrations of drug inhibited over 400 μM lipid peroxide, a ratio of drug to lipid peroxide inhibition of >1:4000. This remarkable effect indicates that the drug combination is capable of interfering with the lipid peroxide chain reaction.

To understand the basis for the antioxidant effects of these compounds, the chemical structure of these molecules was evaluated (Figures 2,3). In Figure 2A, the covalent structure of amlodipine includes two hydrogen atoms associated with its dihydropyridine ring that can be donated to unstable free radicals associated with lipids. Once a hydrogen atom is donated, an unpaired free electron remains associated with the amlodipine molecule. This unpaired electron, a potential source of free radical damage, can be safely stabilized in the resonance structure calculated for amlodipine in Figures 2B and 2C. Similar chemical analyses were conducted for a key hydroxyl metabolite of atorvastatin, as indicated in Figure 3. In addition to the chemical properties of these agents, these compounds have strong affinity for the membrane lipid bilayer, resulting in an alteration in the intermolecular diffusion of free radicals, as previously reported for amlodipine ¹.

The antioxidant activity of amlodipine may contribute to its benefit in patients with documented coronary artery disease, as recently reported in the Prospective Randomized

Evaluation of the Vascular Effects of Norvasc Trial (PREVENT)³. Patients randomized to amlodipine showed a significant reduction in angina (-33%, $p < 0.01$), major vascular procedures (-45%, $p < 0.001$), and the composite of any major event (-30%, $p > 0.01$), as compared to placebo, independent of changes in lipid levels⁴. The benefits of amlodipine in coronary artery disease have not been observed with other drugs in this class, suggesting that the additional antioxidant properties of this compound contribute to direct antithrombotic effects¹.

Role of Antioxidants in Reducing Coronary Artery Disease: The results of animal and clinical investigations have indicated an important role for lipophilic antioxidants in reducing cardiovascular morbidity and mortality, especially coronary artery disease. The rationale for this observation is attributed to the ability of these compounds to interfere with the peroxidation of lipids associated with LDL and vascular cell membranes. In particular, it has been demonstrated that LDL particles with greater resistance to oxidative damage, due to the presence of lipophilic antioxidants, have reduced cytotoxicity, interfere less with endothelium-derived nitric oxide production, and do not contribute to foam cell formation. Supplementation with α -tocopherol (i.e., vitamin E) has been shown to increase LDL resistance to oxidative modification, and thus, reduce cytotoxicity toward endothelial cells⁵. The lipophilic antioxidant probucol has also been demonstrated to attenuate the formation of atherosclerotic plaques in cholesterol-fed primates, an effect that correlated with an increased resistance of LDL to oxidative damage⁶. In addition, probucol inhibited atheroma formation of lesions in Watanabe hereditary hyperlipidemic (WHHL) rabbits, a well-characterized animal model of atherosclerosis, independent of cholesterol-lowering effects⁷. Consistent with these animal studies, clinical investigations have shown that probucol reduced restenosis

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by 47% in patients with coronary artery disease following coronary-artery balloon angioplasty, presumably due to its antioxidant effects ⁸.

Another pathological process associated with oxidized LDL is an inhibition of the constitutive release of endothelium-derived nitric oxide in the arterial wall ⁹⁻¹¹. Release of nitric oxide inhibits the abnormal adhesion of leukocytes and platelets to the endothelial surface early during the development of foam cell formation. Nitric oxide is also an important mediator of vasodilation; loss of nitric oxide can lead to vasospasm associated with acute coronary syndromes ¹². The interference of this process by oxidized LDL is attributed to a disruption in secondary messenger systems, including G-protein-dependent nitric oxide release ^{10, 11}. Patients treated with the antioxidant probucol had improved endothelial function, as assessed by nitric oxide production, when compared to control subjects. LDL isolated from these patients showed greater resistance to oxidation *in vitro* and did not interfere with normal nitric oxide production from the endothelium, as compared to control LDL samples ¹³. An important benefit associated with lipophilic antioxidants is the ability to inhibit endothelial dysfunction associated with oxidized LDL, a key early event in atherosclerosis ¹⁴.

Thus, a comprehensive review of the available data provides a compelling mechanistic rationale for the use of lipophilic antioxidants to prevent cellular changes associated with coronary artery disease. By increasing the resistance of LDL to oxidative damage, agents with antioxidant activity may effectively interfere with pathologic alterations in the vessel wall during atherogenesis, including foam cell formation, endothelial dysfunction and toxicity, leukocyte and platelet adhesion, and arterial vasospasm, secondary to a loss of normal nitric oxide production. These cellular observations also provide

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important insights into the results of recent clinical studies demonstrating a benefit associated with antioxidant use by reducing the expression of established coronary artery disease despite an absence of pronounced changes in atherosclerotic lesion development ¹⁵. Indeed, the results of epidemiological analyses have provided direct evidence for an inverse association between the serum levels of antioxidants (e.g., vitamin E and vitamin C) and adverse outcomes associated with coronary disease ¹⁶⁻²².

CONCLUSION

The results of experimental and computational analyses have demonstrated, for the first time, a synergistic antioxidant effect for amlodipine and atorvastatin under well-controlled experimental conditions. In addition to the established mechanisms of action for these agents, this additional benefit provides a compelling rationale for the combined use of these agents in a *single delivery system* for the treatment of coronary artery disease and hypertension.

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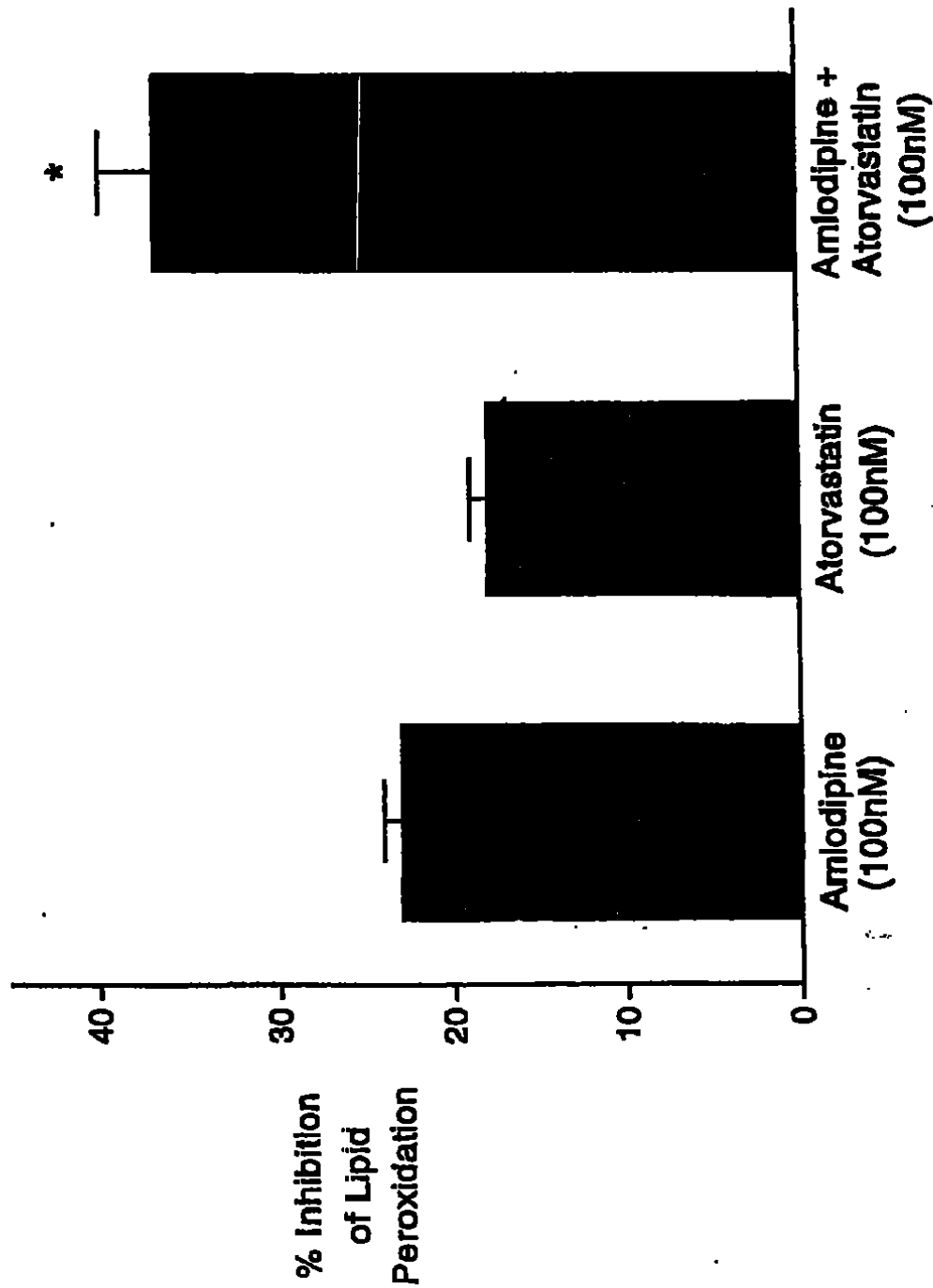
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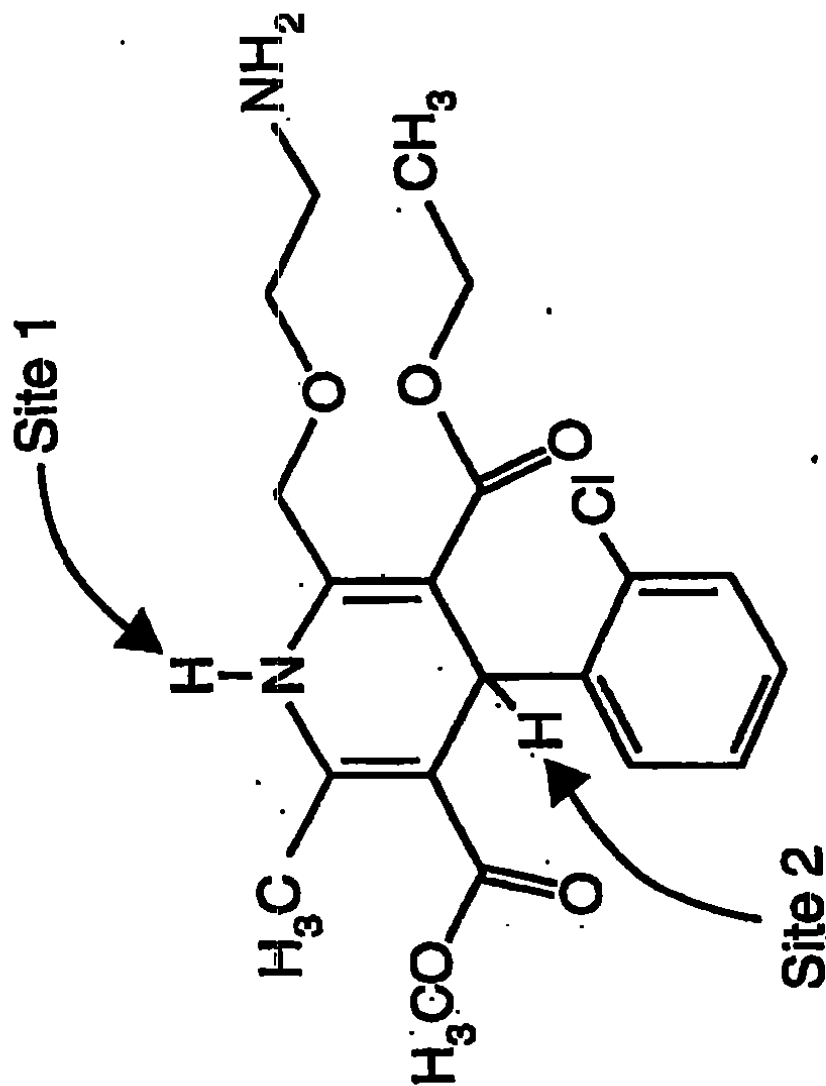
Synergistic Antioxidant Effects of Amlodipine and Atorvastatin Metabolite



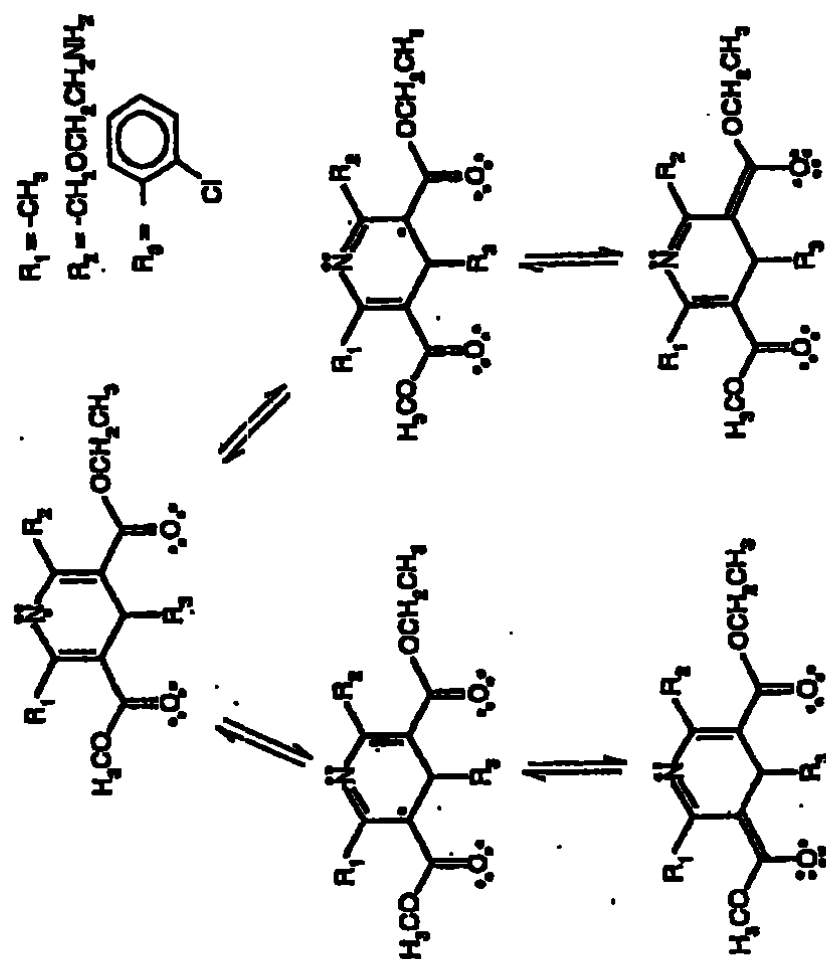
* p<0.001 compared to control or separate drug treatments
Values are mean ± S.D. (n=3)

FIGURE 1

Chemical Antioxidant Mechanisms for Amlodipine: Abstractable Hydrogens



Resonance Stabilization of Free Radical at Site 1 of Amlodipine



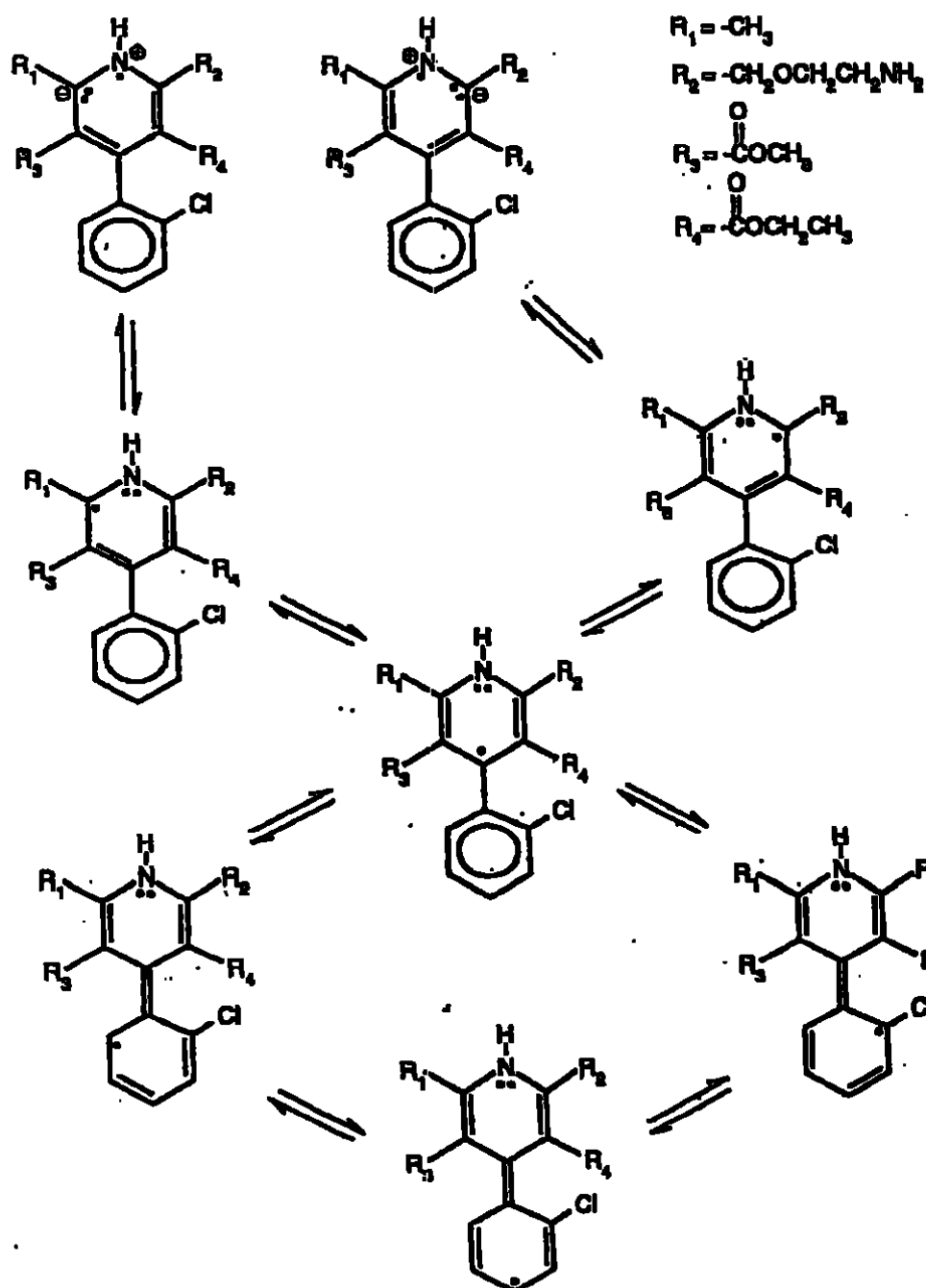
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FIGURE 2B

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Resonance Stabilization of Free Radical at Site 2 of Amlodipine

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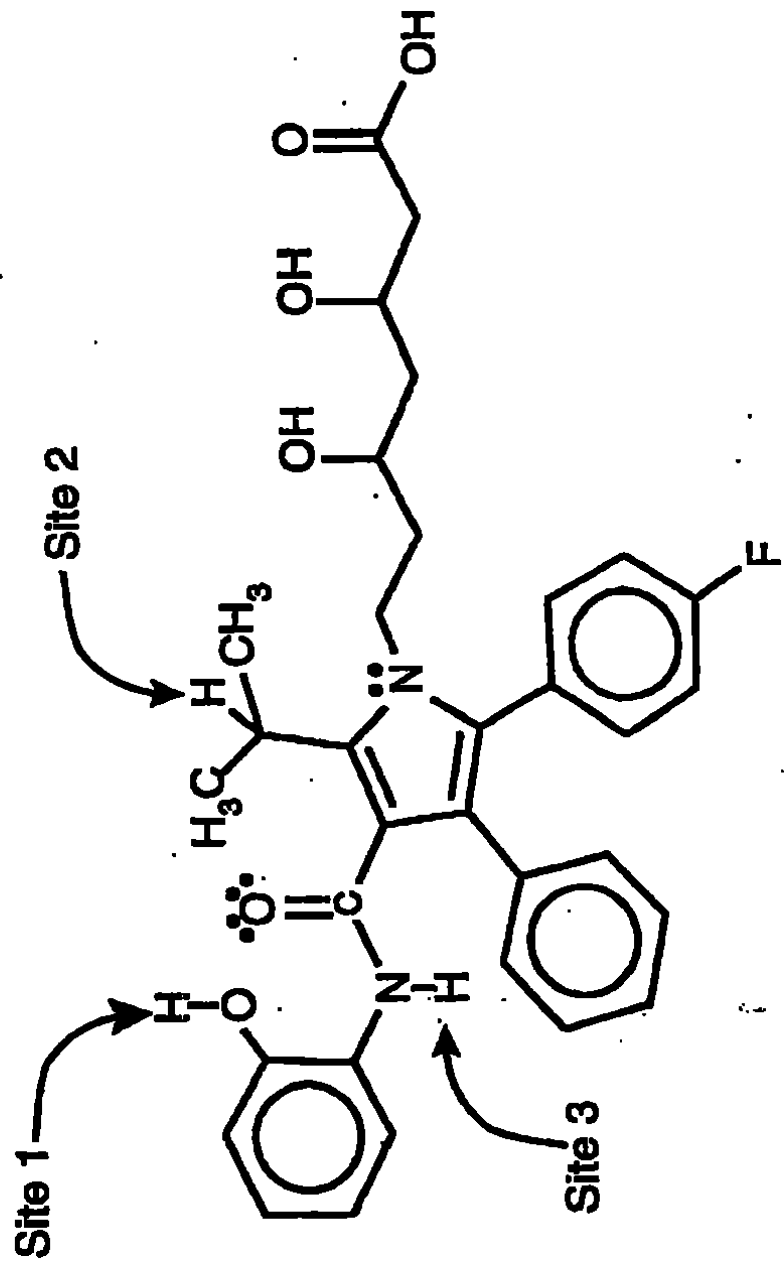


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FIGURE 2C

Antioxidant Mechanisms for Atorvastatin Metabolite: Abstractable Hydrogens



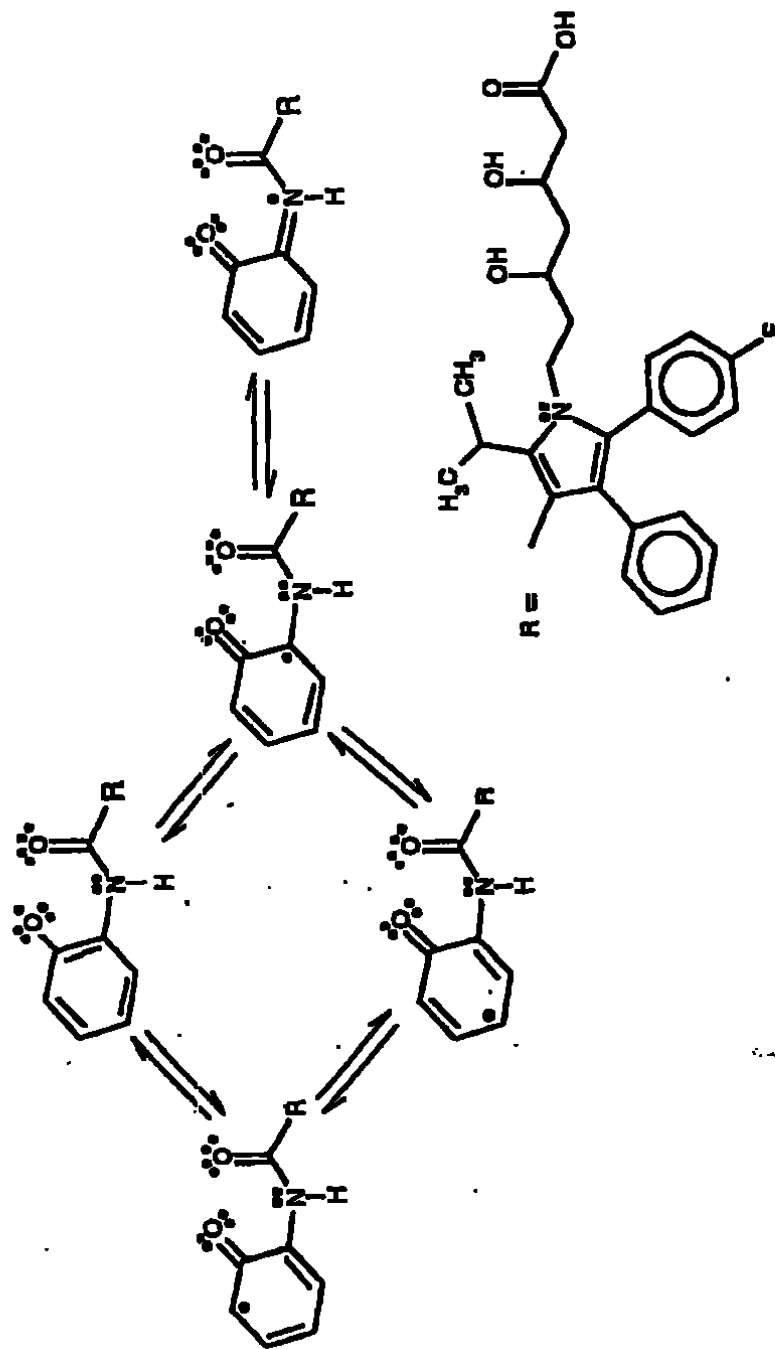
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FIGURE 3A

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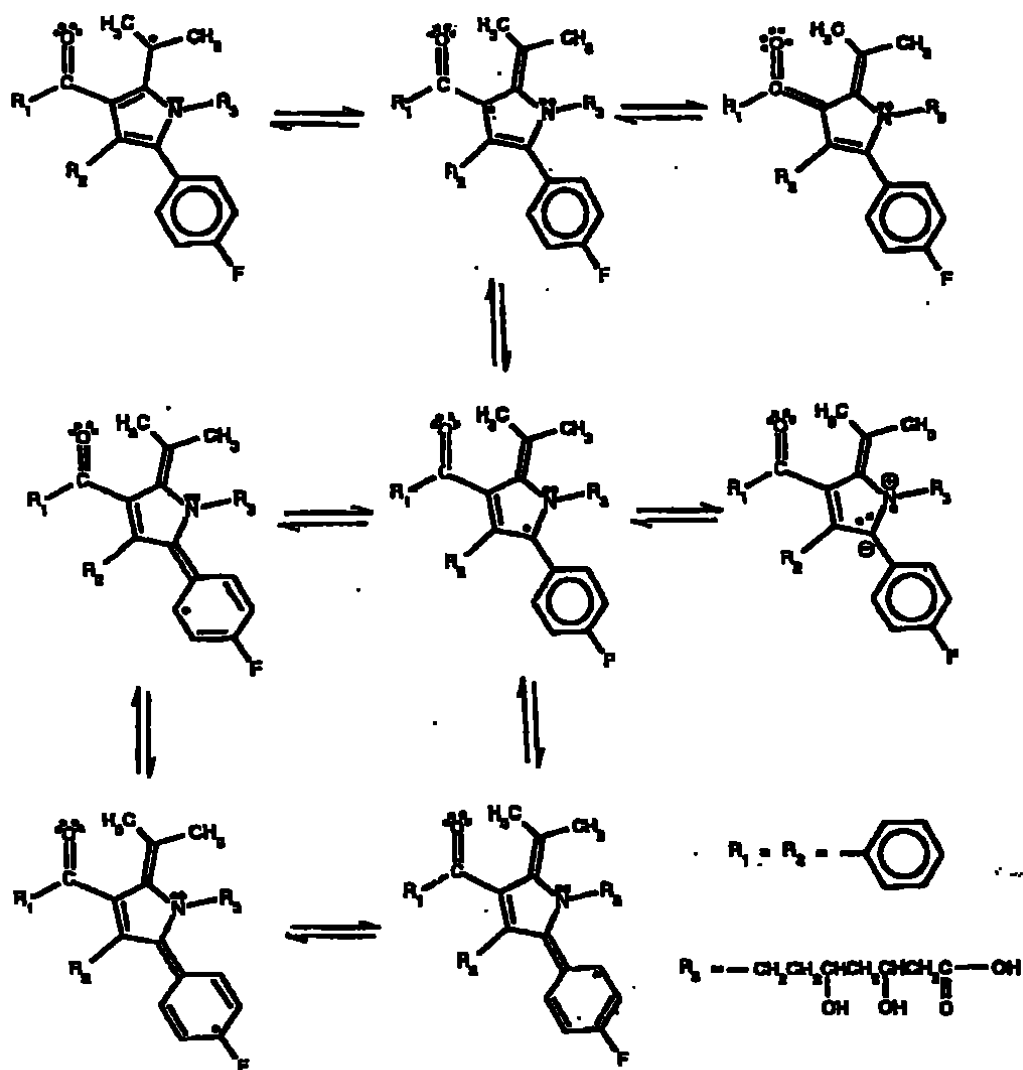
Antioxidant Mechanisms for Atorvastatin Metabolite: Resonance Stabilization of Free Radical at Site 1



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FIGURE 3B

Antioxidant Mechanisms for Atorvastatin
Metabolite: Resonance Stabilization of
Free Radical at Site 2



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FIGURE 3C

Antioxidant Mechanisms for Atorvastatin Metabolite: Resonance Stabilization of Free Radical at Site 3

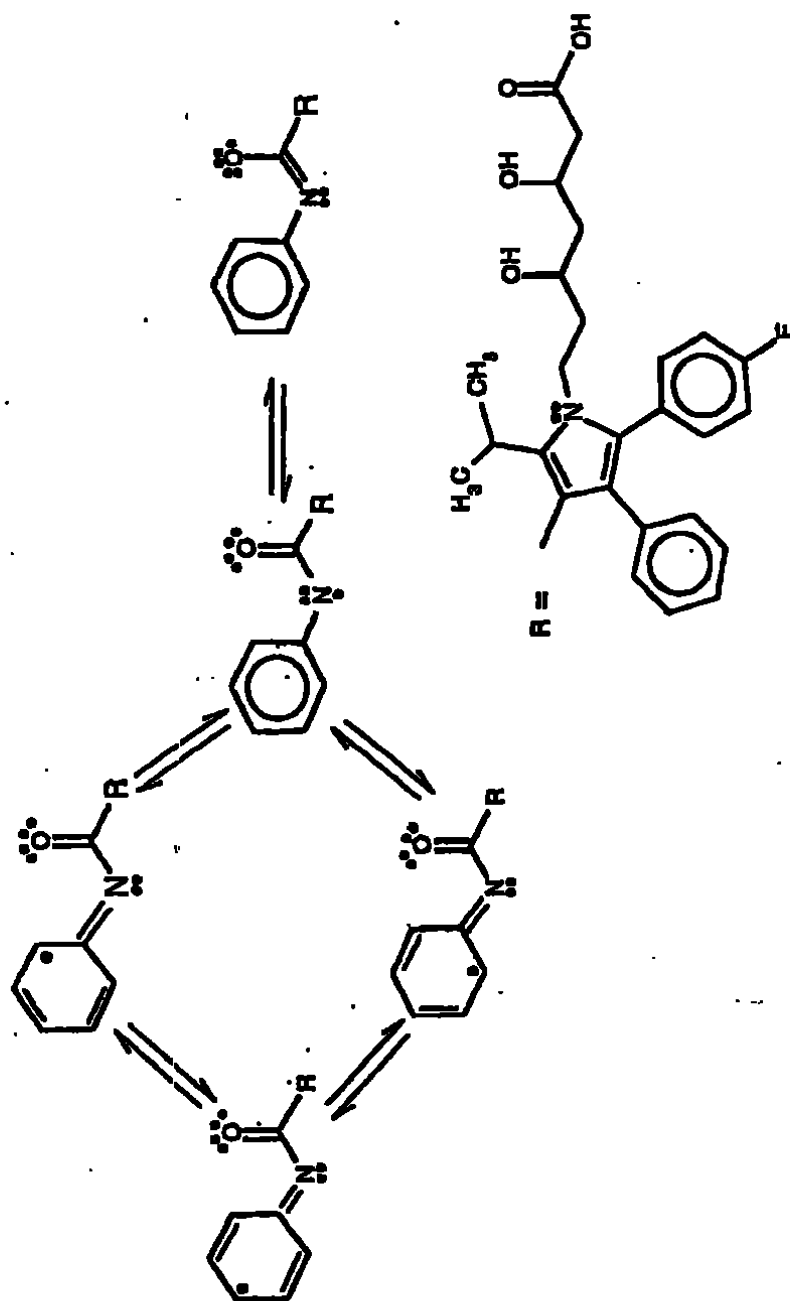


FIGURE 3D

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NUMERO DE SOLICITUD: 60/145.305

FECHA DE RADICACION: 23 de julio de 1999

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Resumen del Invento

Se describen análisis científicos que soportan el uso combinado de besilato de amlodipina (Norvasc®) y atorvastatina cálcica (Lipitor®) en un sistema de liberación única para el tratamiento de enfermedades cardiovasculares. Específicamente, las actividades antioxidantes sinérgicas del bloqueador de canales de calcio (CCB), besilato de amlodipina, y el inhibidor de la HMG-CoA reductasa, atorvastatina cálcica, incluyendo su metabolito hidroxilado, fueron evaluados en un sistema lipídico bien definido en donde las vesículas lípidas son enriquecidas con ácidos grasos poliinsaturados (PUFA), blanco principal del daño oxi-radical en la aterosclerosis. Los efectos sinérgicos de estos agentes fueron demostrados en membranas preparadas en ausencia y presencia de niveles de colesterol elevados que reproducen condiciones del tipo esclerótico. La combinación de la amlodipina con el metabolito hidroxilado de la atorvastatina efectuó una reducción dramática y sostenida del daño oxi-radical lipídico en concentraciones tan bajas como 10.0 nM. Estos experimentos fueron llevados a cabo en membranas con colesterol que reproducen condiciones del tipo fisiológico. La actividad antioxidante catalítica asociada con la combinación de estos fármacos en niveles terapéuticos fue latamente sinérgico y no pudo ser efectivamente

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reproducido por la vitamina E o el carvedilol, un beta bloqueador no selectivo con efectos antioxidantes establecidos. La inhibición sinérgica de la peroxidación lipídica también fue observada con la combinación de la amlodipina y el compuesto precursor de la atorvastatina. La actividad antioxidante no fue demostrada para otros bloqueadores de los canales de calcio (CCBs) que fueron probados (felodipina, verapamil, diltiazem) o los inhibidores de la HMG-CoA reductasa (mevastatina, pravastatina, simvastatina). La propiedad antioxidante diferente, descrita para esta combinación de fármacos, se puede atribuir a las interacciones catalíticas y fisicoquímicas con la bicapa de la membrana que son independientes de los efectos bien caracterizados de estos fármacos sobre el transporte del calcio y el metabolismo del colesterol. Este beneficio antioxidante sinérgico constituye un nuevo mecanismo farmacológico de acción para estos compuestos y una razón fundamental precisa para el uso combinado de los ingredientes activos en Norvasc® y Lipitor® en el tratamiento de enfermedades cardiovasculares, al reducir los niveles de LDL (lipoproteínas de baja densidad) en plasma y mejorar la protección contra la oxidación. Esta nueva propiedad complementa los efectos establecidos de estos fármacos con respecto a la hipertensión y la dislipidemia.

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Descripción Breve de los Dibujos

La Figura 1 muestra la actividad antioxidante dosis-dependiente de la AML.

La Figura 2 muestra los efectos antioxidantes comparativos de la AML y los dos otros bloqueadores de los canales de calcio y un inhibidor del ACE.

La Figura 3 muestra la actividad antioxidante dosis-dependiente del ATM.

La Figura 4 muestra los efectos antioxidantes comparativos de los inhibidores de la HMG-CoA reductasa.

La Figura 5 muestra el efecto del enriquecimiento con colesterol en la formación de peróxido lipídico.

La Figura 6 muestra el efecto del enriquecimiento con colesterol en las actividades antioxidantes del ATM.

La Figura 7 muestra el efecto antioxidante dosis-dependiente de la combinación de la AML y el ATM.

La Figura 8 muestra los efectos antioxidantes sinérgicos de la AML con ATM a 100.0 nM y de AML con AT a 1.0 μ M en vesículas enriquecidas con colesterol.

La Figura 9 muestra los efectos antioxidantes comparativos de AML/ATM y AML/AT a 0.1 y 1.0 μ M en vesículas enriquecidas con colesterol.

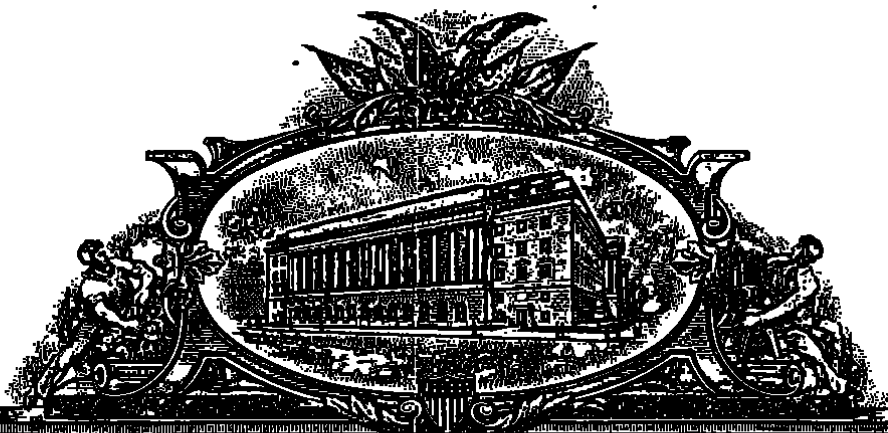
La Figura 10 muestra los efectos antioxidantes comparativos de la vitamina E, carvedilol, probucol, y la combinación de AML/ATM después de varios periodos de incubación a una concentración de 10.0 μ M.

La Figura 11 muestra los mecanismos antioxidantes químicos para la AML.

La Figura 12 muestra los mecanismos antioxidantes químicos para el ATM.

La Figura 13 muestra las interacciones moleculares de la membrana de AML y ATM.

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APPLICATION NUMBER: 60/145,305

FILING DATE: July 23, 1999



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INVENTOR(S)					
Given Name (first and middle (if any))	Family Name or Surname	Residence (City and either State or Foreign Country)			
R. Preston	Mason	Sewickley, PA			
☐ Additional inventors are being named on the _____ separately numbered sheets attached hereto					
TITLE OF THE INVENTION (280 characters max)					
SYNERGISTIC ACTIVITY OF AMLODIPINE BESYLATE (NORVASC) AND ATORVASTATIN CALCIUM (LIPITOR) ON LIPID PEROXIDATION					
Direct all correspondence to: CORRESPONDENCE ADDRESS					
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Address	One Beacon Street				
City	Boston	State	MA	ZIP	02108
Country	US	Telephone	617-854-4000	Fax	617-854-4040
ENCLOSED APPLICATION PARTS (check all that apply)					
☒ Specification	Number of Pages	19	☐ Small Entity Statement		
☒ Drawing(s)	Number of Sheets	21	☐ Other (specify)		
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (check one)					
☒ A check or money order is enclosed to cover the filing fees					FILING FEE AMOUNT (\$)
☒ The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number	03-2410				\$150
The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.					
☒ No.					
☐ Yes, the name of the U.S. Government agency and the Government contract number are _____					

Respectfully submitted,

SIGNATURE

Date 7/27/99

TYPED or PRINTED NAME Jerry Cohen

REGISTRATION NO

20,522

TELEPHONE 617-854-4000

(if appropriate)
Docket Number.

2189 P02A PROV

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington D.C., 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C., 20231.

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**SYNERGISTIC ACTIVITY OF AMLODIPINE BESYLATE (NORVASC®) AND
ATORVASTATIN CALCIUM (LIPITOR®) ON LIPID PEROXIDATION**

Background of the Invention

Coronary artery disease (CAD) is a leading cause of mortality worldwide, and is associated with substantial morbidity as well. Typically, the patient with CAD has several concomitant conditions, including hypertension, diabetes, and dyslipidemia, increasing overall risk for poor outcomes and complicating treatment.

Among antihypertensive therapies, the lipophilic calcium channel blocker (CCB) amlodipine besylate (AML) is a very well-tolerated agent with an established record of safety and effectiveness for the treatment of hypertension and angina. A potential therapeutic role for AML in the treatment of patients with CAD was recently shown in the Prospective Randomized Evaluation of the Vascular Effects of Norvasc Trial (PREVENT). This three-year trial evaluated the effects of AML compared to placebo on the development and progression of atherosclerotic lesions in coronary and carotid arteries in patients with documented CAD⁴. The results of PREVENT showed impressive clinical benefits with AML therapy as compared to placebo, including reduced risk of cardiovascular morbidity¹. AML therapy was also associated with a significant slowing of the progression of carotid atherosclerosis according to B-mode ultrasonographic assessments. The clinical benefit seen with AML in CAD has not been previously reported with other CCB analogs, including dihydropyridine-type agents, that have examined this question in similar patients⁵⁻⁷. This observation has led to interest in other anti-atherogenic properties of AML, including antioxidant effects that are independent of calcium channel modulation^{2, 8-10}.

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Hypolipidemic therapy has also been demonstrated to be very useful in reducing morbidity and mortality associated with CAD. Among HMG-CoA reductase inhibitors, atorvastatin calcium (AT) has been shown to be very effective as hypolipidemic therapy 11, 12. In addition, the hydroxylated metabolites of atorvastatin have been shown to exhibit antioxidant effects in lipoprotein preparations 13.

Thus, a therapeutic goal for the treatment of CAD is the development of drugs that can simultaneously target multiple underlying disease processes that contribute to atherosclerosis, thereby altering the course of the disease. Therefore, CAD therapy may have increased positive outcomes if the use of an antihypertensive agent and HMG-CoA reductase inhibitor was combined in a single delivery system.

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Unexpectedly, when AML and AT were combined, they had a synergistic effect in preventing peroxidation in lipid membranes that occurred in addition to their well-established therapeutic effects on both dyslipidemia and hypertension. Therefore, these agents have heretofore unrecognized synergistic antioxidant effects, a property that would enable these agents to increase the resistance of lipoproteins and vascular cell membranes to oxidative modification. Indeed, oxidative modification of lipids associated with LDL and vascular cell membranes is a well-established cause of injury to the endothelium and underlying smooth muscle 14, 15. Lipophilic antioxidants that protect against lipid peroxidation have been shown to reduce lesion development in various models of atherosclerosis (for review, see reference 6). Moreover, the benefit associated with hypolipidemic therapy is attributed to both its effect on plasma VLDL, LDL and HDL levels and to a reduction in the potential formation of atherogenic oxidized lipoproteins.

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Summary of the Invention

Scientific analyses that support the combined use of amlodipine besylate (Norvasc®) and atorvastatin calcium (Lipitor®) in a single delivery system for the treatment of cardiovascular disease are described. Specifically, the synergistic antioxidant activities of the calcium channel blocker (CCB), amlodipine besylate, and the HMG-CoA reductase inhibitor, atorvastatin calcium, including its active hydroxylated metabolite, were evaluated in a well-defined lipid system in which lipid vesicles are enriched with polyunsaturated fatty acids (PUFA), the key target for oxy-radical damage in atherosclerosis. The synergistic effects of these agents were demonstrated in membranes prepared in the absence and presence of elevated cholesterol levels that reproduce atherosclerotic-like conditions. The combination of amlodipine with the hydroxylated atorvastatin metabolite effected a dramatic and sustained reduction in lipid oxy-radical damage at concentrations as low as 10.0 nM. These experiments were conducted in membranes with cholesterol that reproduce physiologic-like conditions. The catalytic antioxidant activity associated with the combination of these drugs at therapeutic levels was highly synergistic and could not be effectively reproduced by vitamin E or carvedilol, a non-selective β -blocker with established antioxidant effects. Synergistic inhibition of lipid peroxidation was also observed with the combination of amlodipine and the atorvastatin parent compound. Antioxidant activity was not demonstrated for other CCBs that were tested (felodipine, verapamil, diltiazem) or HMG-CoA reductase inhibitors (mevastatin, pravastatin, simvastatin). The distinct antioxidant property described for this drug combination can be attributed to catalytic, physico-chemical interactions with the membrane bilayer that are independent of the well-characterized effects of these drugs on calcium transport and cholesterol metabolism. This synergistic antioxidant benefit constitutes a new pharmacologic mechanism of

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action for these compounds and a compelling rationale for the combined use of the active ingredients in Norvasc® and Lipitor® in the treatment of cardiovascular disease by reducing the levels of LDL in plasma and improving protection against its oxidation. This new property complements the established effects of these drugs on hypertension and dyslipidemia.

Brief Description of the Drawings

Figure 1 shows the dose-dependent antioxidant activity of AML.

Figure 2 shows the comparative antioxidant effects of AML and both other calcium channel blockers and an ACE-inhibitor.

Figure 3 shows the dose-dependent antioxidant activity of ATM.

Figure 4 shows the comparative antioxidant effects of HMG-CoA reductase inhibitors.

Figure 5 shows the effect of cholesterol enrichment on lipid peroxide formation.

Figure 6 shows the effect of cholesterol enrichment on the antioxidant activities of ATM.

Figure 7 shows the dose-dependent antioxidant effect of the combination of AML and ATM.

Figure 8 shows the synergistic antioxidant effects of AML with ATM at 100.0 nM and of AML with AT at 1.0 μ M in cholesterol-enriched vesicles.

Figure 9 shows the comparative antioxidant effects of AML/ATM and AML/AT at 0.1 and 1.0 μ M in cholesterol-enriched vesicles.

Figure 10 shows the comparative antioxidant effects of vitamin E, carvedilol, probucol, and the AML/ATM combination after various incubation periods at a 10.0 μ M concentration.

Figure 11 shows the chemical antioxidant mechanisms for AML.

Figure 12 shows the chemical antioxidant mechanisms for ATM.

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Figure 13 shows the molecular membrane interactions of AML and ATM.

Detailed Description of Embodiments

Comparative Antioxidant Effects of Calcium Channel Blockers and HMG-CoA Reductase

Inhibitors: The separate and combined dose-dependent antioxidant activities of AML, AT and ATM were compared to representative CCBs (felodipine, verapamil, diltiazem) and HMG-CoA reductase inhibitors (mevastatin, pravastatin) in membrane vesicles reconstituted from phospholipids enriched with the polyunsaturated fatty acid, dilinoleoyl phosphatidylcholine (DLPC). Membrane vesicles were used in these experiments for the following reasons: 1) this system is well-defined and highly reproducible; 2) linoleic acid represents the primary target for oxidative damage and is common in vascular cell membranes and lipoprotein particles; 3) this membrane system does not contain calcium channels or HMG CoA reductase enzymes and; 4) lipid peroxidation in this system can be initiated spontaneously at 37°C in the absence of exogenous chemical initiators, such as high levels of iron and ascorbate. In these experiments, oxidation occurred in a gradual, time-dependent manner that was measured spectrophotometrically.

As demonstrated in Figure 1, AML effectively inhibited membrane lipid peroxide formation in a highly reproducible and dose-dependent manner over a broad range of concentrations, beginning at 10.0 nM. Inhibition of lipid peroxide formation by AML in membrane vesicles enriched with PUFA was measured as a function of drug concentration at 37°C by spectrophotometric methods. In these experiments, the control level of lipid peroxide formation was 1.2 mM after a 48 h incubation period. The fact that inhibition was observed at sub-micromolar levels indicates that the benefit observed with AML is of therapeutic relevance.

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Thus, inhibition of oxy-radical damage by AML was highly catalytic: at an AML concentration of 10.0 nM, the mole ratio of lipid peroxide inhibition: drug concentration was greater than 10,000:1.

The antioxidant effects of AML could not be reproduced by other representative CCBs, including felodipine (a dihydropyridine), verapamil, or diltiazem under identical experimental conditions, even at micromolar levels. Figure 2 shows the antioxidant activity of amlodipine compared to other representative CCBs (felodipine, verapamil, diltiazem) and the ACE-inhibitor, captopril, at a drug concentration of 1.0 μ M. Under identical conditions, only AML produced a significant inhibition of lipid peroxidation. Again, the control level of lipid peroxide formation was 1.2 mM. Thus, the distinct effects of amlodipine can be attributed to its high affinity for the membrane lipid bilayer, resulting in biophysical changes in the intermolecular packing of phospholipid molecules.

In a similar fashion, ATM also inhibited lipid peroxide formation in these lipid preparations in a dose-dependent manner (see Figure 3). This activity was also observed with AT at much higher, micromolar levels but not with other statins that were tested in these experiments (pravastatin, mevastatin), as shown in Figure 4. Thus, AML, AT and ATM separately produced a measurable inhibition of lipid peroxidation in vesicles enriched with PUFA, the key substrate for oxy-radical damage.

Synergistic Antioxidant Effects of Drugs in Cholesterol-Enriched Membrane

Preparations: As cholesterol is a primary constituent of vascular cell plasma membranes that increases in content during atherogenesis¹³, the synergistic antioxidant activity of these agents in membranes composed of cholesterol and phospholipid at a 0.5:1 mole ratio were examined. As shown in Figure 5, the addition of cholesterol to these vesicles resulted in a pronounced, 26%

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increase in the amount of lipid peroxide formation. This effect of cholesterol is attributed to its ability to increase the membrane bilayer packing constraints, contributing to its pathologic activity in atherosclerosis ¹⁹. Under these conditions, the antioxidant effectiveness of ATM was not significantly reduced (see Figure 6).

Under conditions of cholesterol enrichment, AML worked synergistically with ATM to effect an inhibition of lipid peroxidation that was dramatically more potent than that observed with either agent separately. The combination of AML and ATM was very effective and dose-dependent with antioxidant effects beginning in the low nanomolar range. Significant inhibition was observed at concentrations as low as 10.0 nM and nearly complete inhibition was observed at 10.0 μ M (see Figure 7). In this membrane system, the combination of AML with either ATM or AT produced a marked synergistic effect (see Figure 8). Although a synergistic effect was observed with the combination of AML and AT, it was less prominent than when the AML/ATM combination was used. Specifically, at both concentrations (0.1 and 1.0 μ M), the combination of AML/ATM was more effective as an antioxidant than AML/AT (see Figure 9).

The effect of other known antioxidants, including vitamin E and carvedilol, were also compared to the combination of AML and ATM. Carvedilol is vasodilating β -blocker and antioxidant approved for treatment of congestive heart failure. It has been shown to scavenge oxygen free radicals and inhibit lipid peroxidation in swine heart ²⁰. Even at a concentration of 10.0 μ M, these compounds were markedly less effective than the combined AML/ATM preparation (see Figure 10). In the case of vitamin E, the activity of this compound was significantly attenuated over time (48 versus 72h), as compared to AML/ATM (Figure 10A). This observation is consistent with a stoichiometric mechanism in which the antioxidant ability of the vitamin E is terminated by the reaction. By contrast, the activity of the AML/ATM

combination persisted, providing evidence that these agents work by a separate, catalytic fashion as antioxidants. Only the very effective antioxidant, probucol, had similar activity to the AML/ATM complex (Figure 10C).

To understand the basis for the antioxidant effects of these compounds, the chemical structure of these molecules was evaluated (Figures 11, 12). In Figure 11, the covalent structure of AML includes two hydrogen atoms associated with its dihydropyridine ring that can be donated to unstable free radicals associated with lipids (Figure 11A). Once a hydrogen atom is donated, an unpaired free electron remains associated with the AML molecule. This unpaired electron, a potential source of free radical damage, can be safely stabilized in resonance structures (Figures 11B and 11C).

Similar chemical analyses were conducted for a key hydroxyl metabolite of AT, as indicated in Figure 12. Similar to AML, the ATM molecule has three potential hydrogen atoms that can be donated to unstable free radical molecules (A). Resonance stabilization structures for the remaining unpaired free electrons at sites 1 (Figure 12B), 2 (Figure 12C) and 3 (Figure 12D) are calculated.

In addition to its chemical properties, AML also has high affinity for membrane lipids under atherosclerotic conditions, as compared to other CCBs ²¹. The distinct lipophilicity of AML is attributed to its amphiphilic chemical structure that directs the molecule to a favorable location in the membrane where it can then interfere with the propagation of free radicals by both biophysical and biochemical mechanisms, as previously described in detail by my laboratory ^{2, 8, 16, 21}. In the same way, the chemical structure of ATM consistent with an amphiphile that could be expected to interact strongly with the membrane bilayer, as illustrated in Figure 13 and supported by x-ray diffraction analyses.

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The antioxidant activity of AML may contribute to its benefit in patients with documented CAD, as recently reported in the Prospective Randomized Evaluation of the Vascular Effects of Norvasc Trial (PREVENT) ⁴. Patients randomized to AML experienced a significant reduction in angina (-33%, $p < 0.01$), major vascular procedures (-45%, $p < 0.001$), and the composite of any major event (-30%, $p > 0.01$), as compared to placebo, independent of changes in lipid levels ¹. The benefits of AML in CAD have not been observed with other CCBs, including dihydropyridine agents ⁵⁻⁷, suggesting that the additional antioxidant properties of AML contribute to direct antiatherosclerotic effects ².

Role of Antioxidants in Reducing Coronary Artery Disease: The results of in vivo investigations have indicated an important role for lipophilic antioxidants in reducing cardiovascular morbidity and mortality, especially coronary artery disease. The rationale for this observation is attributed to the ability of these compounds to interfere with the peroxidation of PUFA associated with LDL and vascular cell membranes. In particular, it has been demonstrated that LDL particles with greater resistance to oxidative damage, due to the presence of lipophilic antioxidants, have reduced cytotoxicity, interfere less with endothelium-derived nitric oxide production, and do not contribute to foam cell formation ¹⁵. Supplementation with vitamin E has been shown to increase LDL resistance to oxidative modification, and thus, reduce cytotoxicity toward endothelial cells ²². The lipophilic antioxidant probucol has also been demonstrated to attenuate the formation of atherosclerotic plaques in cholesterol-fed primates, an effect that correlated with an increased resistance of LDL to oxidative damage and not changes in lipid levels ²³. In addition, probucol inhibited atheroma formation of lesions in Watanabe hereditary hyperlipidemic (WHHL) rabbits, a well-characterized animal model of atherosclerosis, independent of cholesterol-lowering effects ²⁴. Consistent with these animal

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studies, a double-blind, randomized clinical investigation has shown that probucol reduced restenosis by 47% in patients with CAD following coronary-artery balloon angioplasty, presumably due to its antioxidant effects ²⁵.

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Another pathological process associated with oxidized LDL is an inhibition of the constitutive release of endothelium-derived nitric oxide in the arterial wall ²⁶⁻²⁸. Release of nitric oxide inhibits the abnormal adhesion of leukocytes and platelets to the endothelial surface early during the development of foam cell formation. Nitric oxide is also an important mediator of vasodilation; loss of nitric oxide can lead to vasospasm associated with acute coronary syndromes ²⁹. The interference of this process by oxidized LDL is attributed to a disruption in secondary messenger systems, including G-protein-dependent nitric oxide release ^{27, 28}. Patients treated with the antioxidant probucol had improved endothelial function, as assessed by nitric oxide production, when compared to control subjects. LDL isolated from these patients showed greater resistance to oxidation in vitro and did not interfere with normal nitric oxide production from the endothelium, as compared to control LDL samples ³⁰. An important benefit associated with lipophilic antioxidants is the ability to inhibit endothelial dysfunction associated with oxidized LDL, a key early event in atherosclerosis ¹⁴.

Thus, a comprehensive review of the available data provides a compelling mechanistic rationale for the use of lipophilic antioxidants to prevent cellular changes associated with coronary artery disease. By increasing the resistance of LDL to oxidative damage, agents with antioxidant activity may effectively interfere with pathologic alterations in the vessel wall during atherogenesis, including foam cell formation, endothelial dysfunction and toxicity, leukocyte and platelet adhesion, and arterial vasospasm, secondary to a loss of normal nitric oxide production. These cellular observations also provide important insights into the results of recent clinical

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studies demonstrating a benefit associated with antioxidant use by reducing the expression of established coronary artery disease despite an absence of pronounced changes in atherosclerotic lesion development ³¹. Indeed, the results of epidemiological analyses have provided direct evidence for an inverse association between the serum levels of antioxidants, especially vitamin E, and adverse outcomes associated with coronary disease ³²⁻³⁸.

This invention demonstrates that the combination of amlodipine and atorvastatin produced a powerful, synergistic antioxidant effect that could not be effectively reproduced by well-known antioxidants such as α -tocopherol or carvedilol. While previous studies have shown that these agents separately have antioxidant benefit in both lipoprotein and cell membrane preparations ^{2, 3, 13, 39}, these findings unexpectedly show that the combination of these agents provide a much greater, synergistic effect, especially under atherosclerotic-like condition in which the separate effects of the compounds are especially reduced.

Experimental Methods

Dilinoleoyl phosphatidylcholine (DLPC) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL) and stored at -80°C. Amlodipine besylate (AML) was a gift from Pfizer Central Research (Groton, CT) while atorvastatin calcium (AT) and its hydroxylated metabolite (ATM) were provided by Parke Davis (Ann Arbor, MI). Verapamil, captopril and diltiazem were purchased from RBI Biochemicals (Natick, MA). Mevastatin and vitamin E were purchased from Sigma Chemical Co. (St. Louis, MO).

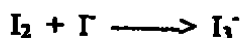
Lipid peroxidation analysis: The separate and combined dose-dependent antioxidant activities of these agents were examined in membranes enriched with PUFA prepared in the absence and presence of cholesterol at a 0.5 sterol to phospholipid mole ratio ². The biological

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lipid (dilinoleoyl phosphatidylcholine) used for these samples was dissolved in HPLC-grade chloroform (25.0 mg/ml). An aliquot of the lipids (1.0 mg) was added to individual glass 13 x 100-mm test tubes and the chloroform was removed under a steady stream of N₂ gas while vortexing. The lipids were then dried in the absence or presence of drug dissolved in ethanol. Residual solvent was removed under vacuum while the samples were shielded from light. Membrane vesicles were produced by vortexing the dried lipids at room temperature with 1.0 ml HEPES buffered saline (0.5 mM HEPES and 154.0 mM NaCl, pH, 7.2). The final phospholipid concentration was 1.0 mg/ml buffer and the concentrations of drug ranged from 10.0 nM through 10.0 μM.

In the lipid peroxidation experiments, aliquots of membrane vesicles (1.0 mg) were added to separate test tubes. Membrane peroxidation was carried out at 37°C in a shaking water bath. At various time intervals (24, 48, 65, 72h), a small aliquot of lipid sample was removed before 25 μl of 5.0 mM ethylenediaminetetracetic acid (EDTA) and 20 μl of 35.0 mM butylated hydroxytoluene (BHT) was immediately added to the sample to stop the peroxidation reaction.

The extent of membrane lipid peroxidation was measured by the CHOD-Iodide assay as previously described in detail ¹⁷. The quantity of I₃⁻ was measured from the following reaction in which L represents a phospholipid molecule:



An aliquot of the membrane sample was removed at various time points and then added to 1.0 ml of CHOD color reagent (E.M. Science, Gibbstown, NJ) that includes 20.0 μM BHT, 24.0 μM EDTA, and 0.2% Triton-X. The sample was then covered with foil and allowed to incubate for 2 h in the absence of light before measuring the absorbance of the sample at 365 nm

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($\epsilon = 2.4 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$). The background sample was run along the test samples in triplicate and contains 76.7 μl of 0.652 mM HEPES, 20 μl of 5.0 mM EDTA and 3.3 μl of DDI water. The extent of lipid peroxidation was measured in triplicate for each drug concentration and compared to control samples that did not contain drug. The statistical significance of these experiments was assessed by the non-paired t-test. Significance was accepted at $p < 0.05$.

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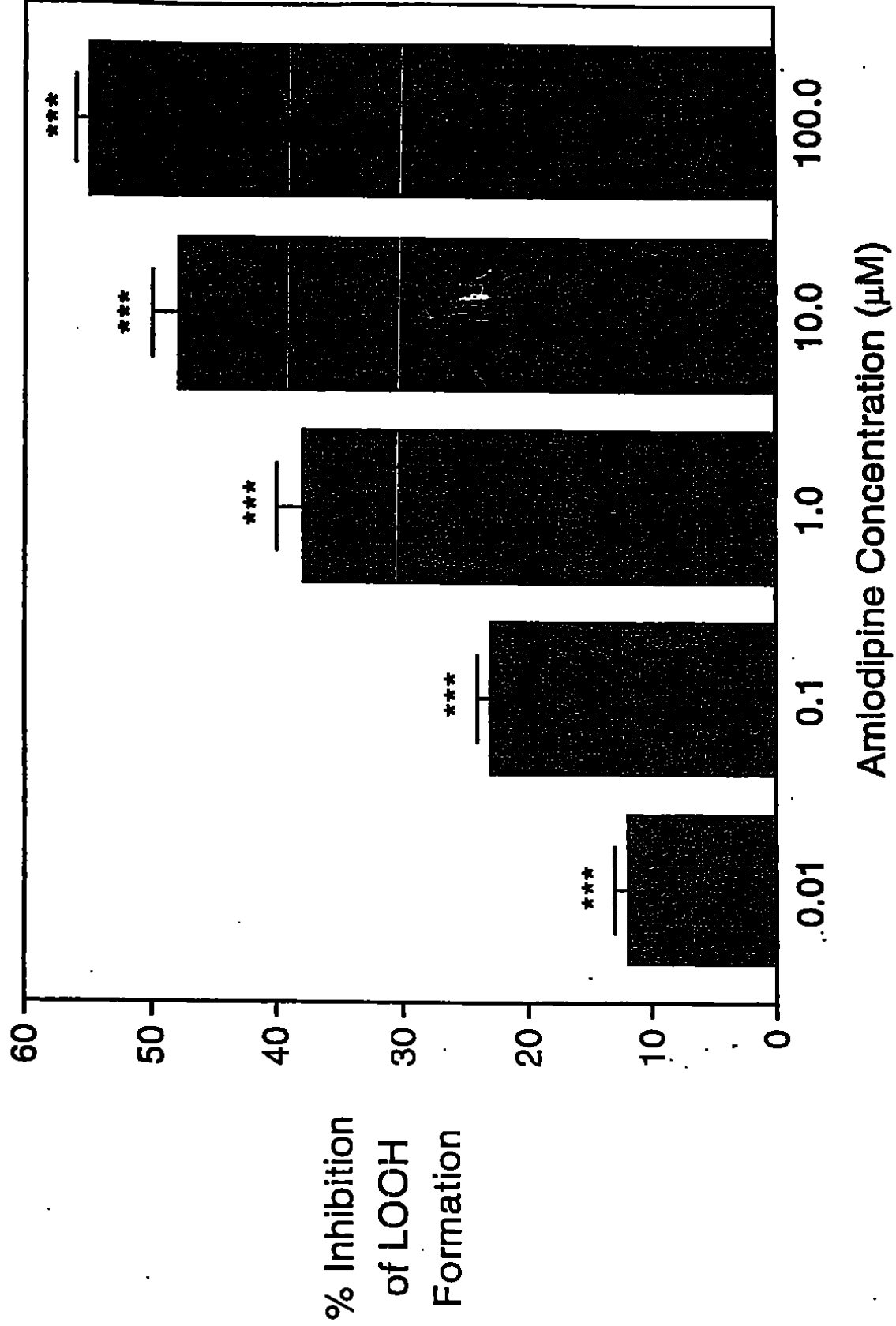
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Abstract

The combination of amlodipine besylate and atorvastatin calcium shows a synergistic antioxidant effect on lipid peroxidation in membrane vesicles enriched with polyunsaturated fatty acids, the key target for free radicals. Inhibition of oxy-radical damage by this drug combination was observed at therapeutic levels and could not be reproduced with other representative CCBs, statins, or even the highly lipophilic antioxidants, vitamin E and carvedilol. This combination therapy can be used to treat cardiovascular disorders, especially coronary artery disease, by reducing the levels of LDL in plasma and improving protection against its oxidation.

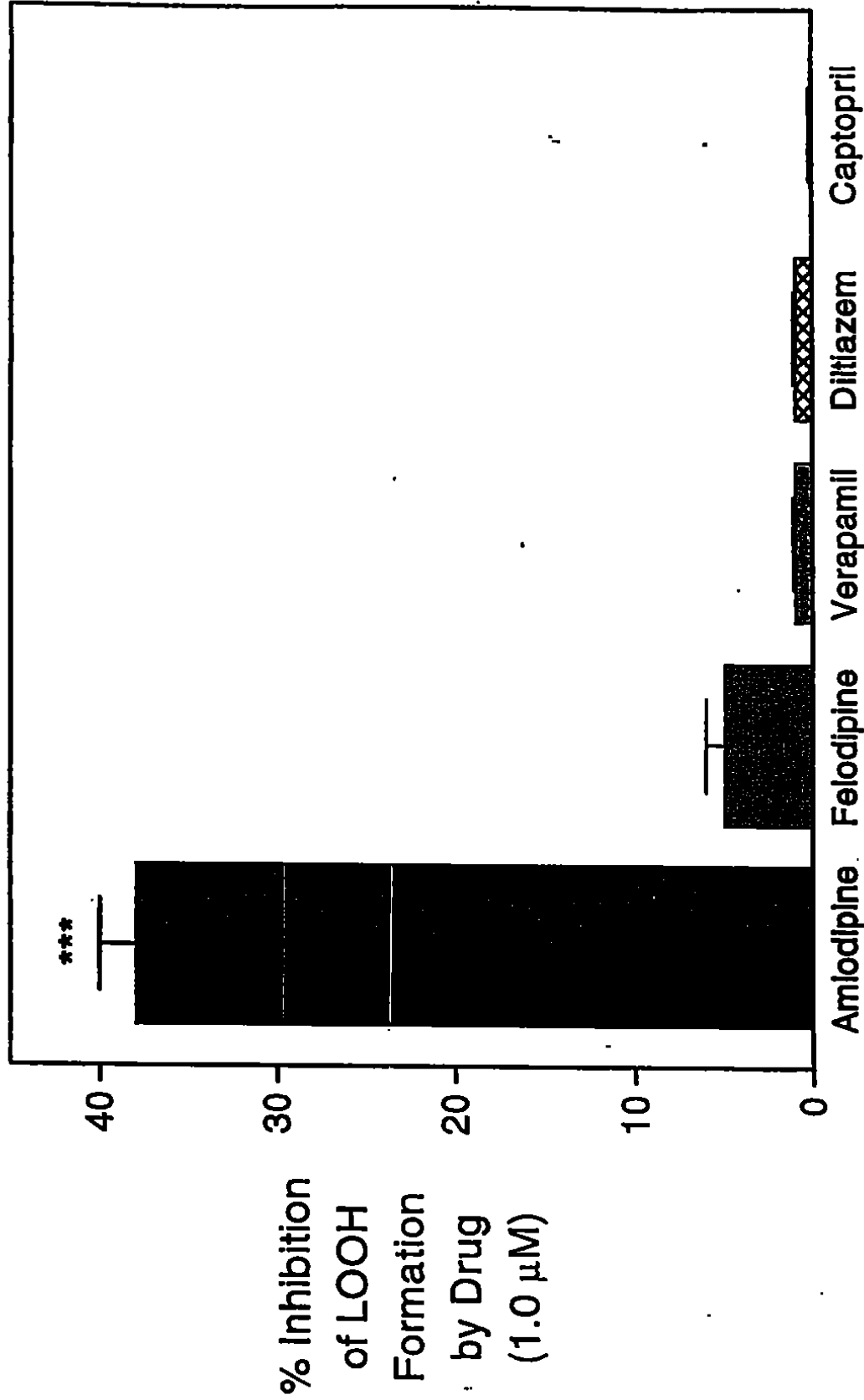
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*** $p < 0.001$ versus control
Values are mean $\pm$ S.D. (n = 3)

Figure 1

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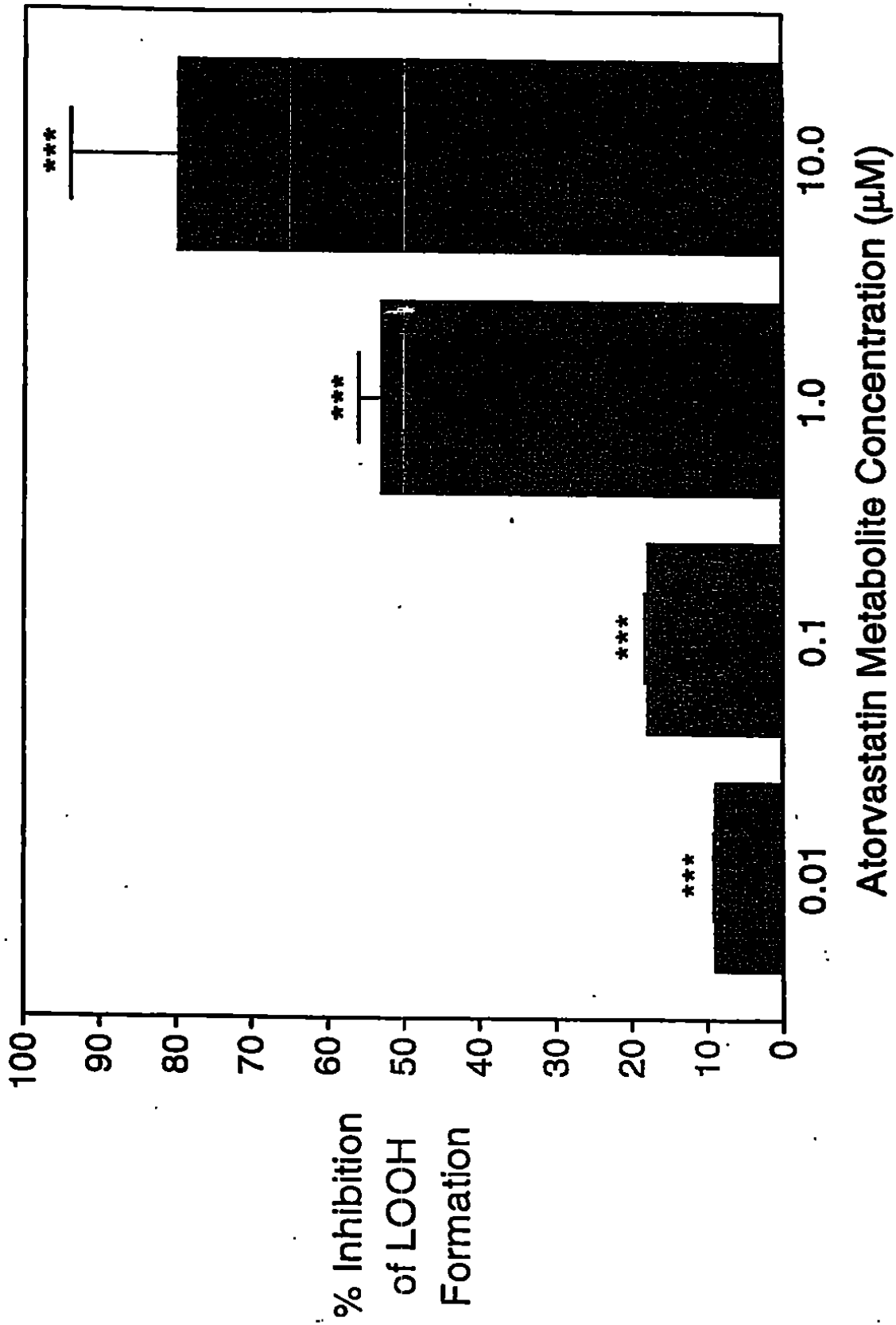


*** $p < 0.001$ versus control and other drugs
 Values are mean $\pm$ S.D. (n = 3)

Figure 2

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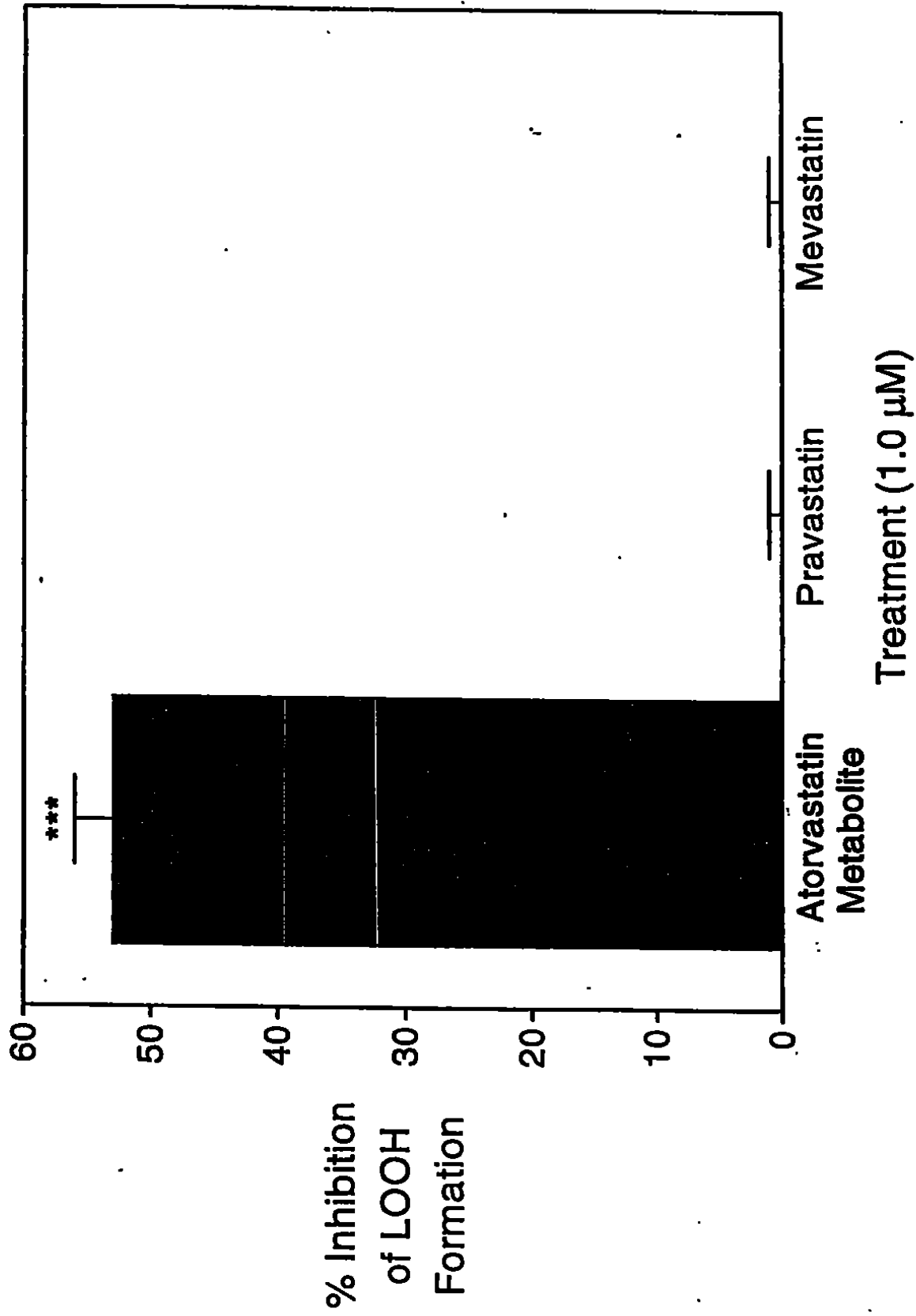


*** $p < 0.001$ versus control
Values are mean $\pm$ S.D. (n = 3)

Figure 3

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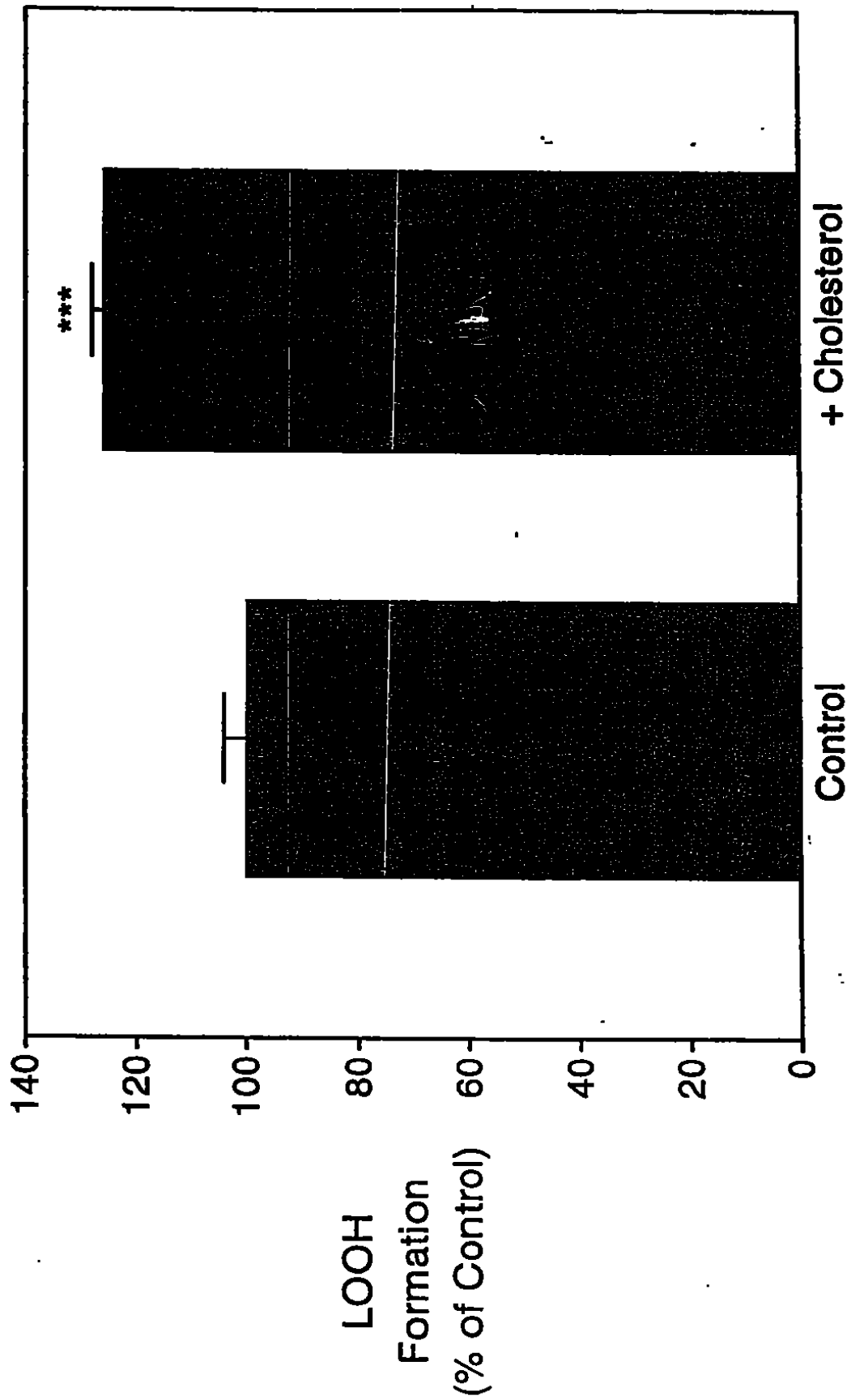
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*** $p < 0.001$ versus control
Values are mean $\pm$ S.D. (n = 3-6)

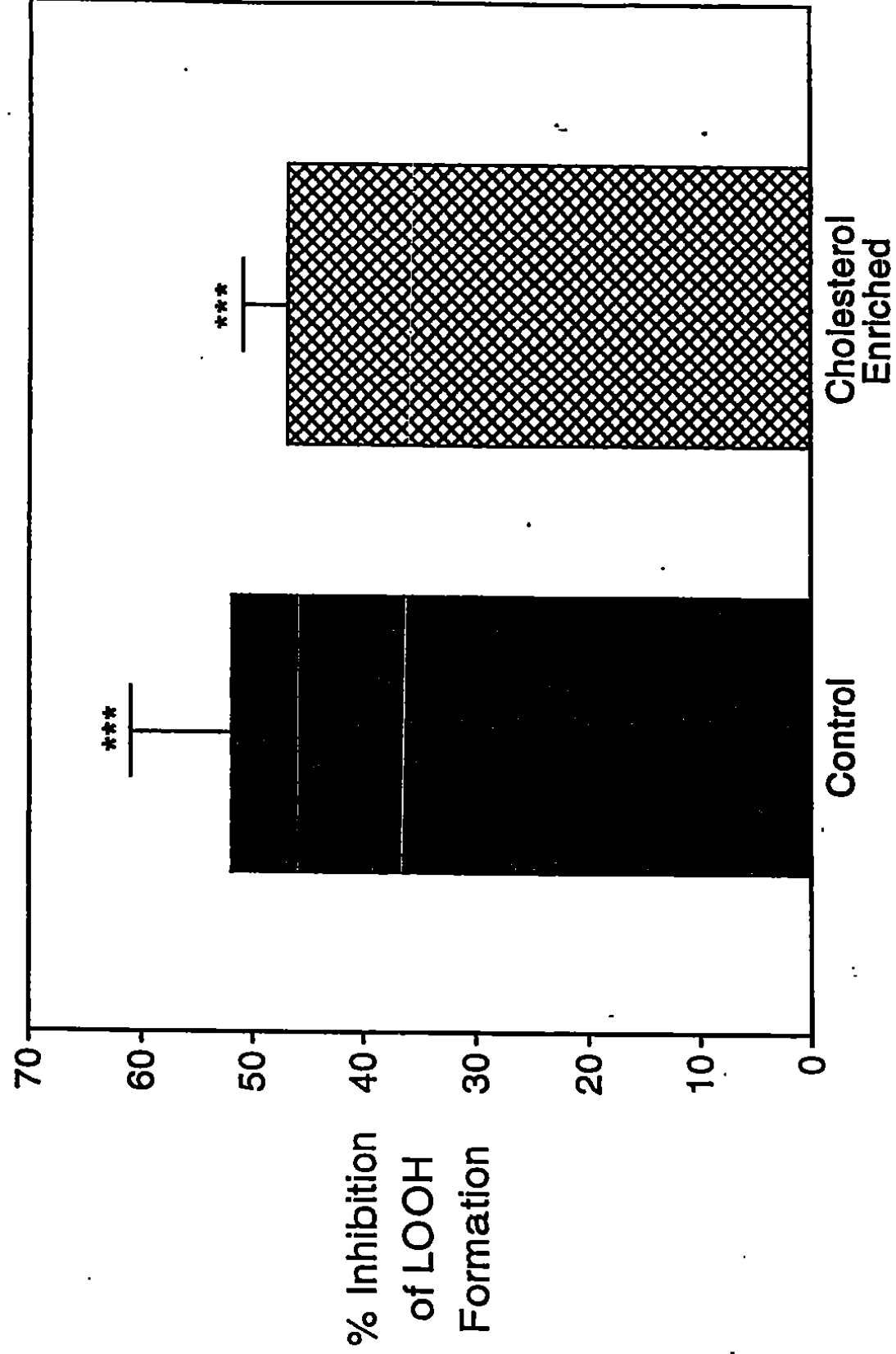
Figure 4

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*** $p < 0.001$ versus control
Values are mean $\pm$ S.D. (n = 4)

Figure 5



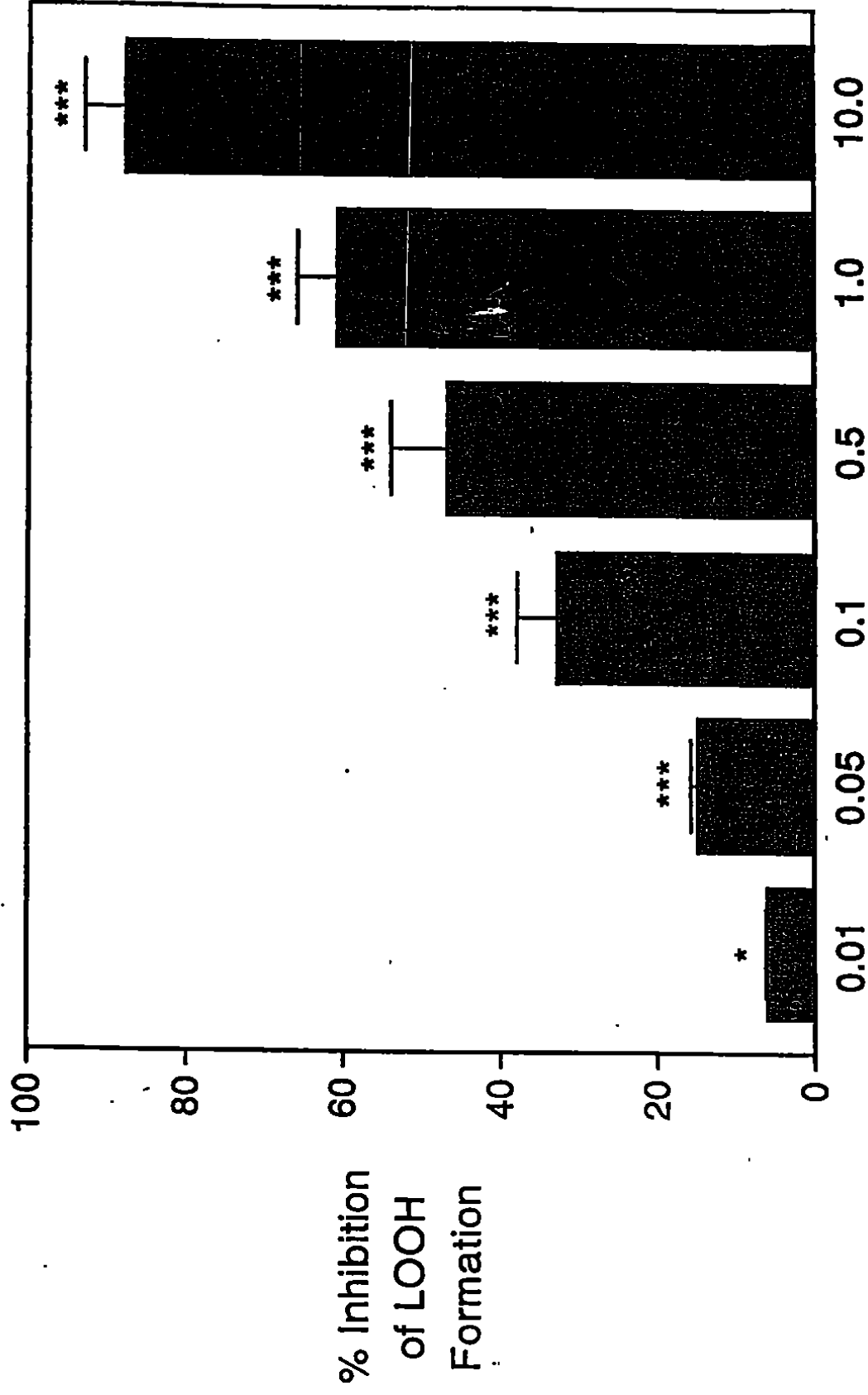
Atorvastatin Metabolite Treatment (1.0 μM)

*** $p < 0.001$ v rsus no treatment
Values are mean ± S.D. (n = 4)

Figure 6

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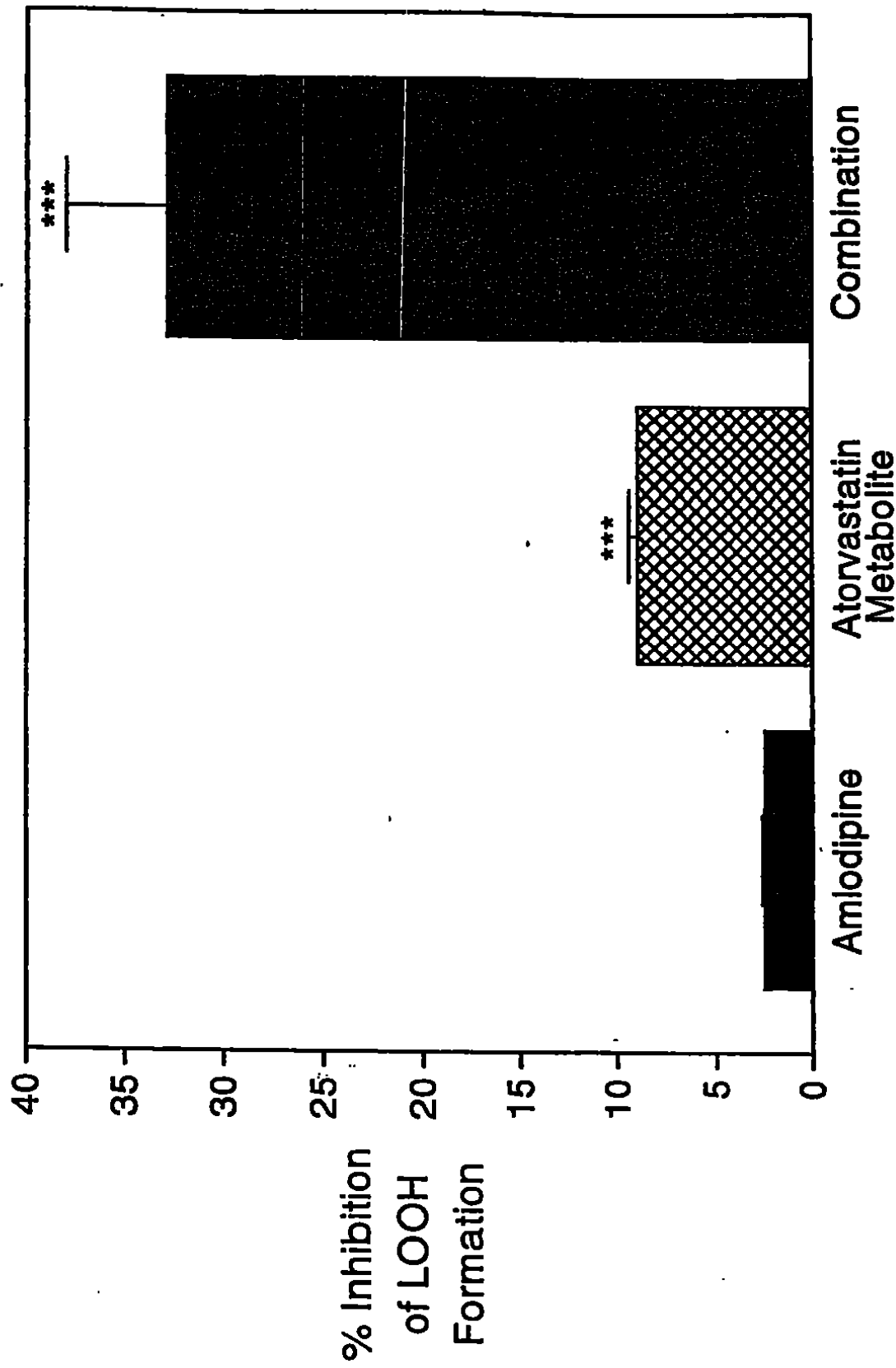
Amlodipine / Atorvastatin Metabolite Combination (μM)

* $p < 0.05$; *** $p < 0.001$ versus control
Values are mean $\pm$ S.D. (n = 6)

Figure 7

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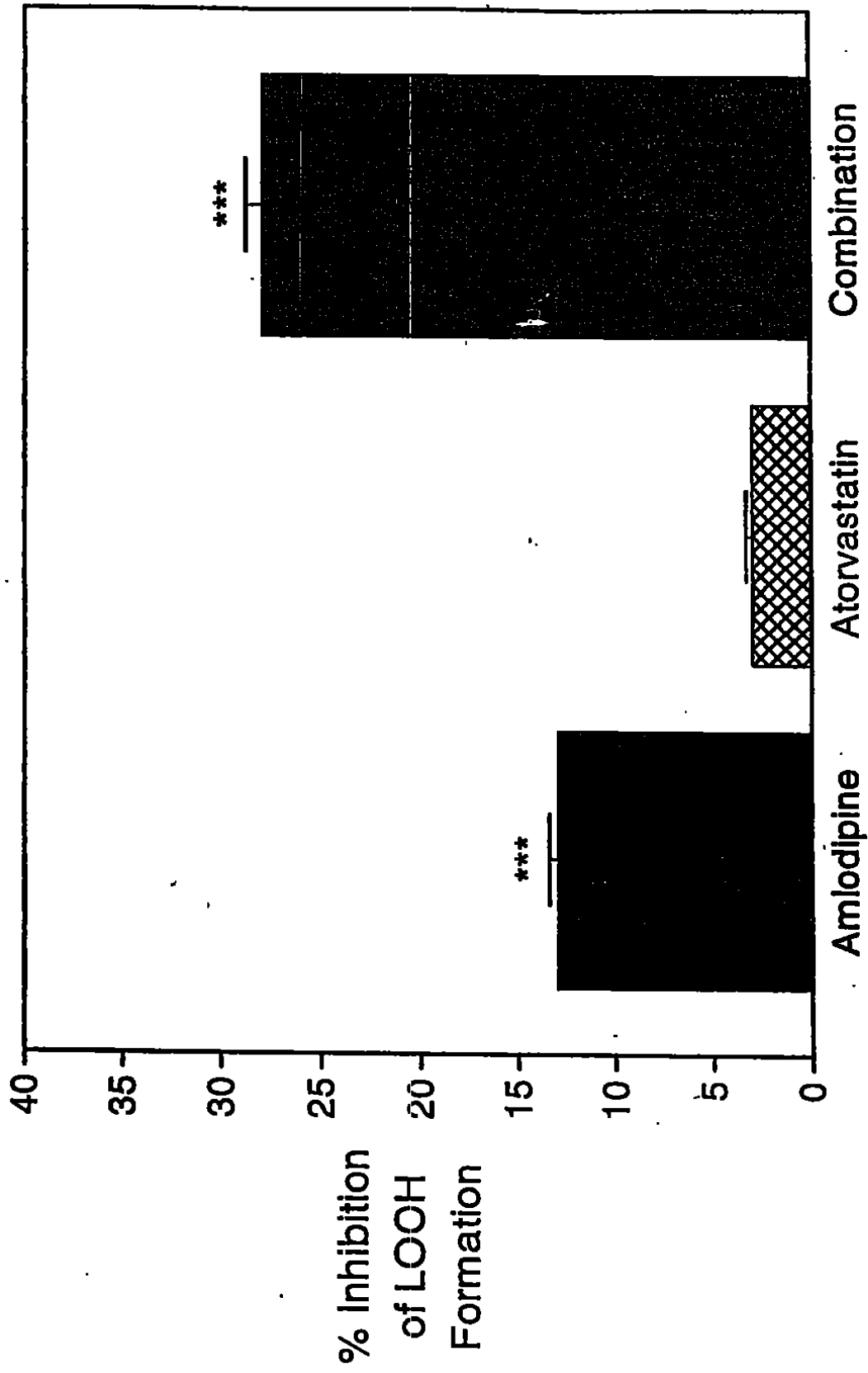
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Treatment (100.0 nM)

*** $p < 0.001$ versus control and other treatments
Values are mean $\pm$ S.D. (n = 6)

Figure 8A



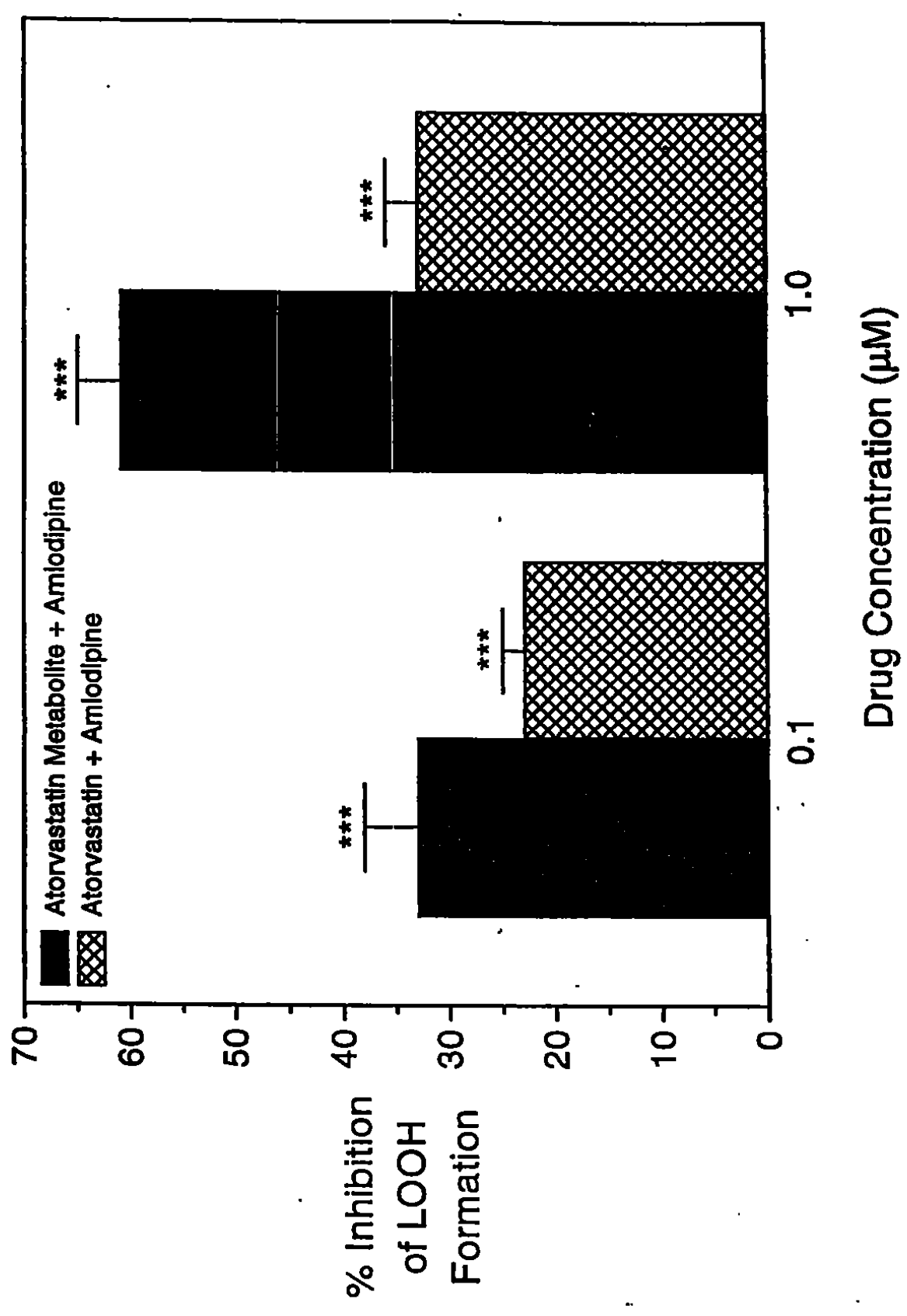
Treatment (1.0 μ M)

*** $p < 0.001$ versus control
Values are mean $\pm$ S.D. (n = 6)

Figure 8B

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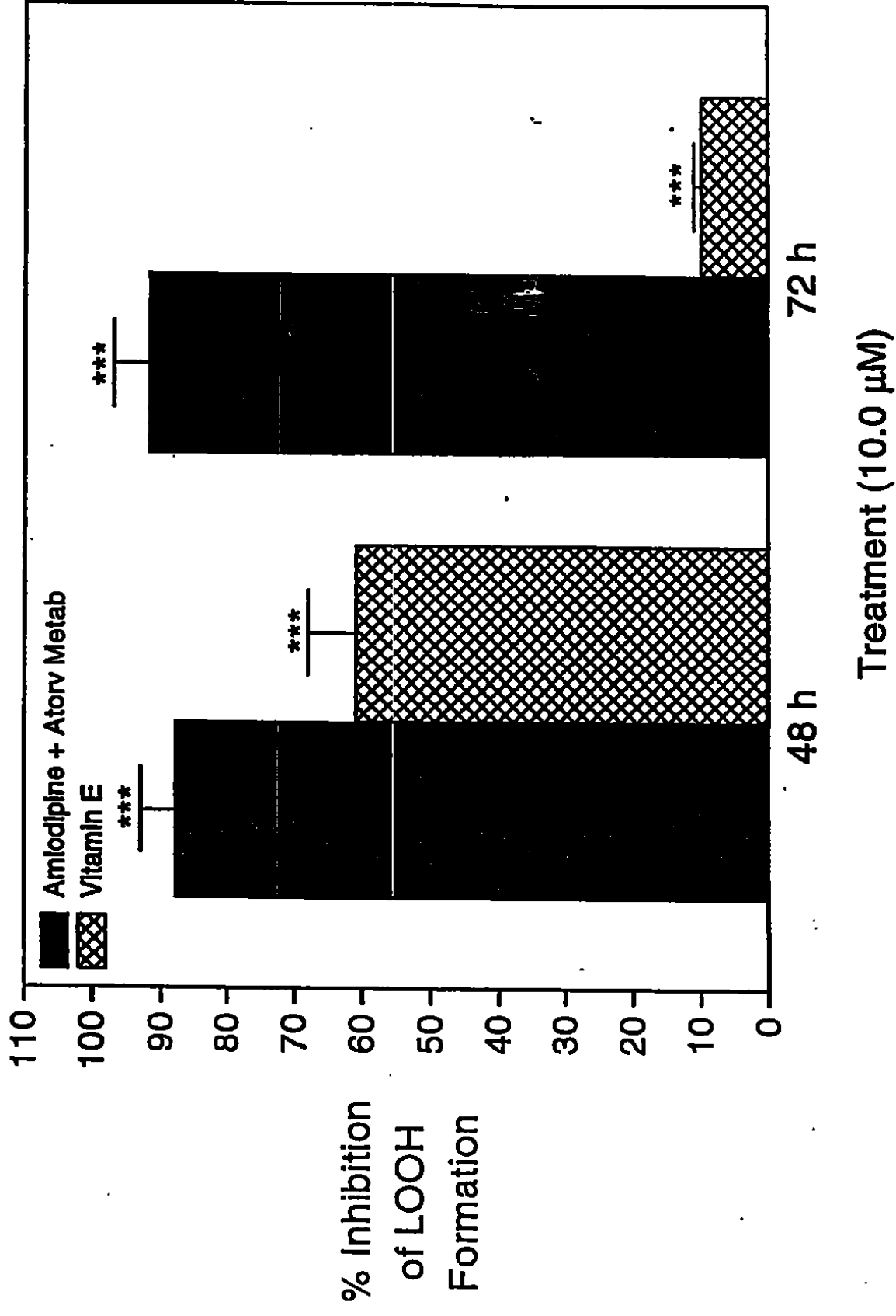


*** $p < 0.001$ versus control
Values are mean $\pm$ S.D. (n = 5-6)

Figure 9

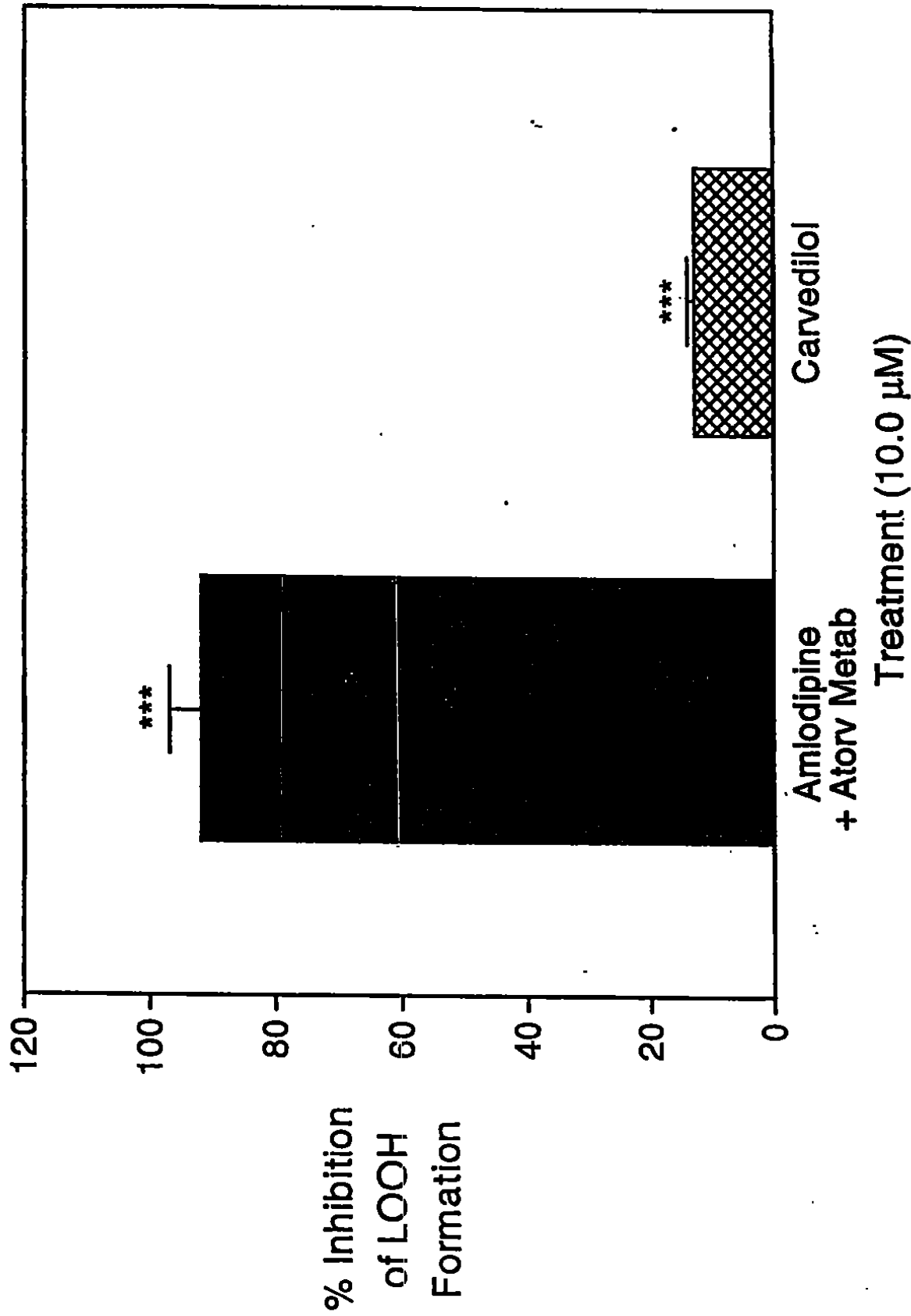
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*** $p < 0.001$ versus control
Values are mean $\pm$ S.D. (n = 6-12)

Figure 10A

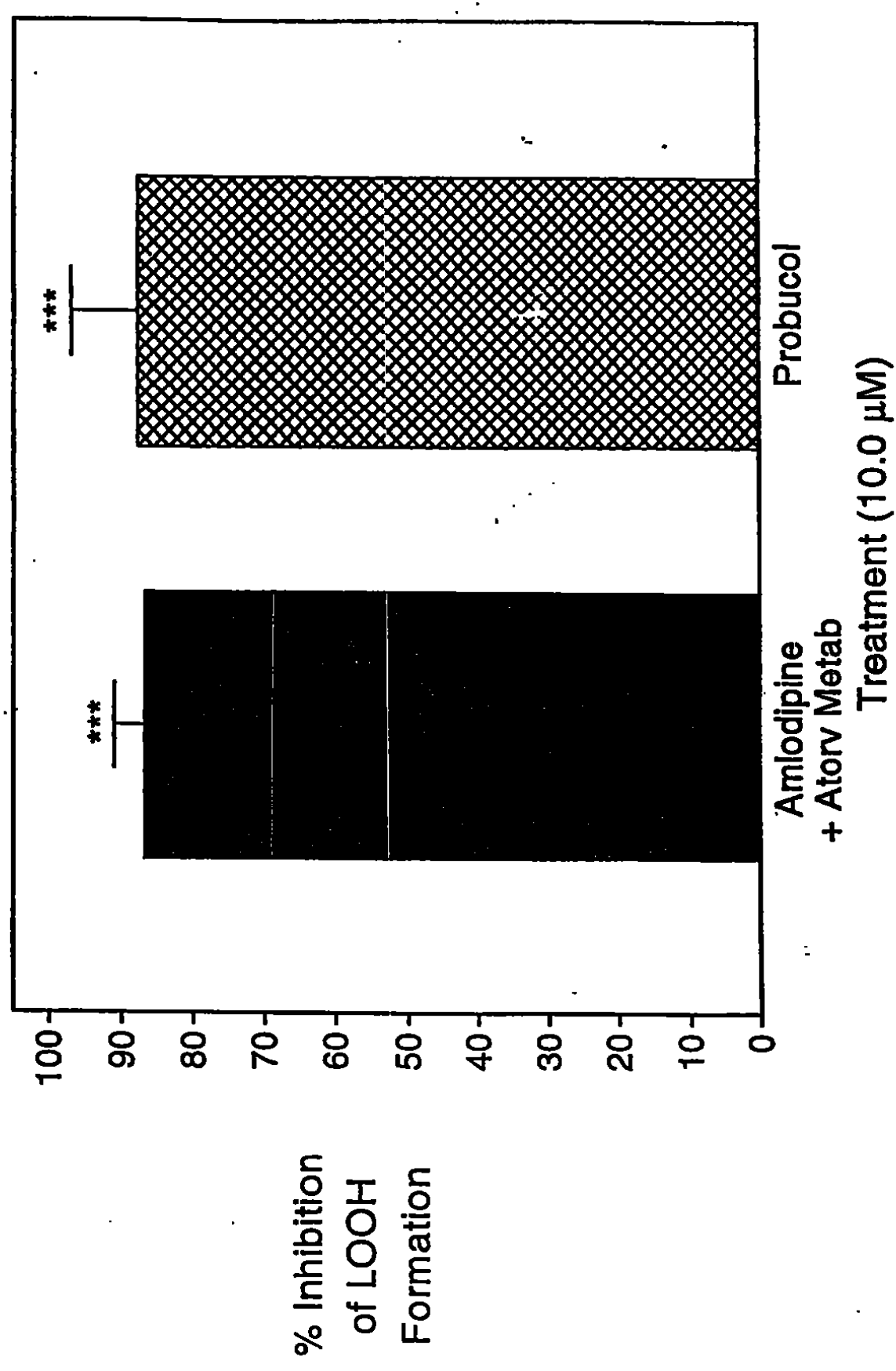


*** $p < 0.001$ versus control
Values are mean $\pm$ S.D. (n = 6)

Figure 10B

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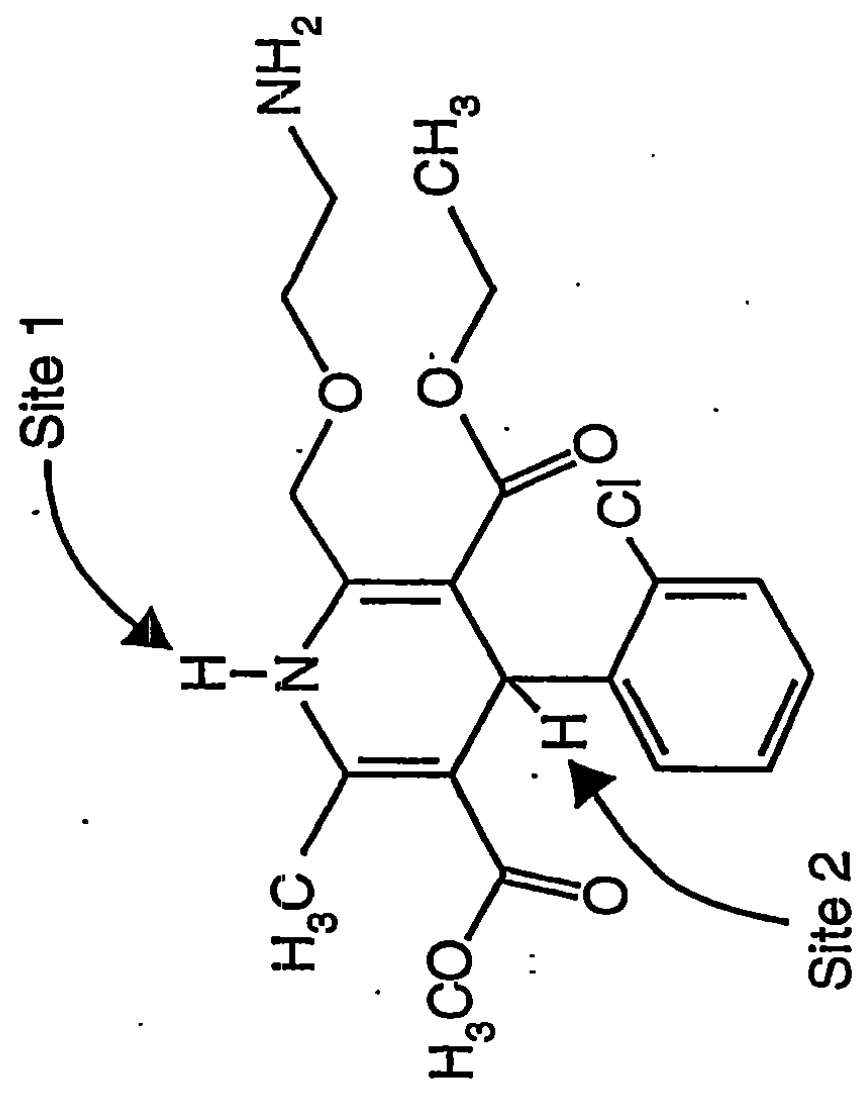
*** $p < 0.001$ versus control
Values are mean $\pm$ S.D. (n = 6)

Figure 10C

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Chemical Antioxidant Mechanisms for Amlodipine: Abstractable Hydrogens



Resonance Stabilization of Free Radical at Site 1 Of Amlodipine

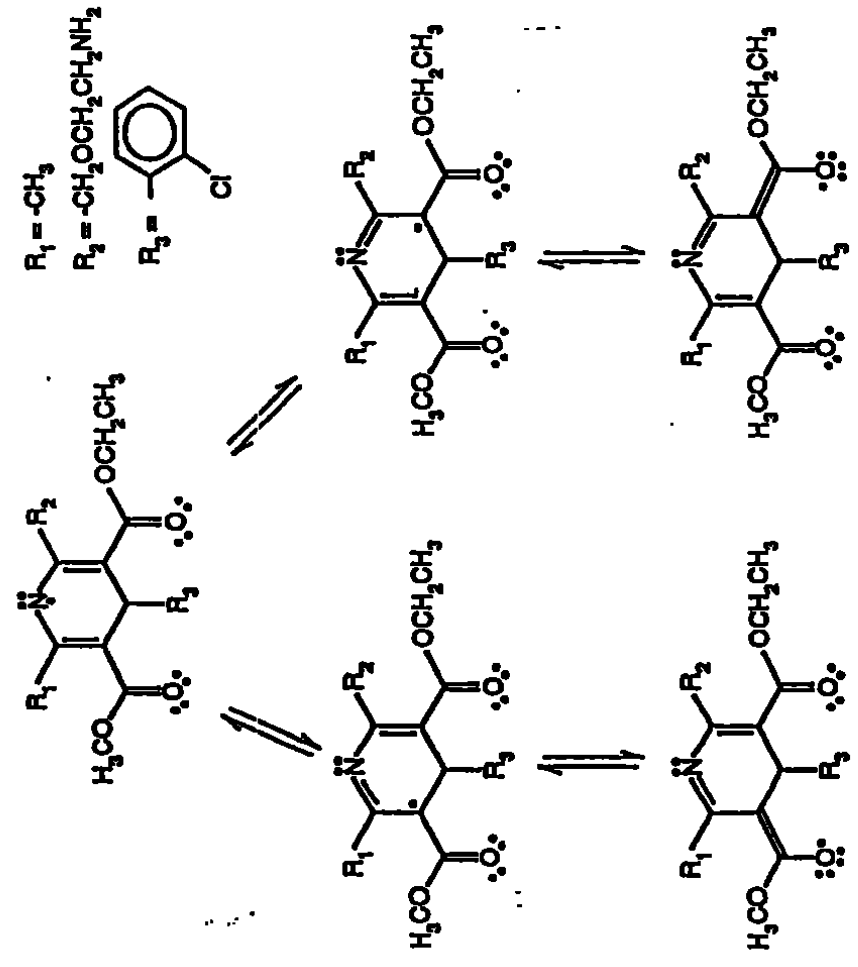


Figure 11B

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Resonance Stabilization of Free Radical at Site 2 of Amlodipine

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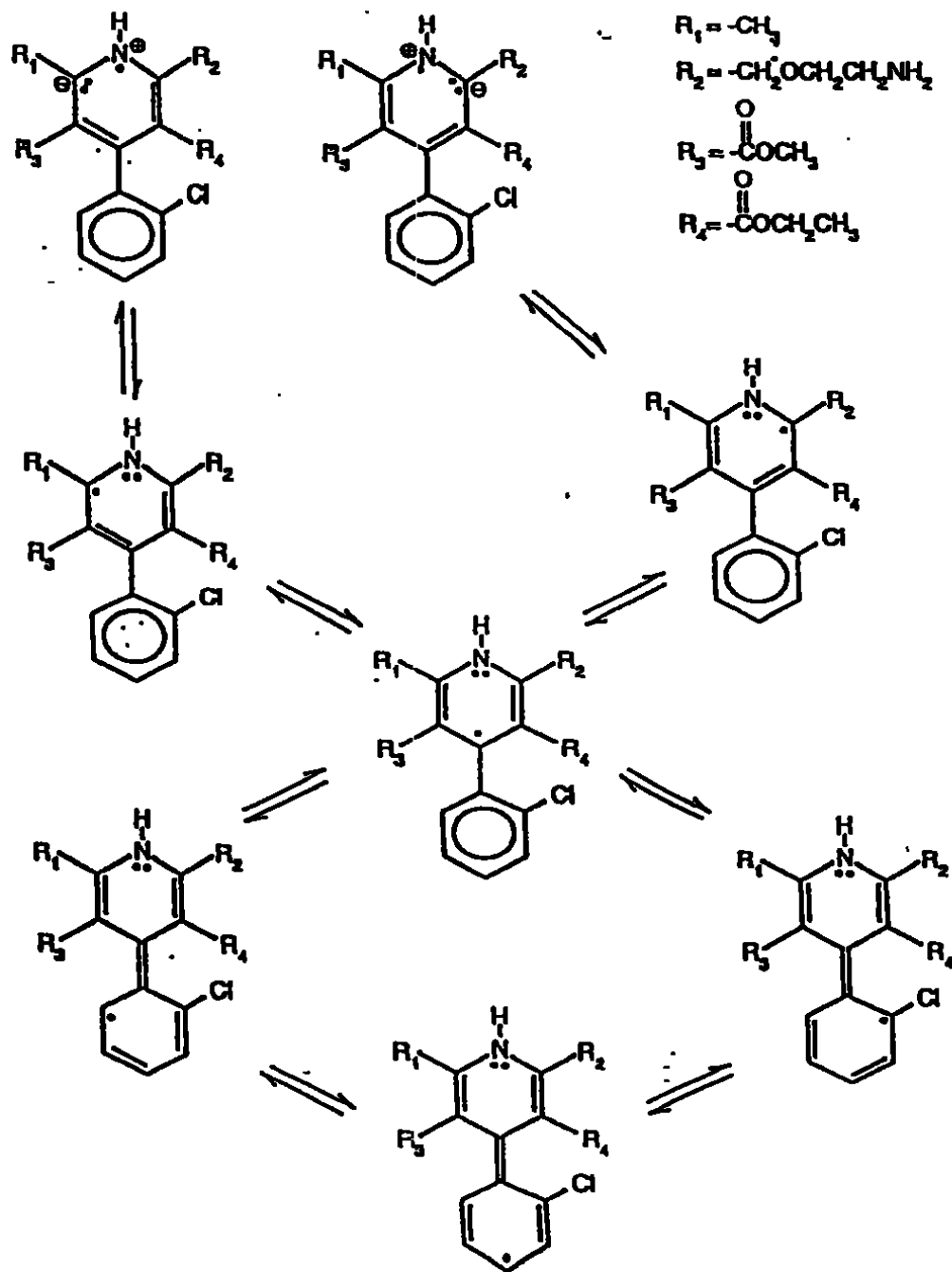
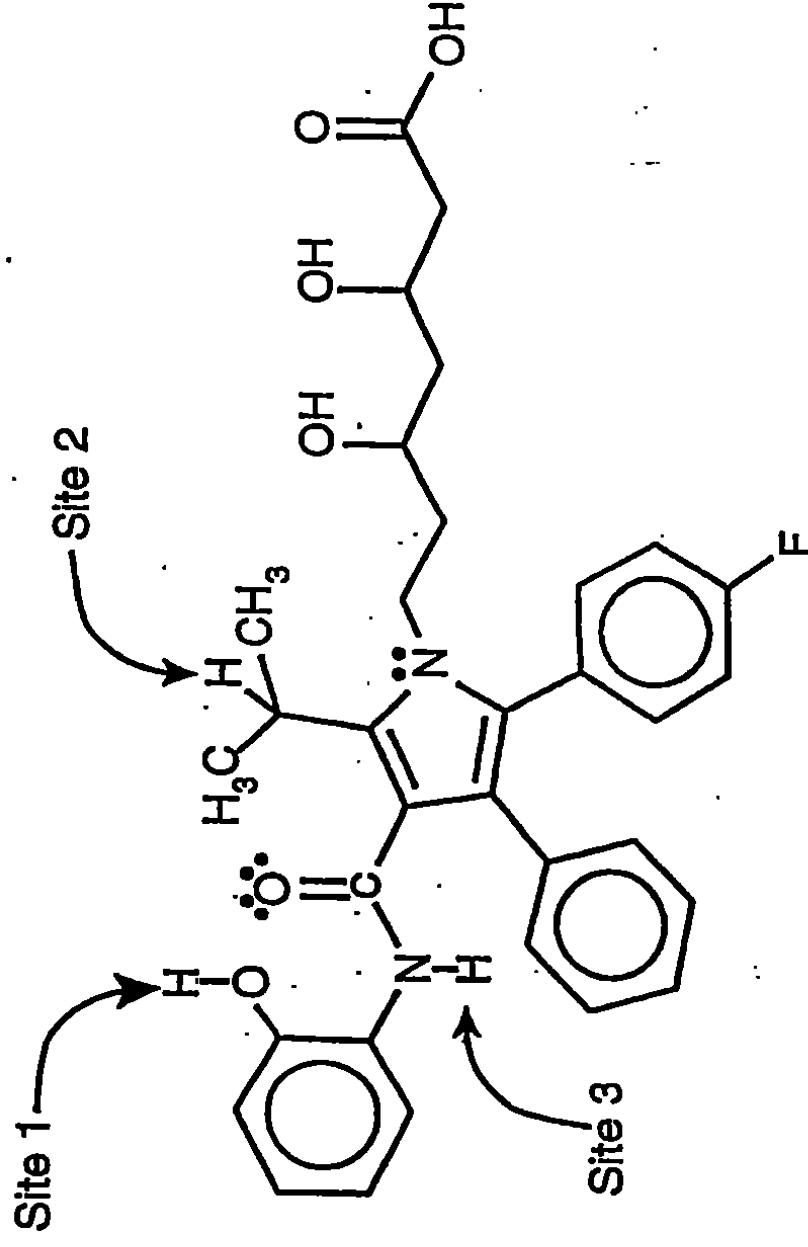


Figure 11C

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Antioxidant Mechanisms for Atorvastatin Metabolite: Abstractable Hydrogens



Antioxidant Mechanisms for Atorvastatin Metabolite: Resonance Stabilization of Free Radical at Site 1

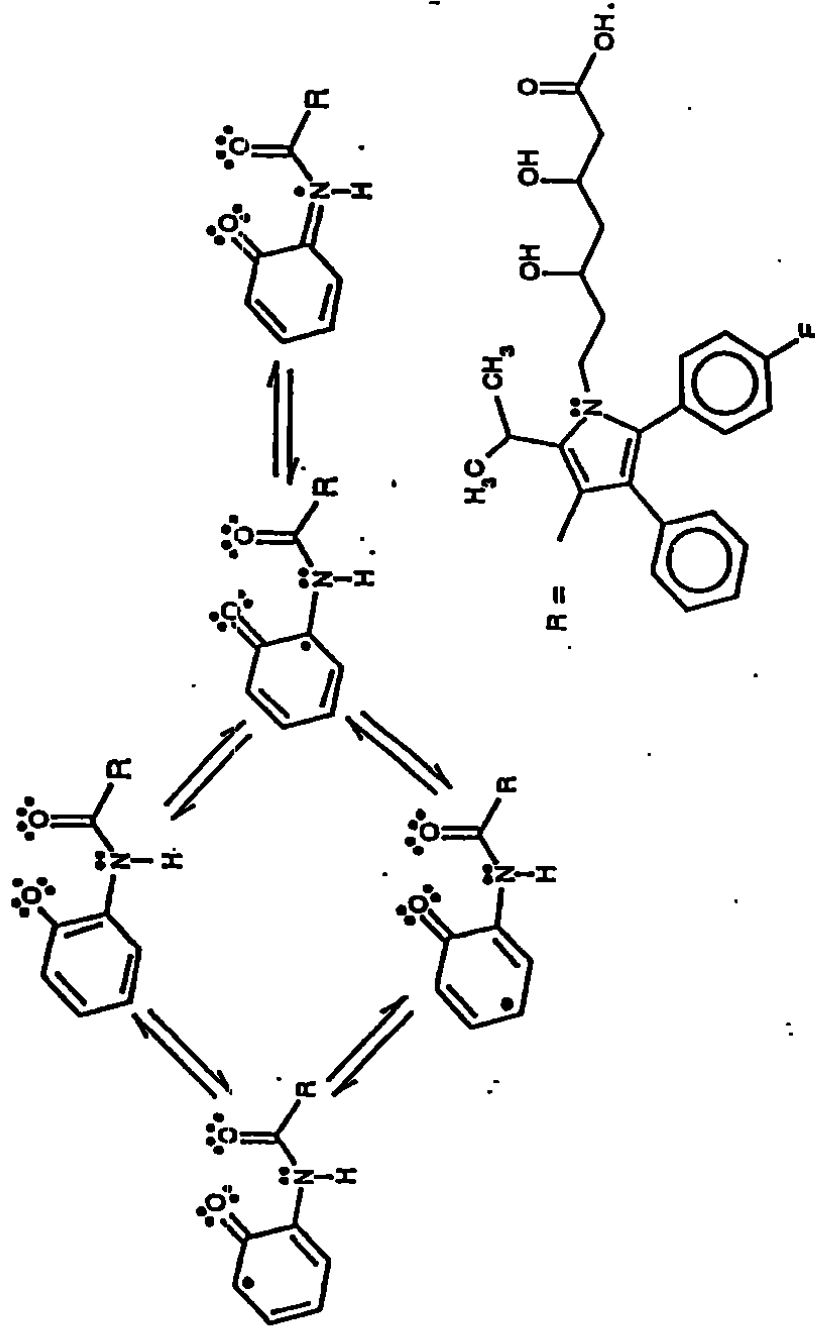


Figure 12B

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Figure 12c

Figure 12D



2H
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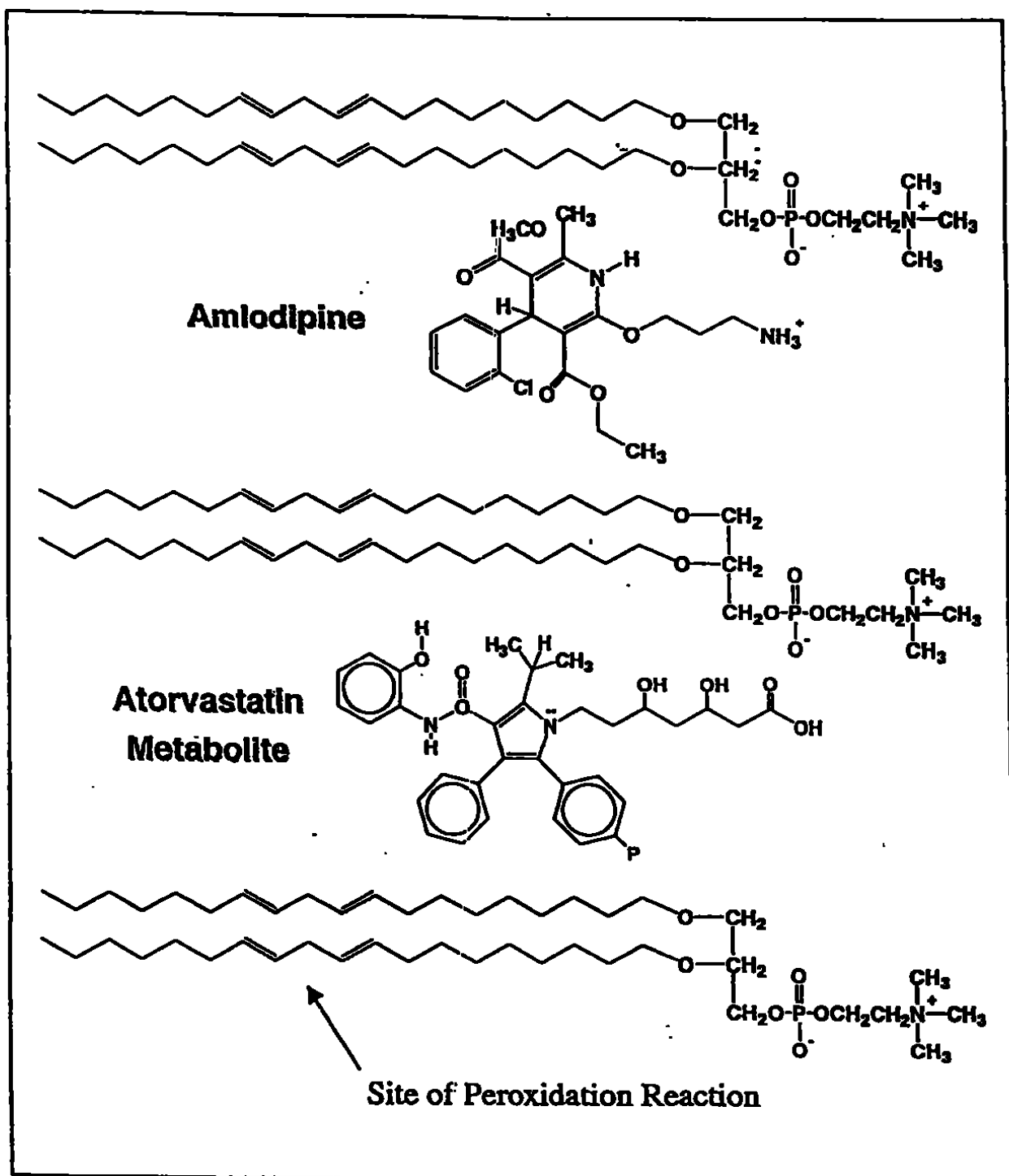


Figure 13

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DEPARTAMENTO DE COMERCIO DE LOS ESTADOS UNIDOS

Oficina de Patentes y Marcas de los Estados Unidos

19 de junio de 2000

ESTO ES PARA CERTIFICAR QUE LO ANEXADO AQUI ES UNA COPIA VERDADERA DE LOS REGISTROS DE LA OFICINA DE PATENTES Y MARCAS DE LOS ESTADOS UNIDOS DE AQUELLOS PAPELES DE ESTA SOLICITUD IDENTIFICADA ENSEGUIDA QUE CUMPLIÓ CON LOS REQUISITOS PARA SER OTORGADA UNA FECHA DE PRESENTACIÓN BAJO 35 USC 111.

NUMERO DE SOLICITUD: 60/166.592

FECHA DE RADICACION: 19 de noviembre de 1999

Por autoridad de

El COMISIONADO DE PATENTES Y

MARCAS

Sello de la Oficina de

(Firmado T. Lawrence)

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Oficial de

de América

Certificación

Resumen del Invento

Se describen análisis científicos que soportan el uso combinado de besilato de amlodipina (Norvasc®) y atorvastatina cálcica (Lipitor®) en un sistema de liberación única para el tratamiento de enfermedades cardiovasculares. Específicamente, las actividades antioxidantes sinérgicas del bloqueador de canales de calcio (CCB), besilato de amlodipina, y el inhibidor de la HMG-CoA reductasa, atorvastatina cálcica, incluyendo su metabolito hidroxilado, fueron evaluados en un sistema lipídico bien definido en donde las vesículas lípidas son enriquecidas con ácidos grasos poliinsaturados (PUFA), blanco principal del daño oxi-radical en la aterosclerosis. Los efectos sinérgicos de estos agentes fueron demostrados en membranas preparadas en ausencia y presencia de niveles de colesterol elevados que reproducen condiciones del tipo esclerótico. La combinación de la amlodipina (AML) con el metabolito hidroxilado de la atorvastatina (ATM) efectuó una reducción dramática y sostenida del daño oxi-radical lipídico en concentraciones tan bajas como 10.0 nM. Estos experimentos fueron llevados a cabo en membranas con colesterol que reproducen condiciones del tipo fisiológico. La actividad antioxidante catalítica asociada con la combinación de estos fármacos en niveles terapéuticos fue latamente sinérgico y no pudo ser

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efectivamente reproducido por la vitamina E o el carvedilol, un beta bloqueador no selectivo con efectos antioxidantes establecidos. La inhibición sinérgica de la peroxidación lipídica también fue observada con la combinación de la amlodipina y el compuesto precursor de la atorvastatina (AT). La actividad antioxidante no fue demostrada para otros bloqueadores de los canales de calcio (CCBs) que fueron probados (felodipina, verapamil, diltiazem) o los inhibidores de la HMG-CoA reductasa (mevastatina, pravastatina, simvastatina). La propiedad antioxidante diferente, descrita para esta combinación de fármacos, se puede atribuir a las interacciones catalíticas y fisicoquímicas con la bicapa de la membrana que son independientes de los efectos bien caracterizados de estos fármacos sobre el transporte del calcio y el metabolismo del colesterol. Este beneficio antioxidante sinérgico constituye un nuevo mecanismo farmacológico de acción para estos compuestos y una razón fundamental precisa para el uso combinado de los ingredientes activos en Norvasc® y Lipitor® en el tratamiento de enfermedades cardiovasculares, al reducir los niveles de LDL (lipoproteínas de baja densidad) en plasma y mejorar la protección contra la oxidación. Esta nueva propiedad complementa los efectos establecidos de estos fármacos con respecto a la hipertensión y la dislipidemia.

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Descripción Breve de los Dibujos

La Figura 1 muestra la actividad antioxidante dosis-dependiente de la AML.

La Figura 2 muestra los efectos antioxidantes comparativos de la AML y los dos otros bloqueadores de los canales de calcio y un inhibidor del ACE.

La Figura 3 muestra la actividad antioxidante dosis-dependiente del ATM.

La Figura 4 muestra los efectos antioxidantes comparativos de los inhibidores de la HMG-CoA reductasa.

La Figura 5 muestra los efectos antioxidantes comparativos de varios inhibidores de la HMG-CoA reductasa combinados con la AML.

La Figura 6 muestra el efecto del enriquecimiento con colesterol en la formación de peróxido lipídico.

La Figura 7 muestra el efecto del enriquecimiento con colesterol en las actividades antioxidantes del ATM.

La Figura 8 muestra el efecto antioxidante dosis-dependiente de la combinación de la AML y el ATM.

La Figura 9 muestra los efectos antioxidantes sinérgicos de la AML con ATM a 100.0 nM y de AML con AT a 1.0 μ M en vesículas enriquecidas con colesterol.

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La Figura 10 muestra los efectos antioxidantes comparativos de AML/ATM y AML/AT a 0.1 y 1.0 μM en vesículas enriquecidas con colesterol.

La Figura 11 muestra los efectos antioxidantes comparativos de la vitamina E, carvedilol, probucol, y la combinación de AML/ATM después de varios periodos de incubación a una concentración de 10.0 μM .

La Figura 12 muestra los mecanismos antioxidantes químicos para la AML.

La Figura 13 muestra los mecanismos antioxidantes químicos para el ATM.

La Figura 14 muestra las interacciones moleculares de la membrana de AML y ATM.

La Figura 15 muestra la determinación de la difracción de los rayos X de distintas interacciones de membrana lipídica con la combinación de la AML y el ATM.

La Figura 16 muestra los efectos de AML, ATM, y la combinación de los fármacos con respecto a las dimensiones de la bicapa de la membrana.

La Figura 17 muestra la inhibición de la oxidación de lipoproteínas de baja densidad humanas mediante la combinación de AML y ATM.

La Figura 18 muestra las entalpías de formación computadas para el ATM y sus radicales.

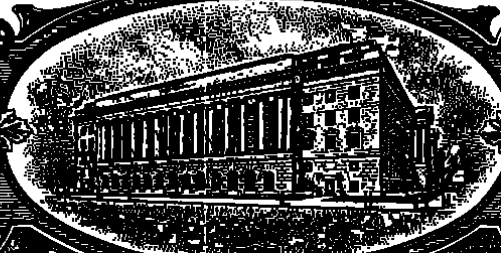
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La Figura 19 muestra las entalpías de formación
computadas para la ALM y sus radicales.

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PA 264777



THE UNITED STATES OF AMERICA

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UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

June 19, 2000

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APPLICATION NUMBER: 60/166,592

FILING DATE: November 19, 1999



By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

T. Lawrence

T. LAWRENCE

Certifying Officer

11/19/99
10662 U.S. PTO

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

INVENTOR(S)					
Given Name (first and middle (if any))		Family Name or Surname		Residence (City and either State or Foreign Country)	
R. Preston		Mason		Sewickley, Pennsylvania, USA	
☐ Additional inventors are being named on the _____ separately numbered sheets attached hereto					
TITLE OF THE INVENTION (280 characters max)					
SYNERGISTIC ACTIVITY OF AMLODIPINE AND ATORVASTATIN ON LIPIDS					
Direct all correspondence to: CORRESPONDENCE ADDRESS					
☐ Customer Number		IDON001368		Place Customer Number Bar Code Label here	
OR Type Customer Number here					
☒ Firm or Individual Name		Jerry Cohen, Esq.			
Address		Perkins, Smith & Cohen			
Address		One Beacon Street			
City		Boston		State	MA
Country		US		ZIP	02108
		Telephone	617-854-4000		Fax
				617-854-4040	
ENCLOSED APPLICATION PARTS (check all that apply)					
☒ Specification		Number of Pages		25	
☒ Drawing(s)		Number of Sheets		19	
				☒ Small Entity Statement	
				☐ Other (specify)	
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (check one)					
☒ A check or money order is enclosed to cover the filing fees				FILING FEE AMOUNT (\$)	
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.					
☒ No					
☐ Yes, the name of the U.S. Government agency and the Government contract number are _____					

10662 U.S. PTO
60/166592
11/19/99

60166592-111999

Respectfully submitted,

[Signature]

Date 11/19/99

SIGNATURE _____

TYPED or PRINTED NAME Jerry Cohen

TELEPHONE (617) 854 4000

REGISTRATION NO (if appropriate) 20,522

Docket Number 2189 P04 PROV

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

+

This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington D.C., 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C., 20231.

SYNERGISTIC ACTIVITY OF AMLODIPINE AND ATORVASTATIN ON LIPIDS

Background of the Invention

Coronary artery disease (CAD) is a leading cause of mortality worldwide, and is associated with substantial morbidity as well. Typically, the patient with CAD has several concomitant conditions, including hypertension, diabetes, and dyslipidemia, increasing overall risk for poor outcomes and complicating treatment.

Among antihypertensive therapies, the lipophilic calcium channel blocker (CCB) amlodipine besylate (AML) is a very well-tolerated agent with an established record of safety and effectiveness for the treatment of hypertension and angina. A potential therapeutic role for AML in the treatment of patients with CAD was recently shown in the Prospective Randomized Evaluation of the Vascular Effects of Norvasc Trial (PREVENT). This three-year trial evaluated the effects of AML compared to placebo on the development and progression of atherosclerotic lesions in coronary and carotid arteries in patients with documented CAD⁴. The results of PREVENT showed impressive clinical benefits with AML therapy as compared to placebo, including reduced risk of cardiovascular morbidity¹. AML therapy was also associated with a significant slowing of the progression of carotid atherosclerosis according to B-mode ultrasonographic assessments. The clinical benefit seen with AML in CAD has not been previously reported with other CCB analogs, including dihydropyridine-type agents, that have examined this question in similar patients⁵⁻⁷. This observation has led to interest in other anti-atherogenic properties of AML, including antioxidant effects that are independent of calcium channel modulation^{2, 8-10}.

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Hypolipidemic therapy has also been demonstrated to be very useful in reducing morbidity and mortality associated with CAD. Among HMG-CoA reductase inhibitors, atorvastatin calcium (AT) has been shown to be very effective as hypolipidemic therapy 11, 12. In addition, the hydroxylated metabolites of AT have been shown to exhibit antioxidant effects in lipoprotein preparations 13.

Thus, a therapeutic goal for the treatment of CAD is the development of drugs that can simultaneously target multiple underlying disease processes that contribute to atherosclerosis, thereby altering the course of the disease. Therefore, CAD therapy may have increased positive outcomes if the use of an antihypertensive agent and HMG-CoA reductase inhibitor was combined in a single delivery system.

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Unexpectedly, when AML and AT were combined, they had a synergistic effect in preventing peroxidation in lipid membranes that occurred in addition to their well-established therapeutic effects on both dyslipidemia and hypertension. Therefore, these agents have heretofore unrecognized synergistic antioxidant effects, a property that would enable these agents to increase the resistance of lipoproteins and vascular cell membranes to oxidative modification. Indeed, oxidative modification of lipids associated with LDL and vascular cell membranes is a well-established cause of injury to the endothelium and underlying smooth muscle 14, 15. Lipophilic antioxidants that protect against lipid peroxidation have been shown to reduce lesion development in various models of atherosclerosis (for review, see reference 6). Moreover, the benefit associated with hypolipidemic therapy is attributed to both its effect on plasma VLDL, LDL and HDL levels and to a reduction in the potential formation of atherogenic oxidized lipoproteins.

Summary of the Invention

Scientific analyses that support the combined use of amlodipine besylate (Norvasc®) and atorvastatin calcium (Lipitor®) in a single delivery system for the treatment of cardiovascular disease are described. Specifically, the synergistic antioxidant activities of the calcium channel blocker (CCB), amlodipine besylate, and the HMG-CoA reductase inhibitor, atorvastatin calcium, including its active hydroxylated metabolite, were evaluated in a well-defined lipid system in which lipid vesicles are enriched with polyunsaturated fatty acids (PUFA), the key target for oxy-radical damage in atherosclerosis. The synergistic effects of these agents were demonstrated in membranes prepared in the absence and presence of elevated cholesterol levels that reproduce atherosclerotic-like conditions. The combination of AML with the hydroxylated atorvastatin metabolite (ATM) effected a dramatic and sustained reduction in lipid oxy-radical damage at concentrations as low as 10.0 nM. These experiments were conducted in membranes with cholesterol that reproduce physiologic-like conditions. The catalytic antioxidant activity associated with the combination of these drugs at therapeutic levels was highly synergistic and could not be effectively reproduced by vitamin E or carvedilol, a non-selective β -blocker with established antioxidant effects. Synergistic inhibition of lipid peroxidation was also observed with the combination of AML and the AT parent compound. Antioxidant activity was not demonstrated for other CCBs that were tested (felodipine, verapamil, diltiazem) or HMG-CoA reductase inhibitors (mevastatin, pravastatin, simvastatin). The distinct antioxidant property described for this drug combination can be attributed to catalytic, physico-chemical interactions with the membrane bilayer that are independent of the well-characterized effects of these drugs on calcium transport and cholesterol metabolism. This synergistic antioxidant benefit constitutes a new pharmacologic mechanism of action for these compounds and a compelling rationale for

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the combined use of the active ingredients in Norvasc® and Lipitor® in the treatment of cardiovascular disease by reducing the levels of LDL in plasma and improving protection against its oxidation. This new property complements the established effects of these drugs on hypertension and dyslipidemia.

Brief Description of the Drawings

Figure 1 shows the dose-dependent antioxidant activity of AML.

Figure 2 shows the comparative antioxidant effects of AML and both other calcium channel blockers and an ACE-inhibitor.

Figure 3 shows the dose-dependent antioxidant activity of ATM.

Figure 4 shows the comparative antioxidant effects of HMG-CoA reductase inhibitors.

Figure 5 shows the comparative antioxidant effects of various HMG-CoA reductase inhibitors combined with AML.

Figure 6 shows the effect of cholesterol enrichment on lipid peroxide formation.

Figure 7 shows the effect of cholesterol enrichment on the antioxidant activities of ATM.

Figure 8 shows the dose-dependent antioxidant effect of the combination of AML and ATM.

Figure 9 shows the synergistic antioxidant effects of AML with ATM at 100.0 nM and of AML with AT at 1.0 μ M in cholesterol-enriched vesicles.

Figure 10 shows the comparative antioxidant effects of AML/ATM and AML/AT at 0.1 and 1.0 μ M in cholesterol-enriched vesicles.

Figure 11 shows the comparative antioxidant effects of vitamin E, carvedilol, probucol, and the AML/ATM combination after various incubation periods at a 10.0 μ M concentration.

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Figure 12 shows the chemical antioxidant mechanisms for AML.

Figure 13 shows the chemical antioxidant mechanisms for ATM.

Figure 14 shows the molecular membrane interactions of AML and ATM.

Figure 15 shows the x-ray diffraction determination of distinct lipid membrane interactions with the combination of AML and ATM.

Figure 16 shows the effects of AML, ATM, and the drug combination on membrane bilayer dimensions.

Figure 17 shows the inhibition of human LDL oxidation by the combination of AML and ATM.

Figure 18 shows the computed enthalpies of formation for ATM and its radicals.

Figure 19 shows the computed enthalpies of formation for AML and its radicals.

Detailed Description of Embodiments

Comparative Antioxidant Effects of Calcium Channel Blockers and HMG-CoA Reductase

Inhibitors: The separate and combined dose-dependent antioxidant activities of AML, AT and ATM were compared to representative CCBs (felodipine, verapamil, diltiazem) and HMG-CoA reductase inhibitors (mevastatin, pravastatin) in membrane vesicles reconstituted from phospholipids enriched with the polyunsaturated fatty acid, dilinoleoyl phosphatidylcholine (DLPC). Membrane vesicles were used in these experiments for the following reasons: 1) this system is well-defined and highly reproducible; 2) linoleic acid represents the primary target for oxidative damage and is common in vascular cell membranes and lipoprotein particles; 3) this membrane system does not contain calcium channels or HMG CoA reductase enzymes and; 4) lipid peroxidation in this system can be initiated spontaneously at 37°C in the absence of

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exogenous chemical initiators, such as high levels of iron and ascorbate. In these experiments, oxidation occurred in a gradual, time-dependent manner that was measured spectrophotometrically.

As demonstrated in Figure 1, AML effectively inhibited membrane lipid peroxide formation in a highly reproducible and dose-dependent manner over a broad range of concentrations, beginning at 10.0 nM. Inhibition of lipid peroxide formation by AML in membrane vesicles enriched with PUFA was measured as a function of drug concentration at 37°C by spectrophotometric methods. In these experiments, the control level of lipid peroxide formation was 1.2 mM after a 48 h incubation period. The fact that inhibition was observed at sub-micromolar levels indicates that the benefit observed with AML is of therapeutic relevance. Thus, inhibition of oxy-radical damage by AML was highly catalytic: at an AML concentration of 10.0 nM, the mole ratio of lipid peroxide inhibition: drug concentration was greater than 10,000:1.

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The antioxidant effects of AML could not be reproduced by other representative CCBs, including felodipine (a dihydropyridine), verapamil, or diltiazem under identical experimental conditions, even at micromolar levels. Figure 2 shows the antioxidant activity of AML compared to other representative CCBs (felodipine, verapamil, diltiazem) and the ACE-inhibitor, captopril, at a drug concentration of 1.0 µM. Under identical conditions, only AML produced a significant inhibition of lipid peroxidation. Again, the control level of lipid peroxide formation was 1.2 mM. Thus, the distinct effects of AML can be attributed to its high affinity for the membrane lipid bilayer, resulting in biophysical changes in the intermolecular packing of phospholipid molecules.

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In a similar fashion, ATM also inhibited lipid peroxide formation in these lipid preparations in a dose-dependent manner (see Figure 3). This activity was also observed with AT at much higher, micromolar levels but not with other statins that were tested in these experiments (pravastatin, mevastatin), as shown in Figure 4. Thus, AML, AT and ATM separately produced a measurable inhibition of lipid peroxidation in vesicles enriched with PUFA, the key substrate for oxy-radical damage.

Also, when AML was combined with the various statins (i.e. ATM, AT, mevastatin, and lovastatin), only the combination of AML with either ATM or AT significantly inhibited lipid peroxide formation as shown in Figure 5. Specifically, at a level of 1.0 micromolar, AML significantly interfered with lipid oxidative damage only when combined with ATM or AT, but not with the other representative statins, mevastatin and lovastatin.

Synergistic Antioxidant Effects of Drugs in Cholesterol-Enriched Membrane

Preparations: As cholesterol is a primary constituent of vascular cell plasma membranes that increases in content during atherogenesis ¹⁸, the synergistic antioxidant activity of these agents in membranes composed of cholesterol and phospholipid at a 0.5:1 mole ratio were examined. As shown in Figure 7, the addition of cholesterol to these vesicles resulted in a pronounced, 26% increase in the amount of lipid peroxide formation. This effect of cholesterol is attributed to its ability to increase the membrane bilayer packing constraints, contributing to its pathologic activity in atherosclerosis ¹⁹. Under these conditions, the antioxidant effectiveness of ATM was not significantly reduced (see Figure 7).

Under conditions of cholesterol enrichment, AML worked synergistically with ATM to effect an inhibition of lipid peroxidation that was dramatically more potent than that observed with either agent separately. The combination of AML and ATM was very effective and dose-

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dependent with antioxidant effects beginning in the low nanomolar range. Significant inhibition was observed at concentrations as low as 10.0 nM and nearly complete inhibition was observed at 10.0 μ M (see Figure 8). In this membrane system, the combination of AML with either ATM or AT produced a marked synergistic effect (see Figure 9). Although a synergistic effect was observed with the combination of AML and AT, it was less prominent than when the AML/ATM combination was used. Specifically, at both concentrations (0.1 and 1.0 μ M), the combination of AML/ATM was more effective as an antioxidant than AML/AT (see Figure 10).

The effect of other known antioxidants, including vitamin E and carvedilol, were also compared to the combination of AML and ATM. Carvedilol is vasodilating β -blocker and antioxidant approved for treatment of congestive heart failure. It has been shown to scavenge oxygen free radicals and inhibit lipid peroxidation in swine heart ²⁰. Even at a concentration of 10.0 μ M, these compounds were markedly less effective than the combined AML/ATM preparation (see Figure 11). In the case of vitamin E, the activity of this compound was significantly attenuated over time (48 versus 72h), as compared to AML/ATM (Figure 11A). This observation is consistent with a stoichiometric mechanism in which the antioxidant ability of the vitamin E is terminated by the reaction. By contrast, the activity of the AML/ATM combination persisted, providing evidence that these agents work by a separate, catalytic fashion as antioxidants. Only the very effective antioxidant, probucol, had similar activity to the AML/ATM complex (Figure 11C).

To understand the basis for the antioxidant effects of these compounds, the chemical structure of these molecules was evaluated (Figures 12, 13). In Figure 12, the covalent structure of AML includes two hydrogen atoms associated with its dihydropyridine ring that can be donated to unstable free radicals associated with lipids (Figure 12A). Once a hydrogen atom is

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donated, an unpaired free electron remains associated with the AML molecule. This unpaired electron, a potential source of free radical damage, can be safely stabilized in resonance structures (Figures 12B and 12C).

Similar chemical analyses were conducted for a key hydroxyl metabolite of AT, as indicated in Figure 13. Similar to AML, the ATM molecule has three potential hydrogen atoms that can be donated to unstable free radical molecules (A). Resonance stabilization structures for the remaining unpaired free electrons at sites 1 (Figure 13B), 2 (Figure 13C) and 3 (Figure 13D) are calculated.

In addition to its chemical properties, AML also has high affinity for membrane lipids under atherosclerotic conditions, as compared to other CCBs ²¹. The distinct lipophilicity of AML is attributed to its amphiphilic chemical structure that directs the molecule to a favorable location in the membrane where it can then interfere with the propagation of free radicals by both biophysical and biochemical mechanisms ^{2, 8, 16, 21}. In the same way, the chemical structure of ATM consistent with an amphiphile that could be expected to interact strongly with the membrane bilayer, as illustrated in Figure 14.

This conclusion is supported by x-ray diffraction analyses. As indicated in Figure 15, x-ray diffraction measurements show that lipid membranes have distinct interactions with the combination of AML and ATM. One-dimensional electron density profiles, measured in angstroms versus electrons per cubic angstrom, show that these profiles were superimposed for control membranes, indicated by solid lines, and the experimental groups, indicated by dotted lines. This was true for AML (Figure 15A), ATM (Figure 15B), and the combination of AML and ATM (Figure 15C). The two peaks of electron density on either side in the Figure corresponds to the electron-dense phospholipid headgroups while the minimum electron density

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is associated with the terminal methylene segments at the center of the membrane bilayer. In the absence of drug, the overall membrane bilayer width, including surface hydration, was 55.5 angstroms with an intrabilayer headgroup separation of 44 angstroms. In the presence of the drug combination, the overall membrane bilayer width, including surface hydration, was reduced to 53.5 angstroms with an intrabilayer headgroup separation of 41 angstroms. Separately, the membrane bilayer widths of membranes containing AML and ATM were 54.8 angstroms and 58.0 angstroms, respectively.

Direct subtraction of the membrane electron density profiles (angstroms versus electrons per cubic angstrom) demonstrated large differences in lipid structure that could be attributed to the presence of the drugs. Specifically, the addition of the drug combination produced a broad increase in electron density associated with the upper hydrocarbon core/hydrated headgroup of the membrane bilayer as ± 11 to 21 angstroms from the center of the bilayer. This large increase in electron density distributed over 10 angstroms is attributed to the equilibrium location of the drugs in the membrane. Concomitant with this change was an observed disordering effect associated with the central hydrocarbon core region of the membrane, as ± 0 to 11 angstroms. This decrease in electron density is due to an increase in molecular volume resulting from the insertion of the drug molecules into a region of high molecular density near the membrane hydrocarbon core/water interface. Such changes in biophysical properties have been previously shown to interfere with the propagation of free radicals through the lipid bilayer matrix ¹⁹.

In addition, Figure 16 shows the effects of AML, ATM, and the drug combination on membrane bilayer dimensions. As determined by x-ray diffraction analyses, the separate and combined contribution of these agents produced distinct effects on the structure of the phospholipid bilayer at a 1:15 drug to phospholipid mole ratio. Overall membrane width

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measurements included surface hydration. Separately, AML and ATM affected distinct changes in membrane structure, as compared with the drug combination, due to specific interactions with constituent phospholipid molecules. While the drug combination caused a 2 angstrom or 4 percent decrease in overall membrane width, the ATM produced a 2.5 angstrom or 5 percent increase in width. In addition, AT separately produced a 4 angstrom or 7 percent increase in width while AML alone did not significantly alter membrane dimensions, including the intrabilayer headgroup separation. The control membrane width was 55 angstroms.

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The antioxidant activity of AML may contribute to its benefit in patients with documented CAD, as recently reported in the Prospective Randomized Evaluation of the Vascular Effects of Norvasc Trial (PREVENT) ⁴. Patients randomized to AML experienced a significant reduction in angina (-33%, $p < 0.01$), major vascular procedures (-45%, $p < 0.001$), and the composite of any major event (-30%, $p > 0.01$), as compared to placebo, independent of changes in lipid levels ¹. The benefits of AML in CAD have not been observed with other CCBs, including dihydropyridine agents ⁵⁻⁷, suggesting that the additional antioxidant properties of AML contribute to direct antiatherosclerotic effects ².

Synergistic Antioxidant Effects of Amlodipine with the Hydroxylated Atorvastatin Metabolite in Human LDL Preparations: The separate and combined antioxidant effects of AML and the ATM were evaluated in human LDL preparations. The ability of these agents to inhibit LDL peroxidation was assessed in vitro following addition of copper (10 μ M) in order to stimulate oxidative damage. The rate of LDL oxidation was consistent with sigmoidal curve kinetics, as described by Esterbauer et al., characterized by an initial lag phase followed by a sharp propagation and final plateau phase (Figure 17) ²².

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After a 4 h incubation period, the differential effects of these compounds could be clearly observed at a concentration of 3.0 μ M. Separately, AML and the ATM inhibited LDL oxidation by 7 and 33%, respectively. When combined, however, these agents produced 73% inhibition. This unexpected *synergistic* effect persisted at the 6 h time point when the agents separately did not inhibit peroxidation by more than 11% while the combination reduced TBARS-formation by 32%.

Oxidative modification of lipids associated with low-density lipoproteins (LDL) contributes significantly to inflammatory process associated with atherosclerosis, including foam cell formation, endothelial dysfunction and toxicity, leukocyte and platelet adhesion. The results of these experiments demonstrated a strong and unexpected synergistic antioxidant effect for the combination of AML and the ATM in human LDL, consistent with what was observed in membrane vesicles. The drug combination produced a level of oxy-radical inhibition that substantially exceeded any additive effect. The antioxidant activity of vitamin E was similar to that observed for the ATM when used alone, but was substantially less than what was observed for the drug combination.

LDL particles that have greater resistance to oxidative damage, due to the presence of lipophilic antioxidants, exhibit reduced cytotoxicity, interfere less with endothelium-derived nitric oxide production, and do not contribute to foam cell formation ²³. Supplementation with vitamin E has been shown to increase LDL resistance to oxidative modification and reduce endothelial cell cytotoxicity ²⁴. In addition, the lipophilic antioxidant probucol has been demonstrated to attenuate the formation of atherosclerotic plaques in cholesterol-fed primates, an effect that correlated with an increased resistance of LDL to oxidative damage ²⁵.

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The synergistic antioxidant activity observed for this drug combination in LDL and reconstituted lipid membranes suggests that these compounds interact directly with each other to scavenge lipid radicals. It is proposed that the ATM reacts more quickly with lipid radicals (equation 1) than AML, as described in equation 2. If these are the only two pathways available for these the drugs when added together to the system, then their combined effect would only be additive. However, the combination of both compounds provides the possibility for a third pathway (equation 3) that is supported by the results of the peroxidation experiments in both LDL and lipid membranes. The combination of pathways 1 and 3 would produce a synergistic effect if it occurs more rapidly than pathway 2. Indeed, semi-empirical calculations suggest that reaction 3 is a favorable, exothermic process with an enthalpy of -40.7 kJ/mol. Thus, the presence of the fast inhibitor (ATM) enables the slower inhibitor (AML) to remove free radicals faster than if reacting with the lipid radicals on its own. The three pathways describing these interactions are as follows, in which "LOO•" represents a lipid radical molecule:

- (1) $\text{LOO}\cdot + \text{ATM} \rightarrow \text{LOOH} + \text{ATM}\cdot$ *FAST*
- (2) $\text{LOO}\cdot + \text{AML} \rightarrow \text{LOOH} + \text{AML}\cdot$ *SLOW*
- (3) $\text{ATM}\cdot + \text{AML} \rightarrow \text{ATM} + \text{AML}\cdot$ *FAST*

As a result of this synergy between AML and the ATM, the recycled metabolite is now made available for additional scavenging of lipid radicals. Overall, differences in the rates of inhibition between AML and the ATM are based, in part, on the calculated enthalpies for these compounds and their respective radicals. As shown in Figures 18 and 19, the calculated enthalpies for the ATM are more favorable than for AML. The more negative the ΔH_f value reported in Figures 18 and 19, the easier to abstract the hydrogen atom from that particular site.

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Role of Antioxidants in Reducing Coronary Artery Disease: The results of in vivo investigations have indicated an important role for lipophilic antioxidants in reducing cardiovascular morbidity and mortality, especially coronary artery disease. The rationale for this observation is attributed to the ability of these compounds to interfere with the peroxidation of PUFA associated with LDL and vascular cell membranes. In particular, it has been demonstrated that LDL particles with greater resistance to oxidative damage, due to the presence of lipophilic antioxidants, have reduced cytotoxicity, interfere less with endothelium-derived nitric oxide production, and do not contribute to foam cell formation ¹⁵. Supplementation with vitamin E has been shown to increase LDL resistance to oxidative modification, and thus, reduce cytotoxicity toward endothelial cells ²⁶. The lipophilic antioxidant probucol has also been demonstrated to attenuate the formation of atherosclerotic plaques in cholesterol-fed primates, an effect that correlated with an increased resistance of LDL to oxidative damage and not changes in lipid levels ²⁷. In addition, probucol inhibited atheroma formation of lesions in Watanabe hereditary hyperlipidemic (WHHL) rabbits, a well-characterized animal model of atherosclerosis, independent of cholesterol-lowering effects ²⁸. Consistent with these animal studies, a double-blind, randomized clinical investigation has shown that probucol reduced restenosis by 47% in patients with CAD following coronary-artery balloon angioplasty, presumably due to its antioxidant effects ²⁹.

Another pathological process associated with oxidized LDL is an inhibition of the constitutive release of endothelium-derived nitric oxide in the arterial wall ³⁰⁻³². Release of nitric oxide inhibits the abnormal adhesion of leukocytes and platelets to the endothelial surface early during the development of foam cell formation. Nitric oxide is also an important mediator of vasodilation; loss of nitric oxide can lead to vasospasm associated with acute coronary

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syndromes ³³. The interference of this process by oxidized LDL is attributed to a disruption in secondary messenger systems, including G-protein-dependent nitric oxide release ^{31, 32}.

Patients treated with the antioxidant probucol had improved endothelial function, as assessed by nitric oxide production, when compared to control subjects. LDL isolated from these patients showed greater resistance to oxidation in vitro and did not interfere with normal nitric oxide production from the endothelium, as compared to control LDL samples ³⁴. An important benefit associated with lipophilic antioxidants is the ability to inhibit endothelial dysfunction associated with oxidized LDL, a key early event in atherosclerosis ¹⁴.

Thus, a comprehensive review of the available data provides a compelling mechanistic rationale for the use of lipophilic antioxidants to prevent cellular changes associated with coronary artery disease. By increasing the resistance of LDL to oxidative damage, agents with antioxidant activity may effectively interfere with pathologic alterations in the vessel wall during atherogenesis, including foam cell formation, endothelial dysfunction and toxicity, leukocyte and platelet adhesion, and arterial vasospasm, secondary to a loss of normal nitric oxide production. These cellular observations also provide important insights into the results of recent clinical studies demonstrating a benefit associated with antioxidant use by reducing the expression of established coronary artery disease despite an absence of pronounced changes in atherosclerotic lesion development ³⁵. Indeed, the results of epidemiological analyses have provided direct evidence for an inverse association between the serum levels of antioxidants, especially vitamin E, and adverse outcomes associated with coronary disease ³⁶⁻⁴².

This invention demonstrates that the combination of AML and atorvastatin produced a powerful, synergistic antioxidant effect that could not be effectively reproduced by well-known antioxidants such as α -tocopherol or carvedilol. While previous studies have shown that these

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agents separately have antioxidant benefit in both lipoprotein and cell membrane preparations ^{2, 3, 13, 43}, these findings unexpectedly show that the combination of these agents provide a much greater, synergistic effect, especially under atherosclerotic-like condition in which the separate effects of the compounds are especially reduced.

Experimental Methods

Dilinoleoyl phosphatidylcholine (DLPC) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL) and stored at -80°C. Amlodipine besylate (AML) was a gift from Pfizer Central Research (Groton, CT) while atorvastatin calcium (AT) and its hydroxylated metabolite (ATM) were provided by Parke Davis (Ann Arbor, MI). Verapamil, captopril and diltiazem were purchased from RBI Biochemicals (Natick, MA). Mevastatin and vitamin E were purchased from Sigma Chemical Co. (St. Louis, MO).

Lipid Peroxidation Analysis: The separate and combined dose-dependent antioxidant activities of these agents were examined in membranes enriched with PUFA prepared in the absence and presence of cholesterol at a 0.5 sterol to phospholipid mole ratio ². The biological lipid (dilinoleoyl phosphatidylcholine) used for these samples was dissolved in HPLC-grade chloroform (25.0 mg/ml). An aliquot of the lipids (1.0 mg) was added to individual glass 13 x 100-mm test tubes and the chloroform was removed under a steady stream of N₂ gas while vortexing. The lipids were shell dried in the absence or presence of drug dissolved in ethanol. Residual solvent was removed under vacuum while the samples were shielded from light. Membrane vesicles were produced by vortexing the dried lipids at room temperature with 1.0 ml HEPES buffered saline (0.5 mM HEPES and 154.0 mM NaCl, pH, 7.2). The final phospholipid

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concentration was 1.0 mg/ml buffer and the concentrations of drug ranged from 10.0 nM through 10.0 μ M.

In the lipid peroxidation experiments, aliquots of membrane vesicles (1.0 mg) were added to separate test tubes. Membrane peroxidation was carried out at 37°C in a shaking water bath. At various time intervals (24, 48, 65, 72h), a small aliquot of lipid sample was removed before 25 μ l of 5.0 mM ethylenediaminetetracetic acid (EDTA) and 20 μ l of 35.0 mM butylated hydroxytoluene (BHT) was immediately added to the sample to stop the peroxidation reaction.

The extent of membrane lipid peroxidation was measured by the CHOD-Iodide assay as previously described in detail ¹⁷. The quantity of I_3^- was measured from the following reaction in which L represents a phospholipid molecule:



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An aliquot of the membrane sample was removed at various time points and then added to 1.0 ml of CHOD color reagent (E.M. Science, Gibbstown, NJ) that includes 20.0 μ M BHT, 24.0 μ M EDTA, and 0.2% Triton-X. The sample was then covered with foil and allowed to incubate for 2 h in the absence of light before measuring the absorbance of the sample at 365 nm ($\epsilon = 2.4 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$). The background sample was run along the test samples in triplicate and contains 76.7 μ l of 0.652 mM HEPES, 20 μ l of 5.0 mM EDTA and 3.3 μ l of DDI water. The extent of lipid peroxidation was measured in triplicate for each drug concentration and compared to control samples that did not contain drug. The statistical significance of these experiments was assessed by the non-paired t-test. Significance was accepted at $p < 0.05$.

In LDL oxidation determination experiments, the separate and combined antioxidant activities of AML and the active ATM were tested in human low-density lipoproteins (LDL), an

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important target for oxy-radical damage in atherosclerosis. Human LDL preserved with EDTA was obtained from Sigma Chemical Co. (St. Louis, MO). The EDTA content of the LDL samples was removed by gel filtration with PD-10 Sephadex G25-M filtration columns; PBS (nitrogen purged) was used as the eluent. The LDL samples (50 μ g of protein/mL) were preincubated with or without the drugs (3.0 or 6.0 μ M) for 30 min at 37°C. Oxidation of LDL was induced by the addition of 10 μ M of CuSO₄ using the colorimetric TBARS method⁴⁴. The time course of LDL oxidation, measured by TBARS formation, was followed for 6 h at 37°C following addition of copper. LDL oxidation, as determined by the TBARS methods, followed sigmoidal curve kinetics, as described by Esterbauer et al. with an initial lag phase followed by a sharp propagation and final plateau phase²². The protein content of the LDL was determined using the Pierce Coumassie Protein Plus assay kit (Pierce Chemical)⁴⁵.

X-Ray Diffraction Analysis of Lipid Interactions: The methodology for performing x-ray diffraction analysis of lipid interactions has been described previously^{16, 46}. For these studies, the combination of AML and ATM was added to membrane vesicles reconstituted from cholesterol and phospholipid at a 0.5:1 mole ratio.

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Abstract

The combination of amlodipine besylate and atorvastatin calcium shows a synergistic antioxidant effect on lipid peroxidation in membrane vesicles enriched with polyunsaturated fatty acids, the key target for free radicals. Inhibition of oxy-radical damage by this drug combination was observed at therapeutic levels and could not be reproduced with other representative CCBs, statins, or even the highly lipophilic antioxidants, vitamin E and carvedilol. This combination therapy can be used to treat cardiovascular disorders, especially coronary artery disease, by reducing the levels of LDL in plasma and improving protection against its oxidation.

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STATEMENT CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) & 1.27(b))—INDEPENDENT INVENTOR

Docket Number (Optional)
2189 P04 PROV

Applicant, Patentee, or Identifier: R. Preston Mason

Application or Patent No.: _____

Filed or Issued: Herewith

Title: SYNERGISTIC ACTIVITY OF AMLODIPINE AND ATORVASTATIN ON LIPIDS

As a below named inventor, I hereby state that I qualify as an independent inventor as defined in 37 CFR 1.9(c) for purposes of paying reduced fees to the Patent and Trademark Office described in:

- ☒ the specification filed herewith with title as listed above.
☐ the application identified above.
☐ the patent identified above.

I have not assigned, granted, conveyed, or licensed, and am under no obligation under contract or law to assign, grant, convey, or license, any rights in the invention to any person who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern, or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

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Separate statements are required from each named person, concern, or organization having rights to the invention stating their status as small entities. (37 CFR 1.27)

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

R. Preston Mason
NAME OF INVENTOR


Signature of Inventor

11/19/99
Date

NAME OF INVENTOR

Signature of Inventor

Date

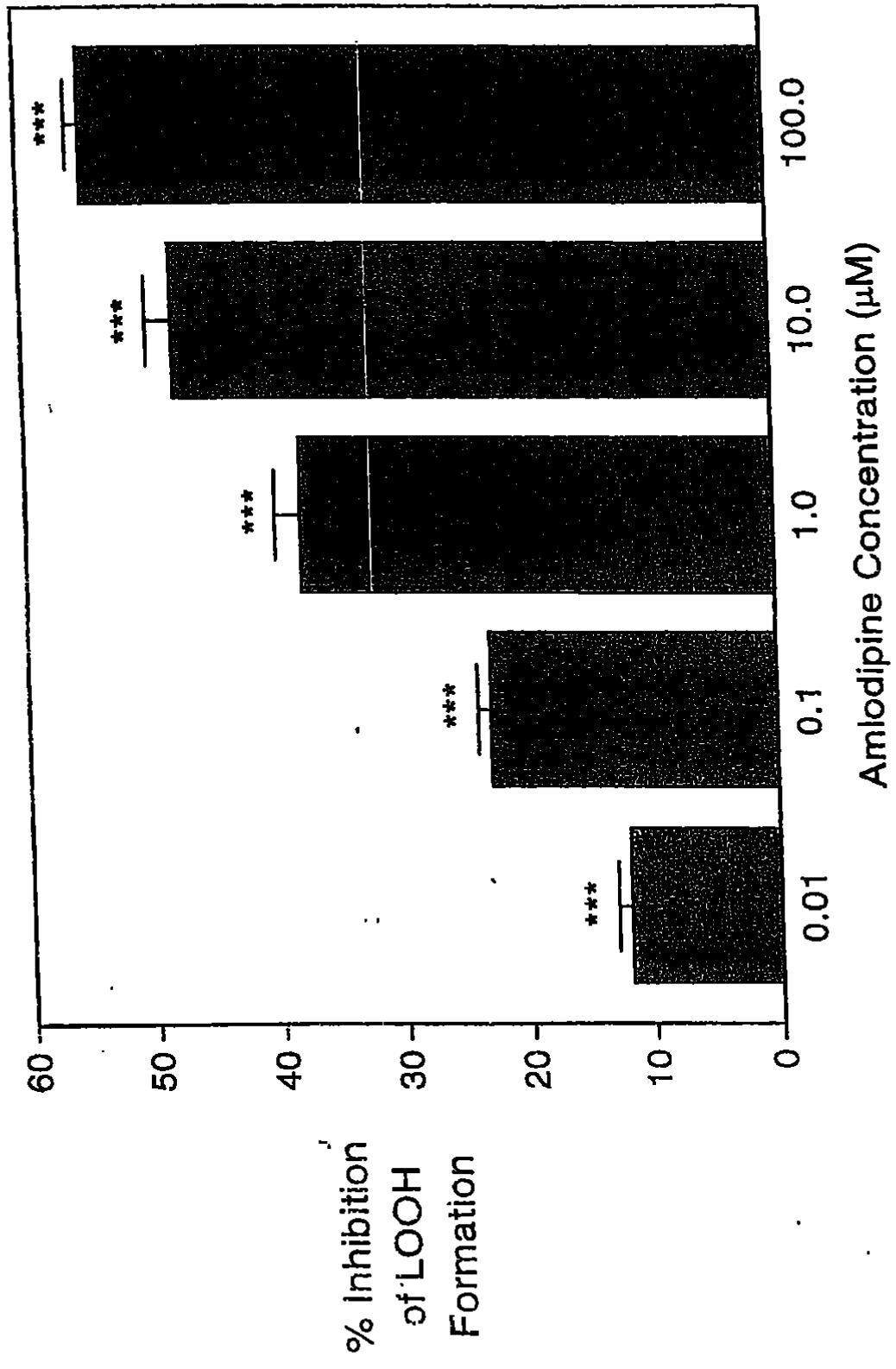
NAME OF INVENTOR

Signature of Inventor

Date

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*** $p < 0.001$ versus control
Values are mean $\pm$ S.D. (n = 3)
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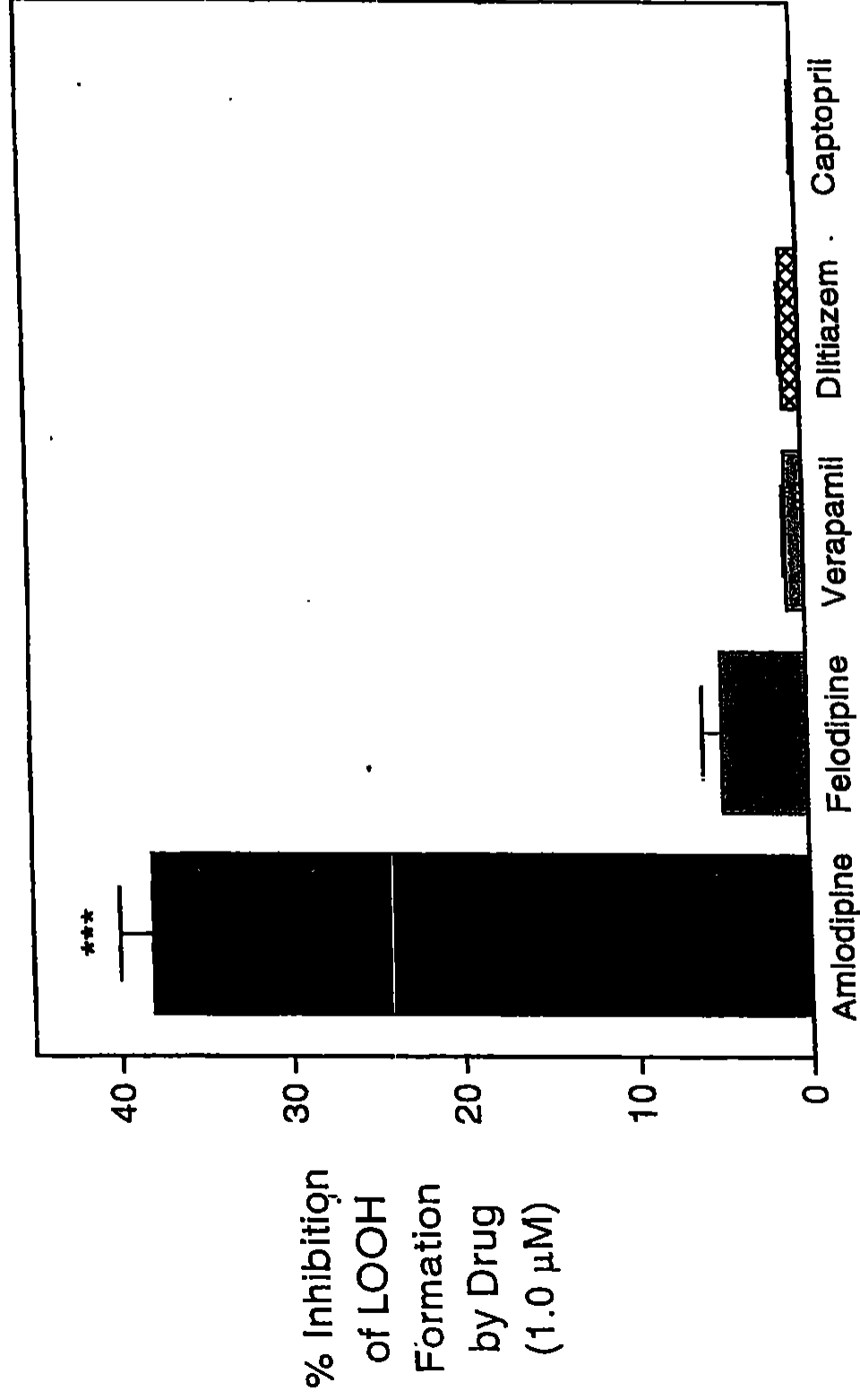
Figure 1

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*** $p < 0.001$ versus control and other drugs
Values are mean $\pm$ S.D. ($n = 3$)

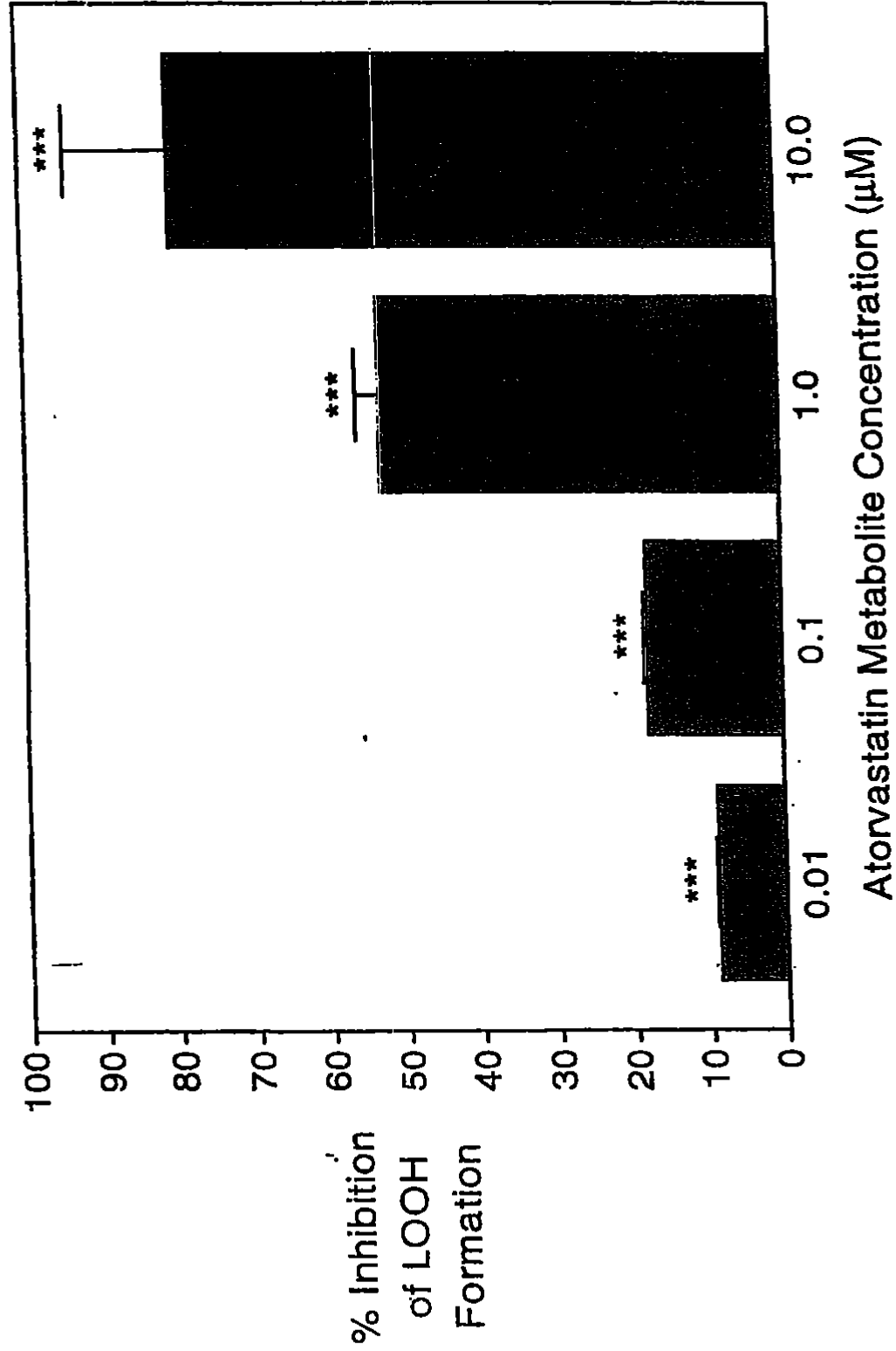
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Figure 2

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*** $p < 0.001$ v rsus control
Values are mean $\pm$ S.D. (n = 3)

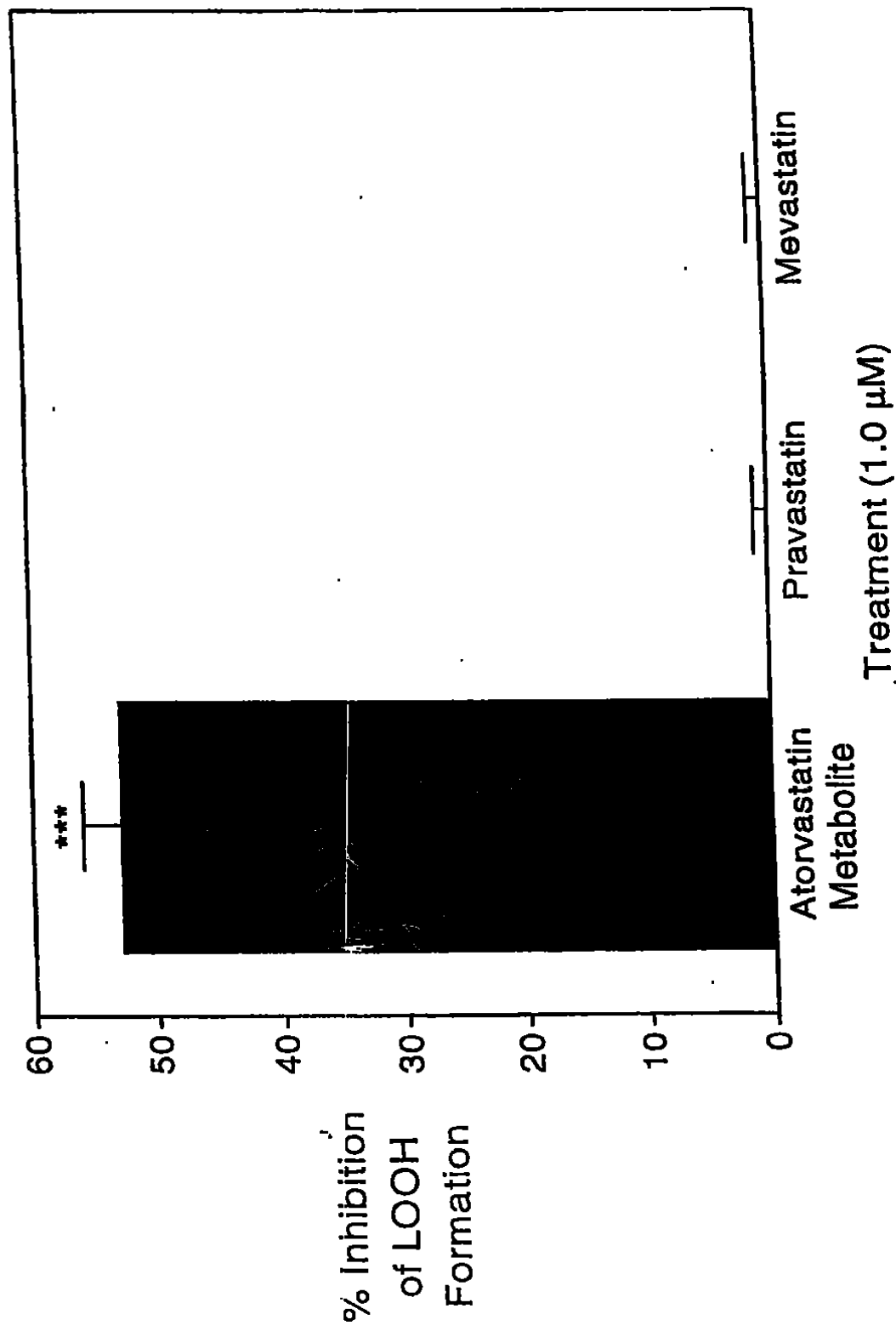
Figure 3

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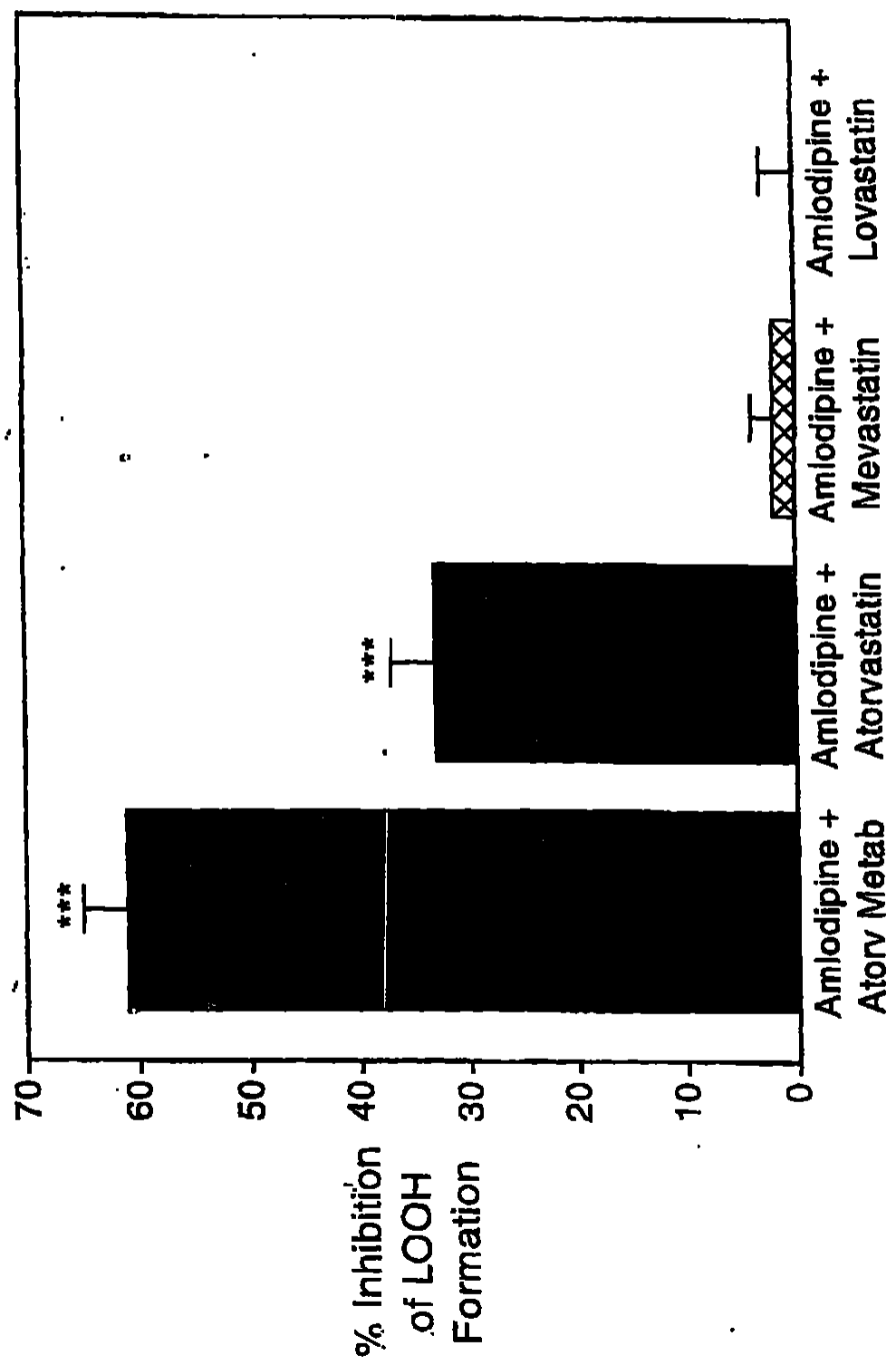


*** $p < 0.001$ versus control
Values are mean $\pm$ S.D. ($n = 3-6$)

Figure 4

Qnt

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Treatment (1.0 μ M)

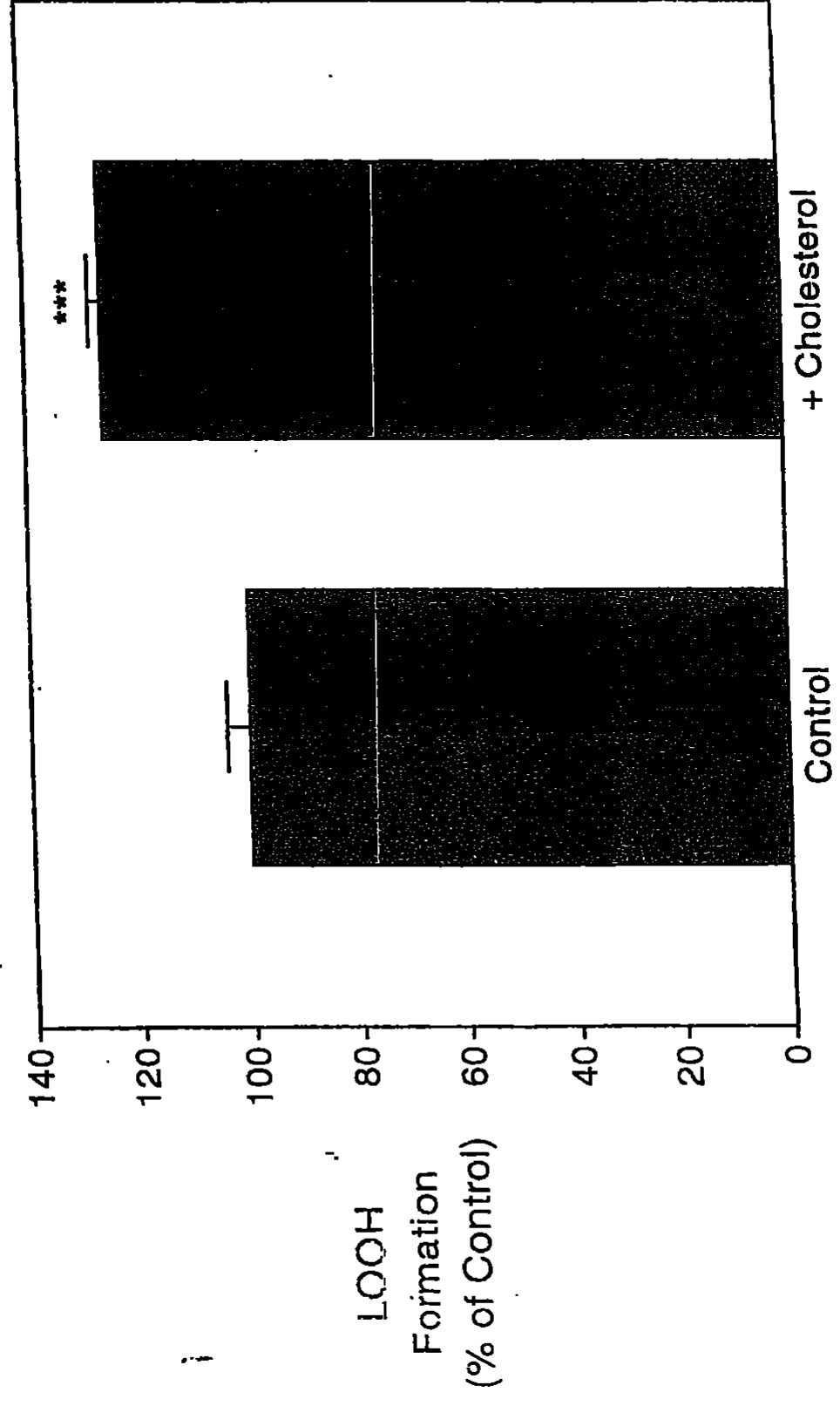
*** $p < 0.001$ versus n treatment

Values are mean $\pm$ S.D. 0116592.111999

Figure 5

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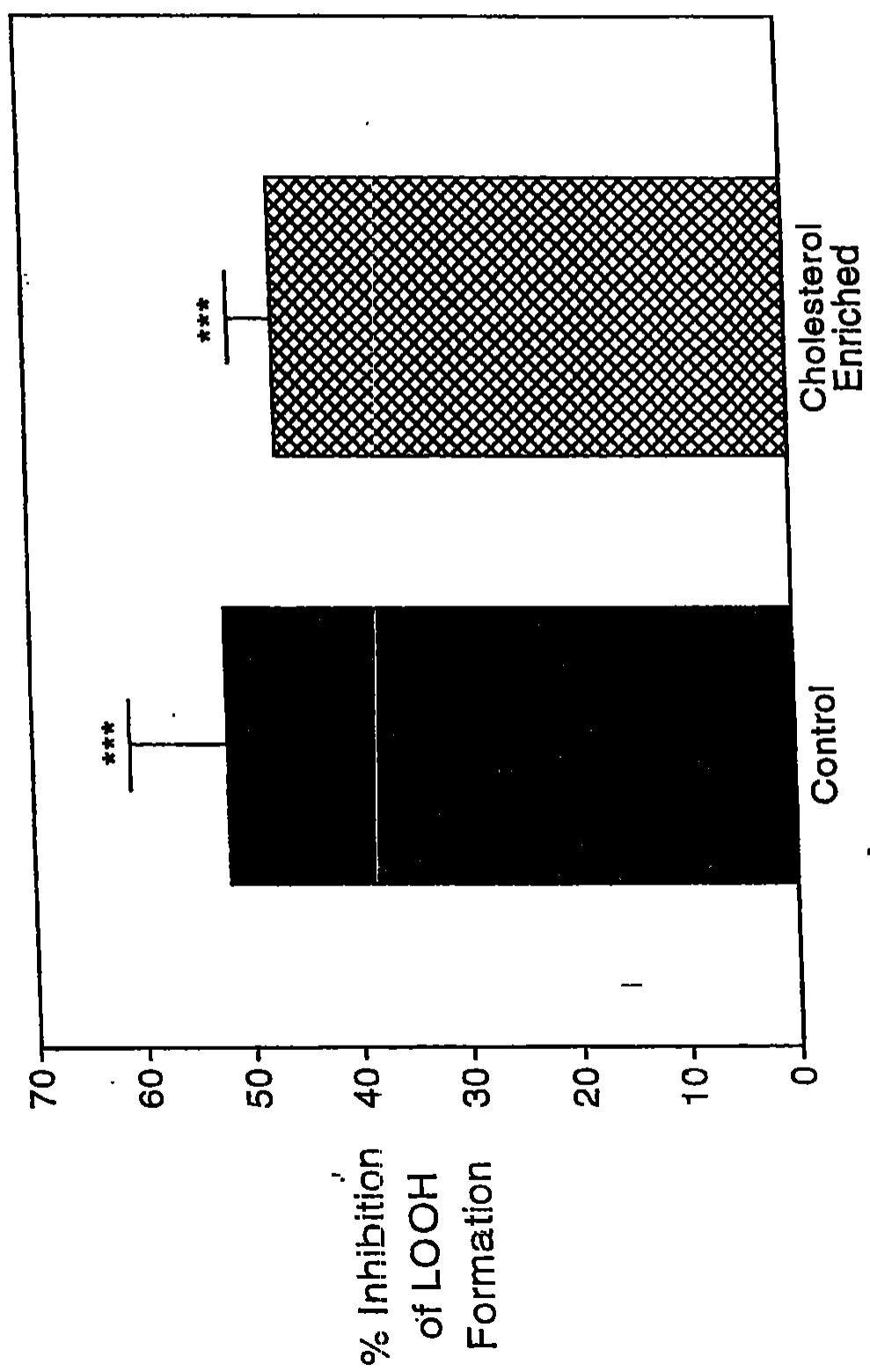


*** $p < 0.001$ versus control
Values are mean $\pm$ S.D. ($n = 4$)
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Figure 6

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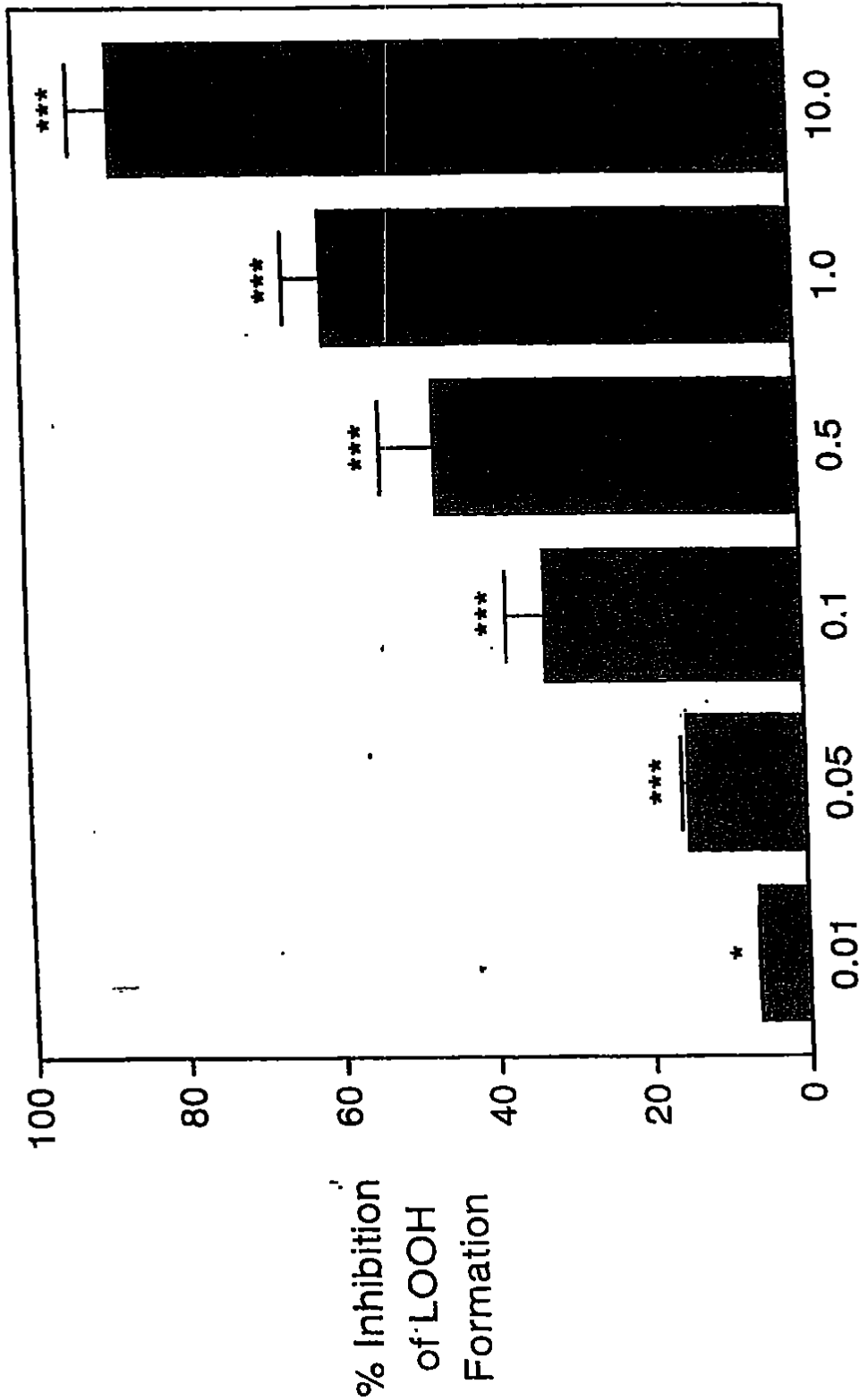
248



Atorvastatin Metabolite Treatment (1.0 μ M)

*** $p < 0.001$ v rsus no treatm nt
Values are mean $\pm$ SD (n = 4) . 111999

Figure 7



Amlodipine / Atorvastatin Metabolite Combination (μM)

* $p < 0.05$; *** $p < 0.001$ versus control

Values are mean $\pm$ S.D. (n = 6)

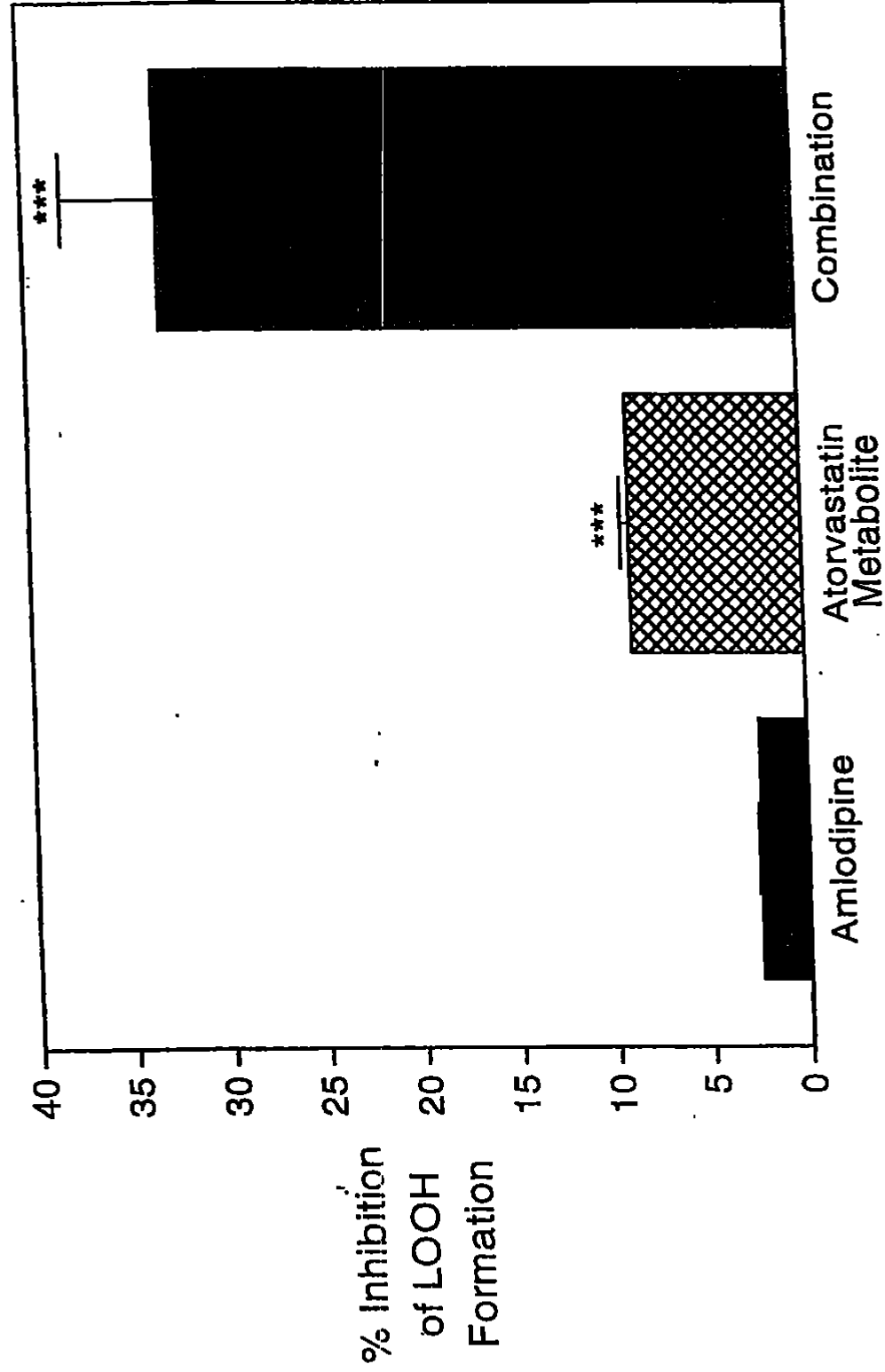
Figure 8

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Treatment (100.0 nM)

*** $p < 0.001$ versus control and other treatments
Values are mean $\pm$ S.D. (n = 6)

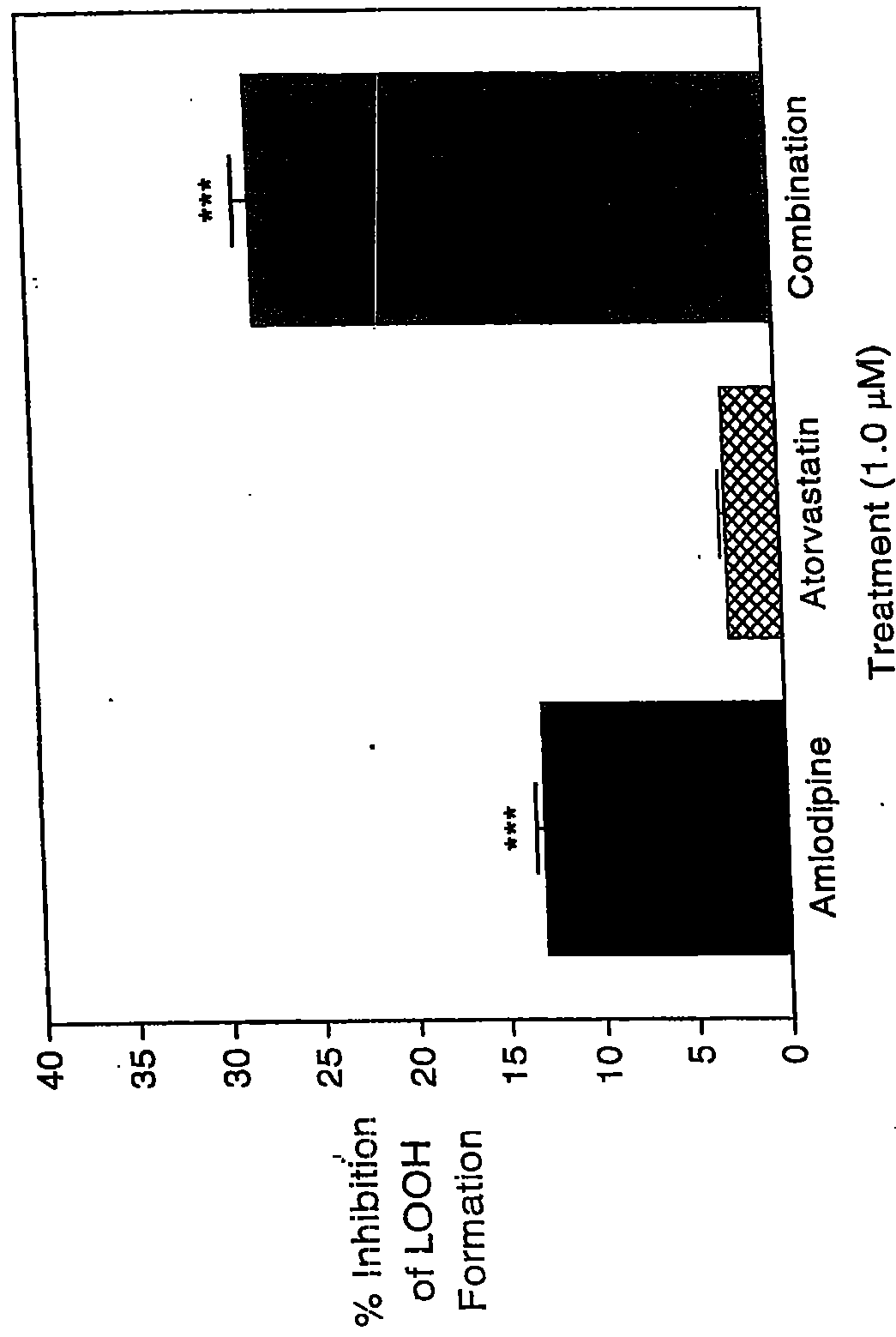
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Figure 9A

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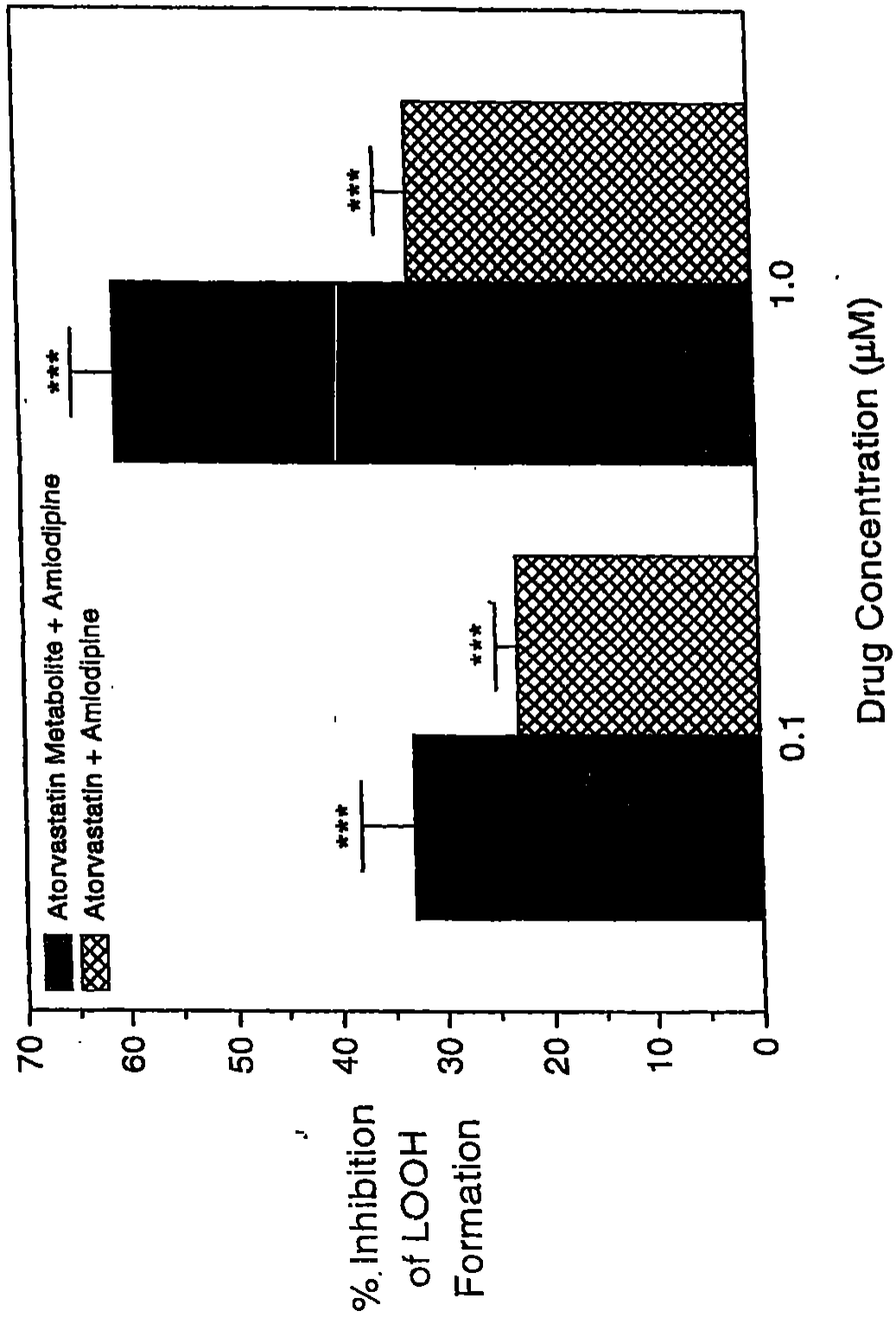
256

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*** $p < 0.001$ versus control
Values are mean $\pm$ S.D. ($n = 6$)
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Figure 9B



*** $p < 0.001$ v rsus control
Values are mean $\pm$ SD (n=5-6)

Figure 10

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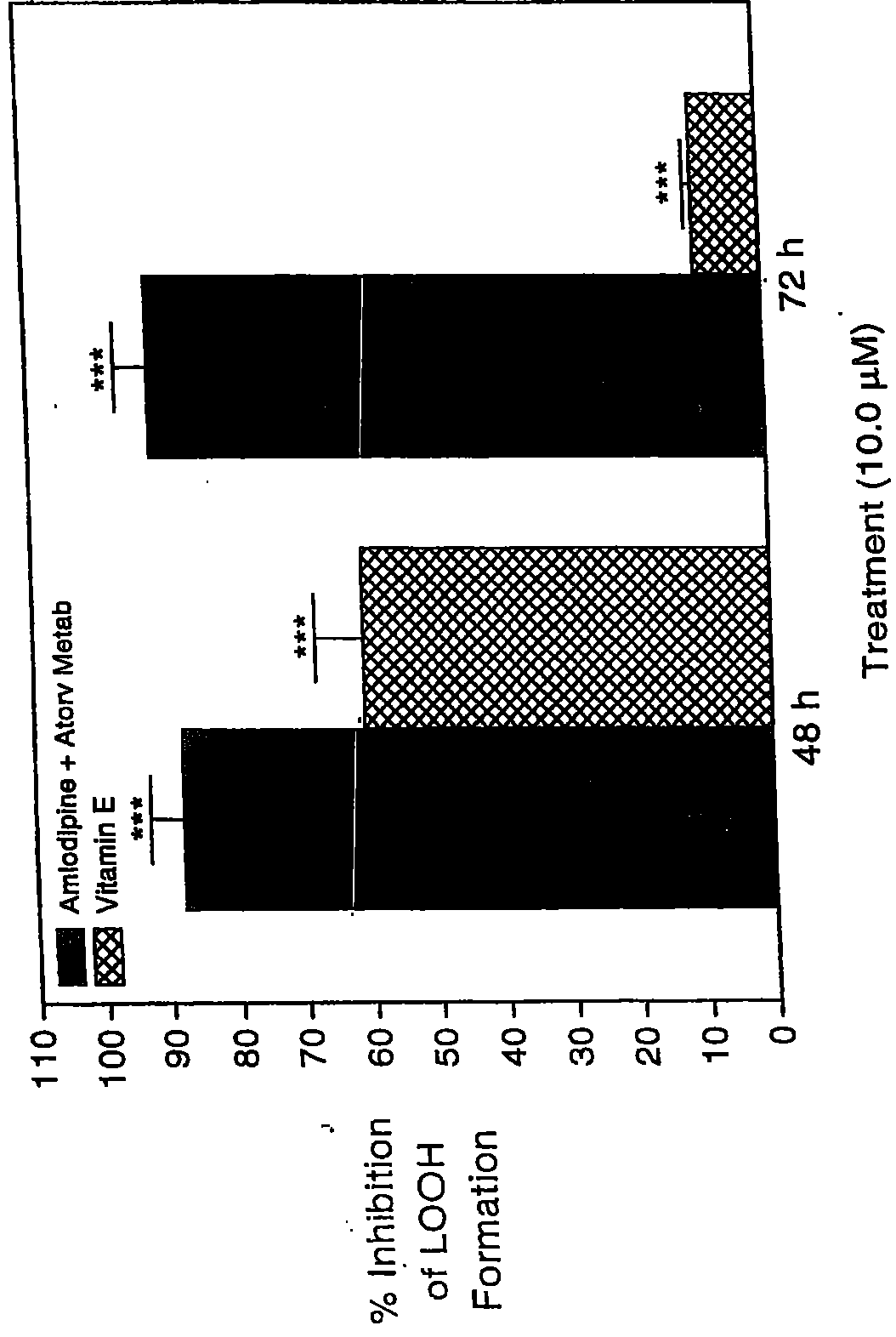


Figure 11A

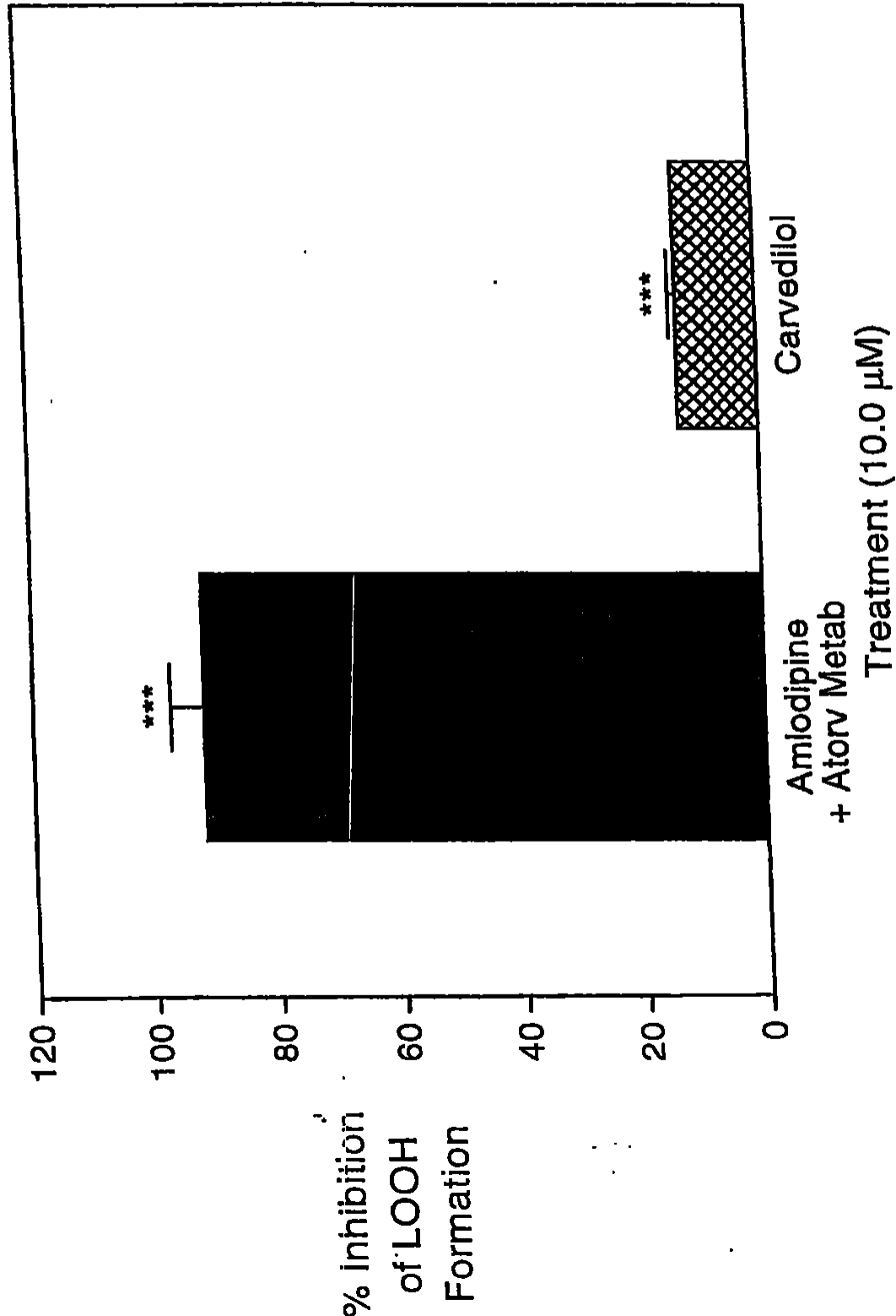


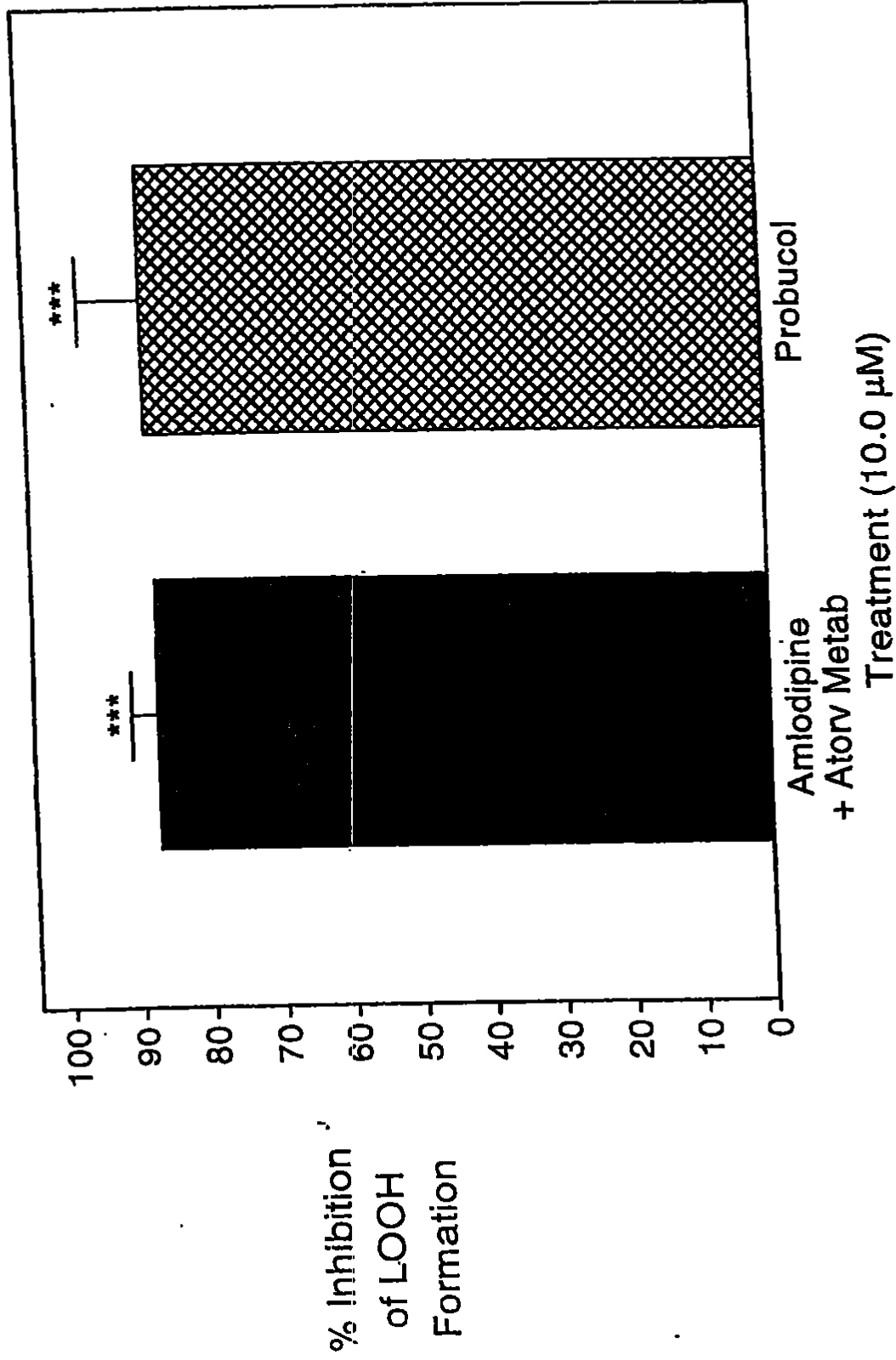
Figure 11B

*** $p < 0.001$ versus control
Values are mean $\pm$ SD (n=6)

254

147

257



*** $p < 0.001$ versus control
 Values are mean $\pm$ S.D. ($n = 6$)

Figure 11 C

255

760
 644

256

Chemical Antioxidant Mechanisms for Amlodipine: Abstractable Hydrogens

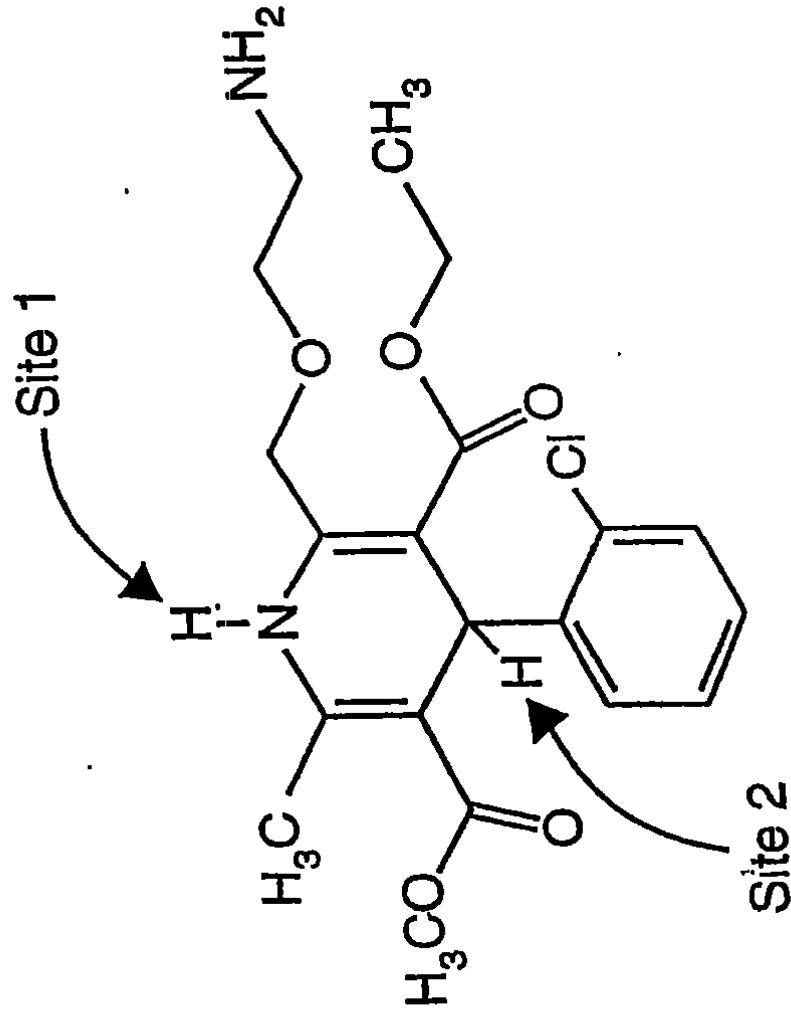


Figure 12 A

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Resonance Stabilization of Free Radical at Site 1 of Amlodipine

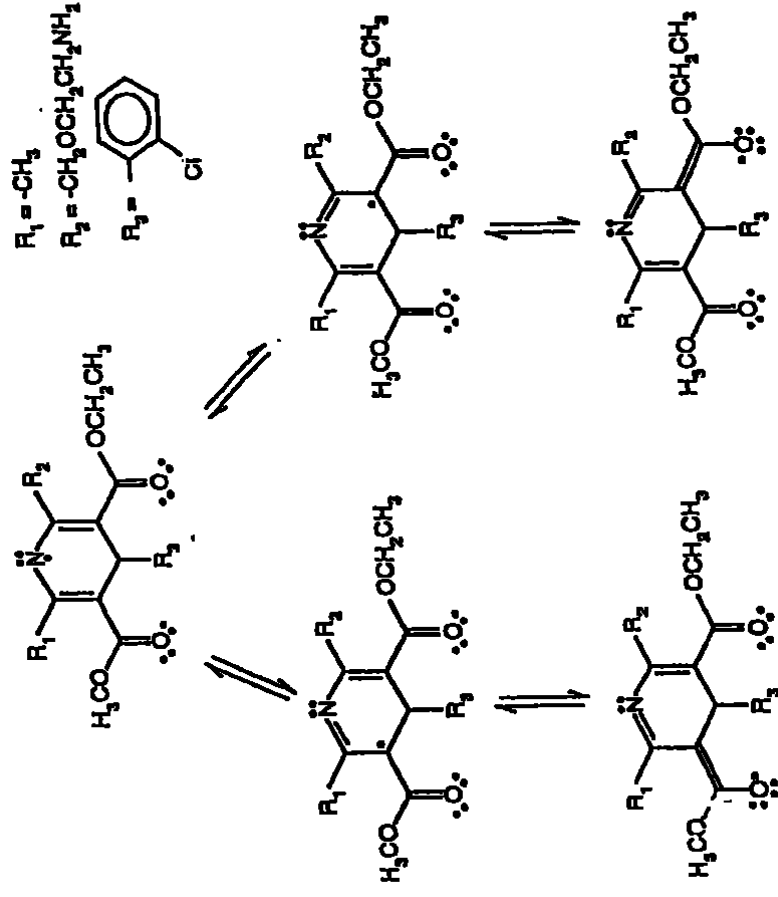


Figure 12B

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Resonance Stabilization of Free Radical at Site 2 of Amlodipine

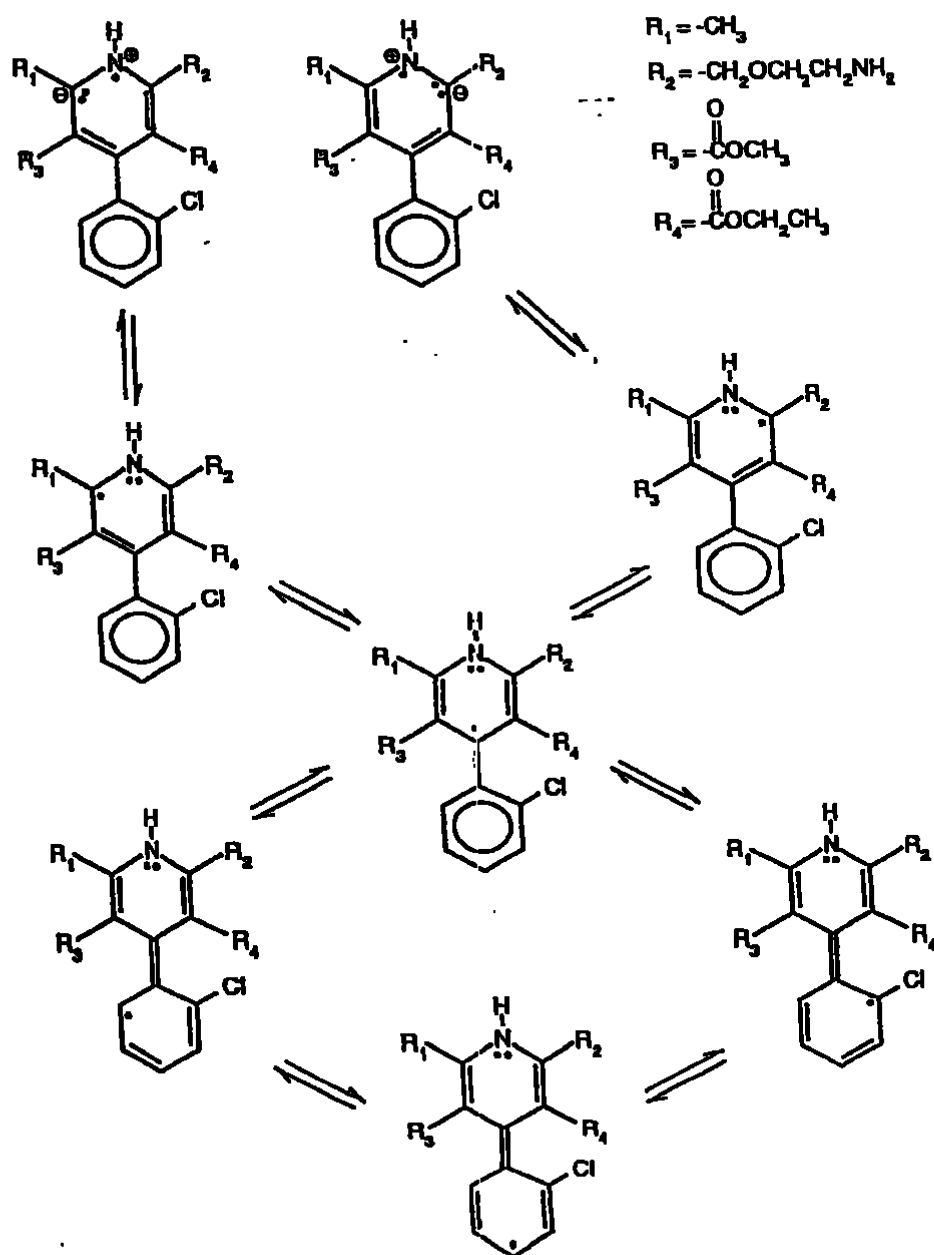


Figure 12C

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Antioxidant Mechanisms for Atorvastatin Metabolite: Abstractable Hydrogens

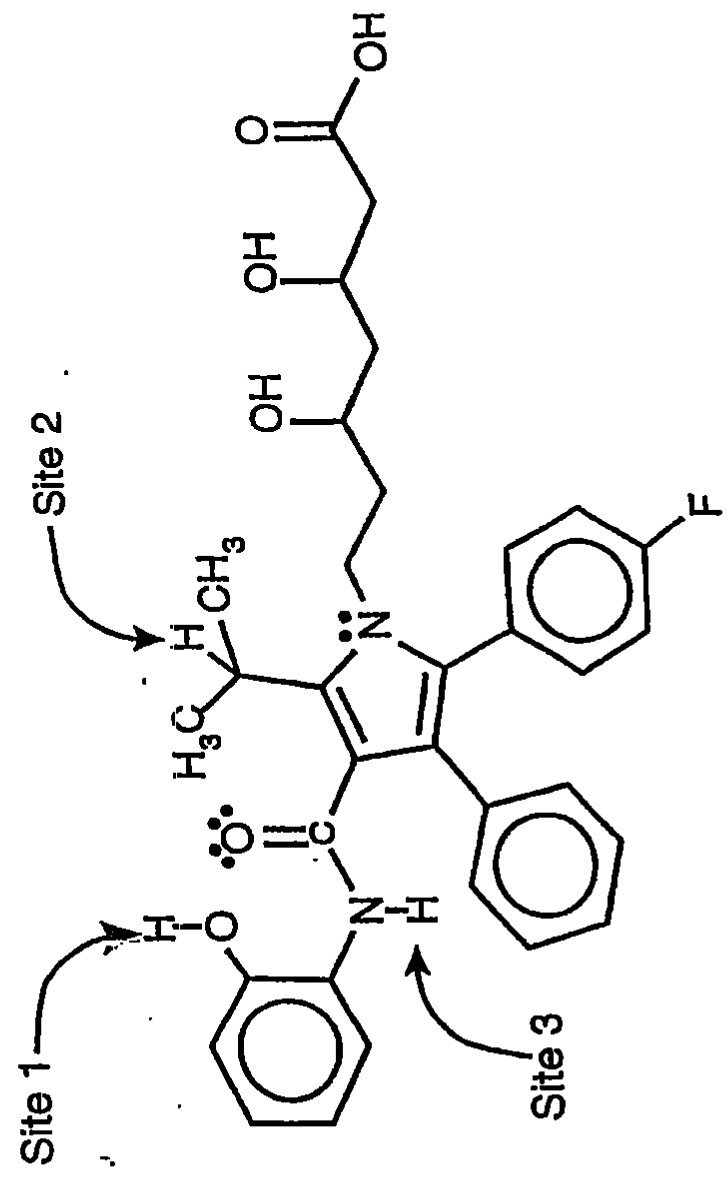


Figure 13A

60165592.111999

Antioxidant Mechanisms for Atorvastatin Metabolite:
Resonance Stabilization of Free Radical at Site 1

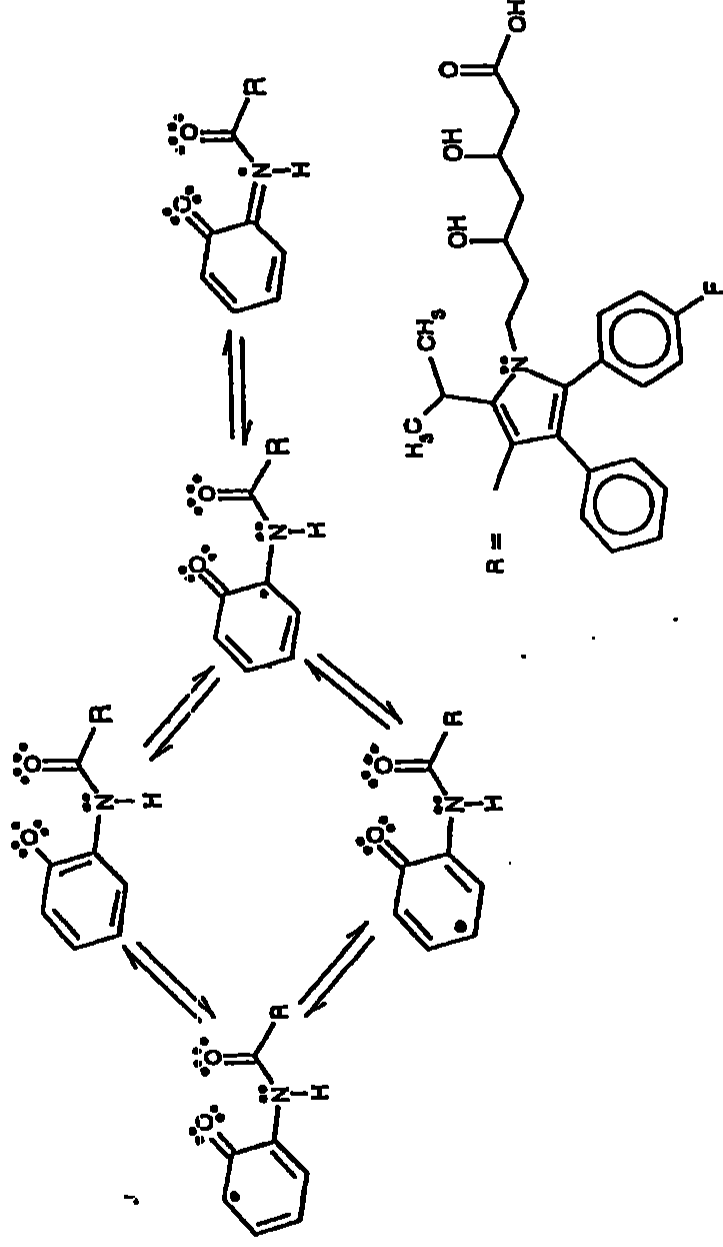


Figure 13B

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761

Antioxidant Mechanisms for Atorvastatin Metabolite: Resonance Stabilization of Free Radical at Site 2

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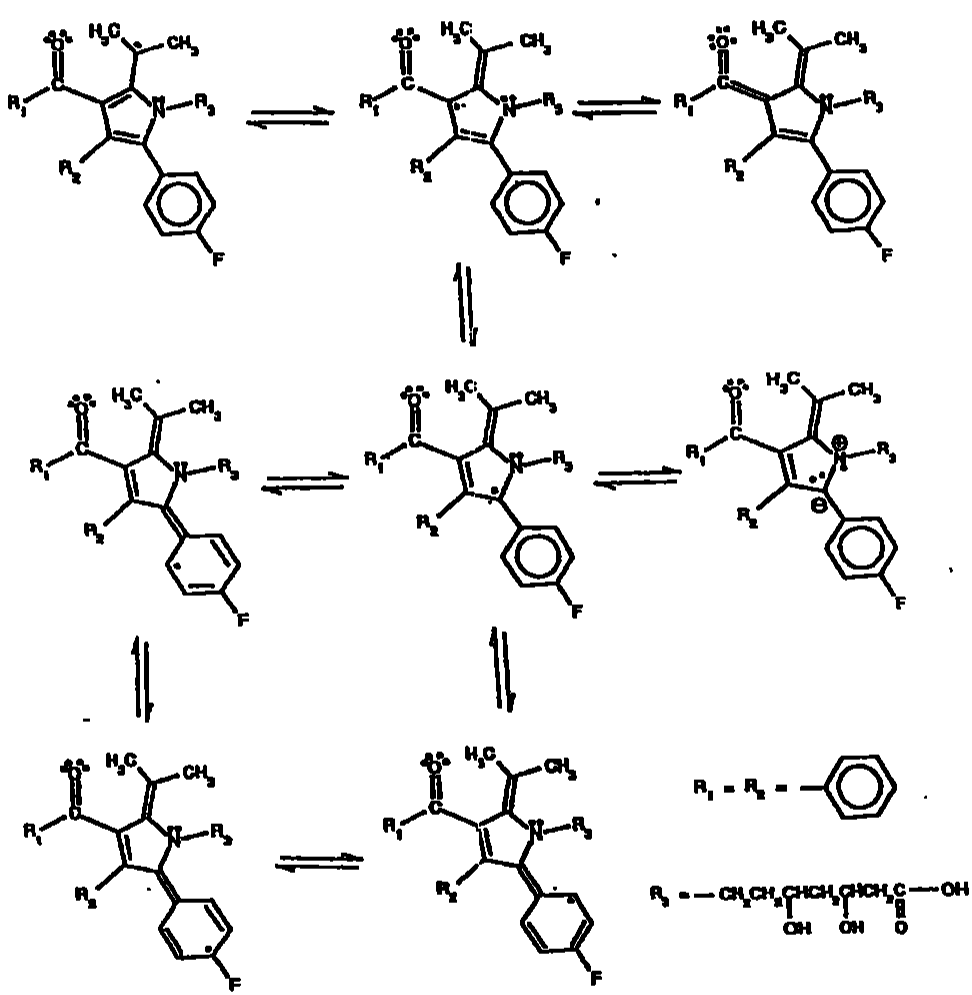


Figure 13C

457
456

262

Antioxidant Mechanisms for Atorvastatin Metabolite: Resonance Stabilization of Free Radical at Site 3

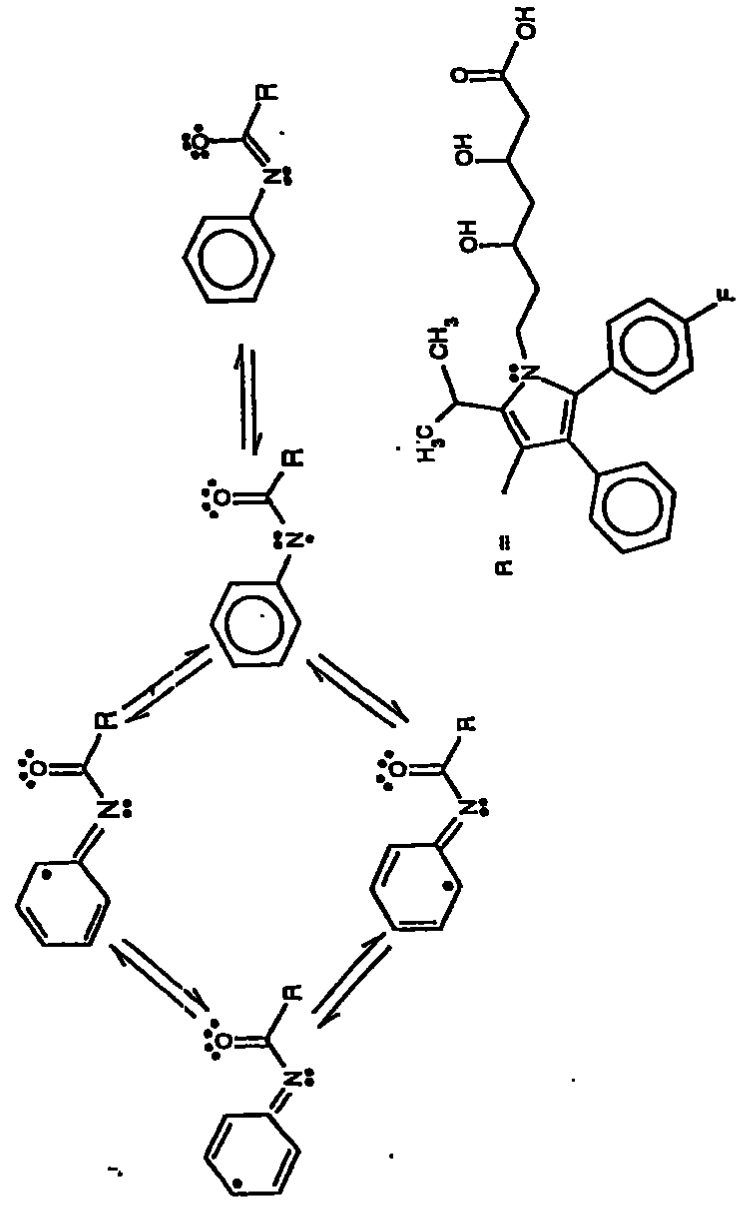


Figure 13D

268
263 4

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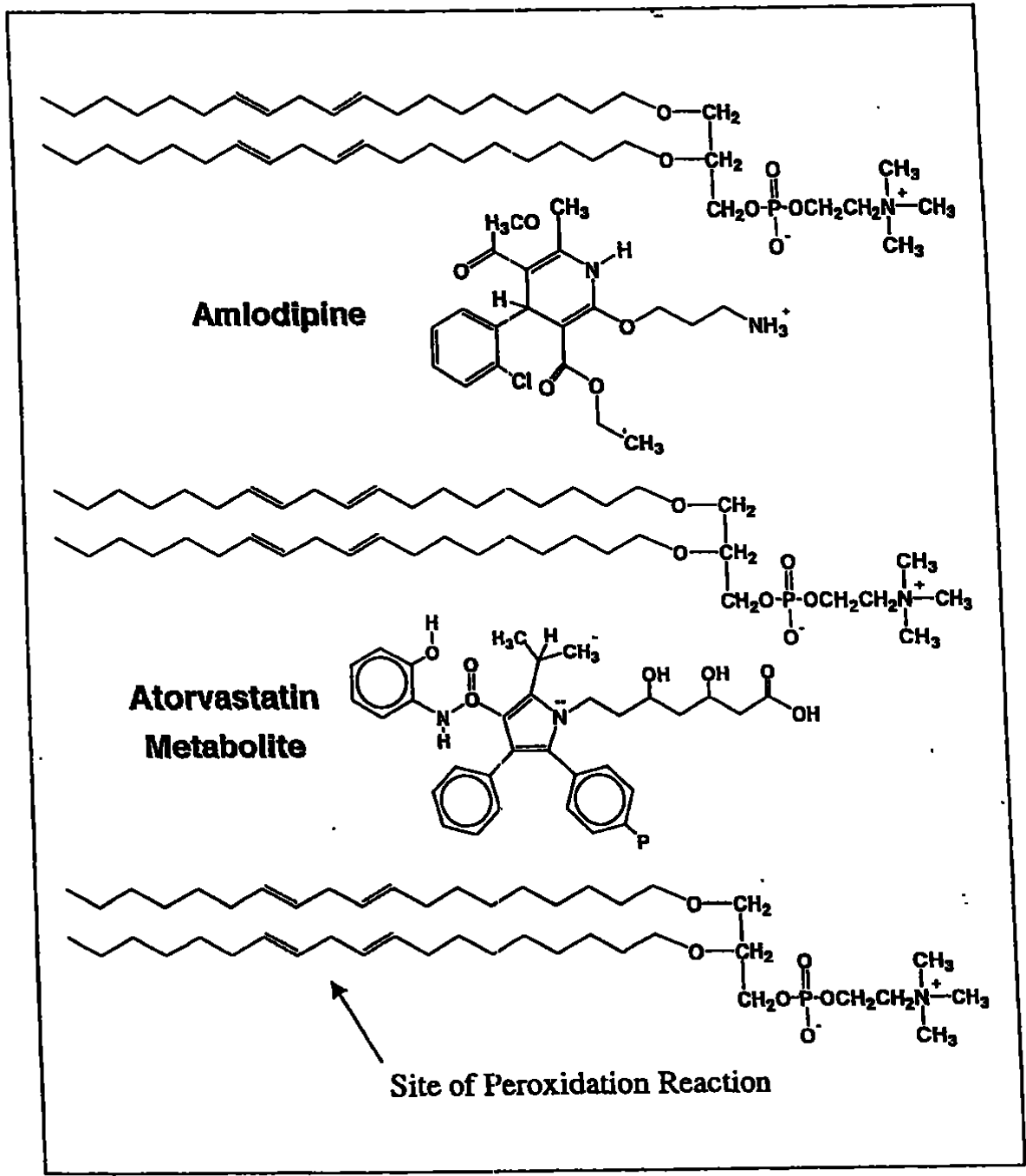


Figure 14

269
264 ~~11~~

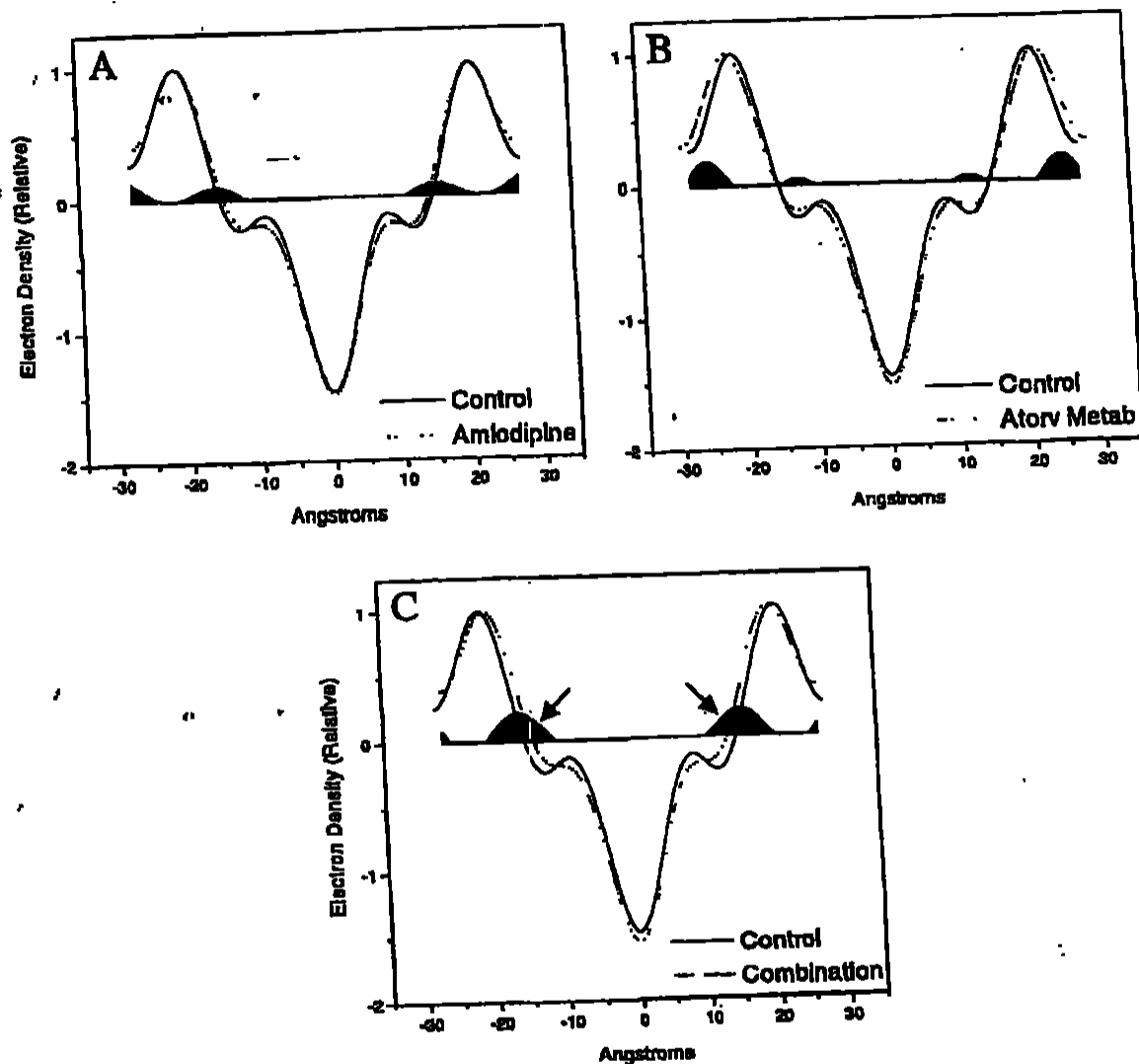


Figure 15

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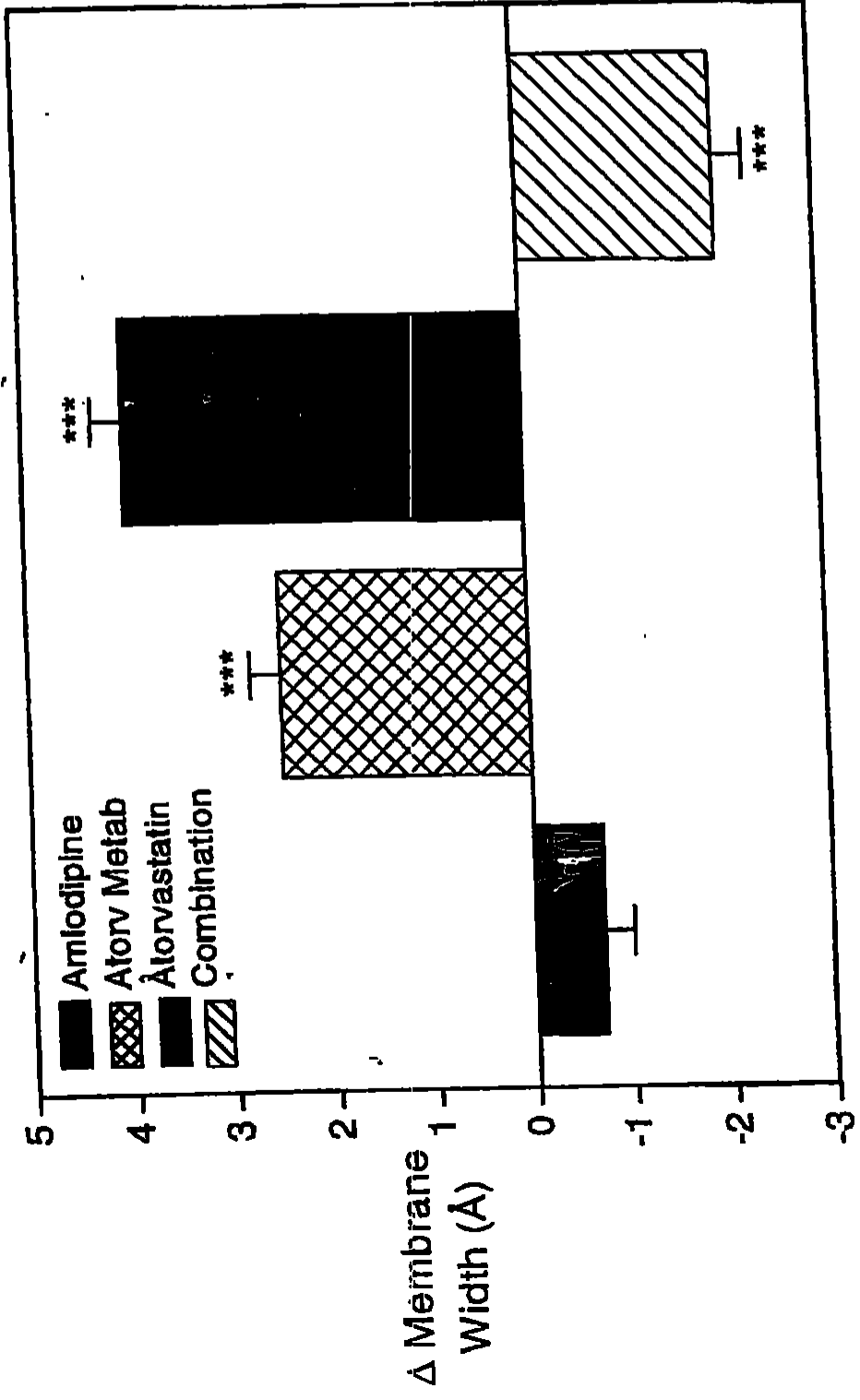


Figure 16

*** $p < 0.001$ versus control

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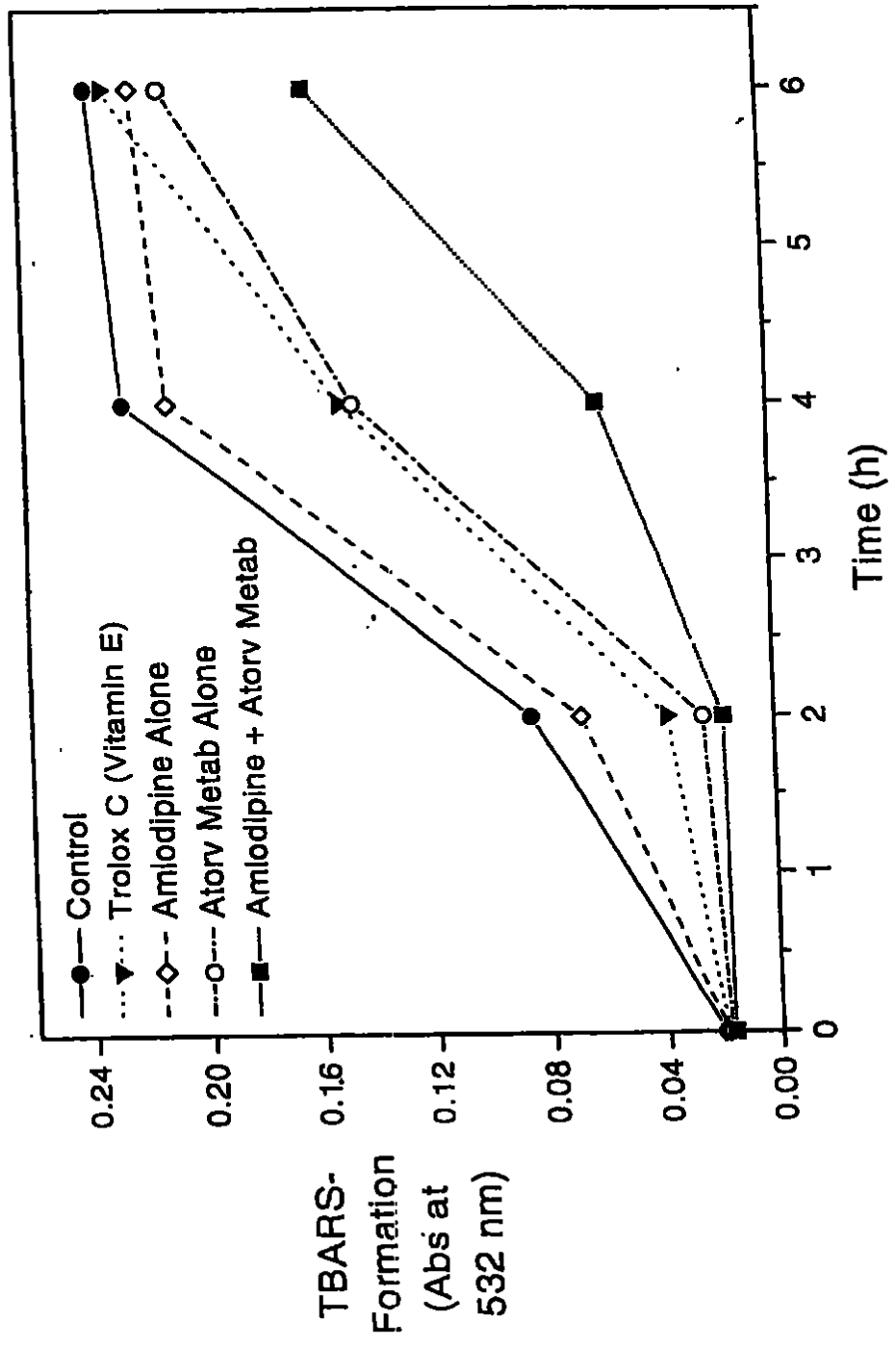


Figure 17

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267 ~~27~~

Figure 18

Computed Enthalpies of Formation for Atorvastatin Metabolite
and its Radicals

Molecule	ΔH_f (kcal/mol)
Atorvastatin Metabolite	-226.10
Carbon radical	-209.50
Oxygen radical	-199.89
Nitrogen radical	-192.55
Methyl radical	-193.51
Phenyl radical	-176.05
Carbon/Oxygen Diradical	-182.07
Carbon/Nitrogen Diradical	-174.25

*The smaller (i.e., more negative) the ΔH_f value, the easier to abstract the H atom from that site.

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268 7

Figure 19

Computed Enthalpies of Formation for Amlodipine
and its Radicals

Molecule	ΔH_f (kcal/mol)
Amlodipine	-171.61
Carbon radical	-164.28
Nitrogen radical	-148.27

*The smaller (i.e., more negative) the ΔH_f value, the easier to abstract the H atom from that site.

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Oficina de Patentes y Marcas de los Estados Unidos

20 de junio de 2000

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NUMERO DE SOLICITUD: 60/151.121

FECHA DE RADICACION: 27 de agosto de 1999

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Certificación

Resumen del Invento

Se describen análisis científicos que soportan el uso combinado de besilato de amlodipina (Norvasc®) y atorvastatina cálcica (Lipitor®) en un sistema de liberación única para el tratamiento de enfermedades cardiovasculares. Específicamente, las actividades antioxidantes sinérgicas del bloqueador de canales de calcio (CCB), besilato de amlodipina, y el inhibidor de la HMG-CoA reductasa, atorvastatina cálcica, incluyendo su metabolito hidroxilado, fueron evaluados en un sistema lipídico bien definido en donde las vesículas lípidas son enriquecidas con ácidos grasos poliinsaturados (PUFA), blanco principal del daño oxi-radical en la aterosclerosis. Los efectos sinérgicos de estos agentes fueron demostrados en membranas preparadas en ausencia y presencia de niveles de colesterol elevados que reproducen condiciones del tipo esclerótico. La combinación de la amlodipina (AML) con el metabolito hidroxilado de la atorvastatina (ATM) efectuó una reducción dramática y sostenida del daño oxi-radical lipídico en concentraciones tan bajas como 10.0 nM. Estos experimentos fueron llevados a cabo en membranas con colesterol que reproducen condiciones del tipo fisiológico. La actividad antioxidante catalítica asociada con la combinación de estos fármacos en niveles terapéuticos fue latamente sinérgico y no pudo ser

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efectivamente reproducido por la vitamina E o el carvedilol, un beta bloqueador no selectivo con efectos antioxidantes establecidos. La inhibición sinérgica de la peroxidación lipídica también fue observada con la combinación de la amlodipina y el compuesto precursor de la atorvastatina (AT). La actividad antioxidante no fue demostrada para otros bloqueadores de los canales de calcio (CCBs) que fueron probados (felodipina, verapamil, diltiazem) o los inhibidores de la HMG-CoA reductasa (mevastatina, pravastatina, simvastatina). La propiedad antioxidante diferente, descrita para esta combinación de fármacos, se puede atribuir a las interacciones catalíticas y fisicoquímicas con la bicapa de la membrana que son independientes de los efectos bien caracterizados de estos fármacos sobre el transporte del calcio y el metabolismo del colesterol. Este beneficio antioxidante sinérgico constituye un nuevo mecanismo farmacológico de acción para estos compuestos y una razón fundamental precisa para el uso combinado de los ingredientes activos en Norvasc® y Lipitor® en el tratamiento de enfermedades cardiovasculares, al reducir los niveles de LDL (lipoproteínas de baja densidad) en plasma y mejorar la protección contra la oxidación. Esta nueva propiedad complementa los efectos establecidos de estos fármacos con respecto a la hipertensión y la dislipidemia.

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Descripción Breve de los Dibujos

La Figura 1 muestra la actividad antioxidante dosis-dependiente de la AML.

La Figura 2 muestra los efectos antioxidantes comparativos de la AML y los dos otros bloqueadores de los canales de calcio y un inhibidor del ACE.

La Figura 3 muestra la actividad antioxidante dosis-dependiente del ATM.

La Figura 4 muestra los efectos antioxidantes comparativos de los inhibidores de la HMG-CoA reductasa.

La Figura 5 muestra el efecto del enriquecimiento con colesterol en la formación de peróxido lipídico.

La Figura 6 muestra el efecto del enriquecimiento con colesterol en las actividades antioxidantes del ATM.

La Figura 7 muestra el efecto antioxidante dosis-dependiente de la combinación de la AML y el ATM.

La Figura 8 muestra los efectos antioxidantes sinérgicos de la AML con ATM a 100.0 nM y de AML con AT a 1.0 μ M en vesículas enriquecidas con colesterol.

La Figura 9 muestra los efectos antioxidantes comparativos de AML/ATM y AML/AT a 0.1 y 1.0 μ M en vesículas enriquecidas con colesterol.

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La Figura 10 muestra los efectos antioxidantes comparativos de la vitamina E, carvedilol, probucol, y la combinación de AML/ATM después de varios periodos de incubación a una concentración de 10.0 μ M.

La Figura 11 muestra los mecanismos antioxidantes químicos para la AML.

La Figura 12 muestra los mecanismos antioxidantes químicos para el ATM.

La Figura 13 muestra las interacciones moleculares de la membrana de AML y ATM.

La Figura 14 muestra la inhibición de la oxidación de lipoproteínas de baja densidad humanas mediante la combinación de AML y ATM.

La Figura 15 muestra las entalpías de formación computadas para el ATM y sus radicales.

La Figura 16 muestra las entalpías de formación computadas para la ALM y sus radicales.

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APPLICATION NUMBER: 60/151,121

FILING DATE: August 27, 1999



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INVENTOR(S)					
Given Name (first and middle (if any))	Family Name or Surname	Residence (City and either State or Foreign Country)			
R. Preston	Mason	Sewickley, PA			
☐ Additional inventors are being named on the _____ separately numbered sheets attached hereto					
TITLE OF THE INVENTION (280 characters max)					
SYNERGISTIC ACTIVITY OF AMLODIPINE BESYLATE (NORVASEC) AND ATORVASTATIN CALCIUM (LIPITOR) ON LIPID PEROXIDATION					
Direct all correspondence to: CORRESPONDENCE ADDRESS					
☐ Customer Number		IDON001368		Place Customer Number Bar Code Label here	
OR Type Customer Number here:					
☒ Firm or Individual Name		Jerry Cohen, Esq.			
Address		Perkins, Smith & Cohen			
Address		One Beacon Street			
City		Boston	State	MA	ZIP 02108
Country		U.S.	Telephone	617 854 4000	Fax 617-854-4040
ENCLOSED APPLICATION PARTS (check all that apply)					
☒ Specification Number of Pages		22	☐ Small Entity Statement		
☒ Drawing(s) Number of Sheets		22	☐ Other (specify) _____		
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (check one)					
☒ A check or money order is enclosed to cover the filing fees				FILING FEE AMOUNT (\$)	
☒ The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number:		03-2410			
The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.					
☒ No.					
☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____					

Respectfully submitted,

SIGNATURE

Jerry Cohen

TYPED or PRINTED NAME

617 854 4000

TELEPHONE

Date

8/27/99

REGISTRATION NO.

20,522

(if appropriate)

Docket Number:

2198 P03PROV

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This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C., 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C., 20231.

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**SYNERGISTIC ACTIVITY OF AMLODIPINE BESYLATE (NORVASC®) AND
ATORVASTATIN CALCIUM (LIPITOR®) ON LIPID PEROXIDATION**

Background of the Invention

Coronary artery disease (CAD) is a leading cause of mortality worldwide, and is associated with substantial morbidity as well. Typically, the patient with CAD has several concomitant conditions, including hypertension, diabetes, and dyslipidemia, increasing overall risk for poor outcomes and complicating treatment.

Among antihypertensive therapies, the lipophilic calcium channel blocker (CCB) amlodipine besylate (AML) is a very well-tolerated agent with an established record of safety and effectiveness for the treatment of hypertension and angina. A potential therapeutic role for AML in the treatment of patients with CAD was recently shown in the Prospective Randomized Evaluation of the Vascular Effects of Norvasc Trial (PREVENT). This three-year trial evaluated the effects of AML compared to placebo on the development and progression of atherosclerotic lesions in coronary and carotid arteries in patients with documented CAD⁴. The results of PREVENT showed impressive clinical benefits with AML therapy as compared to placebo, including reduced risk of cardiovascular morbidity¹. AML therapy was also associated with a significant slowing of the progression of carotid atherosclerosis according to B-mode ultrasonographic assessments. The clinical benefit seen with AML in CAD has not been previously reported with other CCB analogs, including dihydropyridine-type agents, that have examined this question in similar patients⁵⁻⁷. This observation has led to interest in other anti-atherogenic properties of AML, including antioxidant effects that are independent of calcium channel modulation^{2, 8-10}.

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Hypolipidemic therapy has also been demonstrated to be very useful in reducing morbidity and mortality associated with CAD. Among HMG-CoA reductase inhibitors, atorvastatin calcium (AT) has been shown to be very effective as hypolipidemic therapy ^{11, 12}. In addition, the hydroxylated metabolites of AT have been shown to exhibit antioxidant effects in lipoprotein preparations ¹³.

Thus, a therapeutic goal for the treatment of CAD is the development of drugs that can simultaneously target multiple underlying disease processes that contribute to atherosclerosis, thereby altering the course of the disease. Therefore, CAD therapy may have increased positive outcomes if the use of an antihypertensive agent and HMG-CoA reductase inhibitor was combined in a single delivery system.

Unexpectedly, when AML and AT were combined, they had a synergistic effect in preventing peroxidation in lipid membranes that occurred in addition to their well-established therapeutic effects on both dyslipidemia and hypertension. Therefore, these agents have heretofore unrecognized synergistic antioxidant effects, a property that would enable these agents to increase the resistance of lipoproteins and vascular cell membranes to oxidative modification. Indeed, oxidative modification of lipids associated with LDL and vascular cell membranes is a well-established cause of injury to the endothelium and underlying smooth muscle ^{14, 15}. Lipophilic antioxidants that protect against lipid peroxidation have been shown to reduce lesion development in various models of atherosclerosis (for review, see reference ⁶). Moreover, the benefit associated with hypolipidemic therapy is attributed to both its effect on plasma VLDL, LDL and HDL levels and to a reduction in the potential formation of atherogenic oxidized lipoproteins.

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Summary of the Invention

Scientific analyses that support the combined use of amlodipine besylate (Norvasc®) and atorvastatin calcium (Lipitor®) in a single delivery system for the treatment of cardiovascular disease are described. Specifically, the synergistic antioxidant activities of the calcium channel blocker (CCB), amlodipine besylate, and the HMG-CoA reductase inhibitor, atorvastatin calcium, including its active hydroxylated metabolite, were evaluated in a well-defined lipid system in which lipid vesicles are enriched with polyunsaturated fatty acids (PUFA), the key target for oxy-radical damage in atherosclerosis. The synergistic effects of these agents were demonstrated in membranes prepared in the absence and presence of elevated cholesterol levels that reproduce atherosclerotic-like conditions. The combination of AML with the hydroxylated atorvastatin metabolite (ATM) effected a dramatic and sustained reduction in lipid oxy-radical damage at concentrations as low as 10.0 nM. These experiments were conducted in membranes with cholesterol that reproduce physiologic-like conditions. The catalytic antioxidant activity associated with the combination of these drugs at therapeutic levels was highly synergistic and could not be effectively reproduced by vitamin E or carvedilol, a non-selective β -blocker with established antioxidant effects. Synergistic inhibition of lipid peroxidation was also observed with the combination of AML and the AT parent compound. Antioxidant activity was not demonstrated for other CCBs that were tested (felodipine, verapamil, diltiazem) or HMG-CoA reductase inhibitors (mevastatin, pravastatin, simvastatin). The distinct antioxidant property described for this drug combination can be attributed to catalytic, physico-chemical interactions with the membrane bilayer that are independent of the well-characterized effects of these drugs on calcium transport and cholesterol metabolism. This synergistic antioxidant benefit constitutes a new pharmacologic mechanism of action for these compounds and a compelling rationale for

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the combined use of the active ingredients in Norvasc® and Lipitor® in the treatment of cardiovascular disease by reducing the levels of LDL in plasma and improving protection against its oxidation. This new property complements the established effects of these drugs on hypertension and dyslipidemia.

Brief Description of the Drawings

Figure 1 shows the dose-dependent antioxidant activity of AML.

Figure 2 shows the comparative antioxidant effects of AML and both other calcium channel blockers and an ACE-inhibitor.

Figure 3 shows the dose-dependent antioxidant activity of ATM.

Figure 4 shows the comparative antioxidant effects of HMG-CoA reductase inhibitors.

Figure 5 shows the effect of cholesterol enrichment on lipid peroxide formation.

Figure 6 shows the effect of cholesterol enrichment on the antioxidant activities of ATM.

Figure 7 shows the dose-dependent antioxidant effect of the combination of AML and ATM.

Figure 8 shows the synergistic antioxidant effects of AML with ATM at 100.0 nM and of AML with AT at 1.0 μ M in cholesterol-enriched vesicles.

Figure 9 shows the comparative antioxidant effects of AML/ATM and AML/AT at 0.1 and 1.0 μ M in cholesterol-enriched vesicles.

Figure 10 shows the comparative antioxidant effects of vitamin E, carvedilol, probucol, and the AML/ATM combination after various incubation periods at a 10.0 μ M concentration.

Figure 11 shows the chemical antioxidant mechanisms for AML.

Figure 12 shows the chemical antioxidant mechanisms for ATM.

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Figure 13 shows the molecular membrane interactions of AML and ATM.

Figure 14 shows the inhibition of human LDL oxidation by the combination of AML and ATM.

Figure 15 shows the computed enthalpies of formation for ATM and its radicals.

Figure 16 shows the computed enthalpies of formation for AML and its radicals.

Detailed Description of Embodiments

Comparative Antioxidant Effects of Calcium Channel Blockers and HMG-CoA Reductase

Inhibitors: The separate and combined dose-dependent antioxidant activities of AML, AT and ATM were compared to representative CCBs (felodipine, verapamil, diltiazem) and HMG-CoA reductase inhibitors (mevastatin, pravastatin) in membrane vesicles reconstituted from phospholipids enriched with the polyunsaturated fatty acid, dilinoleoyl phosphatidylcholine (DLPC). Membrane vesicles were used in these experiments for the following reasons: 1) this system is well-defined and highly reproducible; 2) linoleic acid represents the primary target for oxidative damage and is common in vascular cell membranes and lipoprotein particles; 3) this membrane system does not contain calcium channels or HMG CoA reductase enzymes and; 4) lipid peroxidation in this system can be initiated spontaneously at 37°C in the absence of exogenous chemical initiators, such as high levels of iron and ascorbate. In these experiments, oxidation occurred in a gradual, time-dependent manner that was measured spectrophotometrically.

As demonstrated in Figure 1, AML effectively inhibited membrane lipid peroxide formation in a highly reproducible and dose-dependent manner over a broad range of concentrations, beginning at 10.0 nM. Inhibition of lipid peroxide formation by AML in

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membrane vesicles enriched with PUFA was measured as a function of drug concentration at 37°C by spectrophotometric methods. In these experiments, the control level of lipid peroxide formation was 1.2 mM after a 48 h incubation period. The fact that inhibition was observed at sub-micromolar levels indicates that the benefit observed with AML is of therapeutic relevance. Thus, inhibition of oxy-radical damage by AML was highly catalytic: at an AML concentration of 10.0 nM, the mole ratio of lipid peroxide inhibition: drug concentration was greater than 10,000:1.

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The antioxidant effects of AML could not be reproduced by other representative CCBs, including felodipine (a dihydropyridine), verapamil, or diltiazem under identical experimental conditions, even at micromolar levels. Figure 2 shows the antioxidant activity of AML compared to other representative CCBs (felodipine, verapamil, diltiazem) and the ACE-inhibitor, captopril, at a drug concentration of 1.0 µM. Under identical conditions, only AML produced a significant inhibition of lipid peroxidation. Again, the control level of lipid peroxide formation was 1.2 mM. Thus, the distinct effects of AML can be attributed to its high affinity for the membrane lipid bilayer, resulting in biophysical changes in the intermolecular packing of phospholipid molecules.

In a similar fashion, ATM also inhibited lipid peroxide formation in these lipid preparations in a dose-dependent manner (see Figure 3). This activity was also observed with AT at much higher, micromolar levels but not with other statins that were tested in these experiments (pravastatin, mevastatin), as shown in Figure 4. Thus, AML, AT and ATM separately produced a measurable inhibition of lipid peroxidation in vesicles enriched with PUFA, the key substrate for oxy-radical damage.

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Synergistic Antioxidant Effects of Drugs in Cholesterol-Enriched Membrane

Preparations: As cholesterol is a primary constituent of vascular cell plasma membranes that increases in content during atherogenesis ¹⁸, the synergistic antioxidant activity of these agents in membranes composed of cholesterol and phospholipid at a 0.5:1 mole ratio were examined. As shown in Figure 5, the addition of cholesterol to these vesicles resulted in a pronounced, 26% increase in the amount of lipid peroxide formation. This effect of cholesterol is attributed to its ability to increase the membrane bilayer packing constraints, contributing to its pathologic activity in atherosclerosis ¹⁹. Under these conditions, the antioxidant effectiveness of ATM was not significantly reduced (see Figure 6).

Under conditions of cholesterol enrichment, AML worked synergistically with ATM to effect an inhibition of lipid peroxidation that was dramatically more potent than that observed with either agent separately. The combination of AML and ATM was very effective and dose-dependent with antioxidant effects beginning in the low nanomolar range. Significant inhibition was observed at concentrations as low as 10.0 nM and nearly complete inhibition was observed at 10.0 μ M (see Figure 7). In this membrane system, the combination of AML with either ATM or AT produced a marked synergistic effect (see Figure 8). Although a synergistic effect was observed with the combination of AML and AT, it was less prominent than when the AML/ATM combination was used. Specifically, at both concentrations (0.1 and 1.0 μ M), the combination of AML/ATM was more effective as an antioxidant than AML/AT (see Figure 9).

The effect of other known antioxidants, including vitamin E and carvedilol, were also compared to the combination of AML and ATM. Carvedilol is vasodilating β -blocker and antioxidant approved for treatment of congestive heart failure. It has been shown to scavenge oxygen free radicals and inhibit lipid peroxidation in swine heart ²⁰. Even at a concentration of

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10.0 μ M, these compounds were markedly less effective than the combined AML/ATM preparation (see Figure 10). In the case of vitamin E, the activity of this compound was significantly attenuated over time (48 versus 72h), as compared to AML/ATM (Figure 10A). This observation is consistent with a stoichiometric mechanism in which the antioxidant ability of the vitamin E is terminated by the reaction. By contrast, the activity of the AML/ATM combination persisted, providing evidence that these agents work by a separate, catalytic fashion as antioxidants. Only the very effective antioxidant, probucol, had similar activity to the AML/ATM complex (Figure 10C).

To understand the basis for the antioxidant effects of these compounds, the chemical structure of these molecules was evaluated (Figures 11, 12). In Figure 11, the covalent structure of AML includes two hydrogen atoms associated with its dihydropyridine ring that can be donated to unstable free radicals associated with lipids (Figure 11A). Once a hydrogen atom is donated, an unpaired free electron remains associated with the AML molecule. This unpaired electron, a potential source of free radical damage, can be safely stabilized in resonance structures (Figures 11B and 11C).

Similar chemical analyses were conducted for a key hydroxyl metabolite of AT, as indicated in Figure 12. Similar to AML, the ATM molecule has three potential hydrogen atoms that can be donated to unstable free radical molecules (A). Resonance stabilization structures for the remaining unpaired free electrons at sites 1 (Figure 12B), 2 (Figure 12C) and 3 (Figure 12D) are calculated.

In addition to its chemical properties, AML also has high affinity for membrane lipids under atherosclerotic conditions, as compared to other CCBs ²¹. The distinct lipophilicity of AML is attributed to its amphiphilic chemical structure that directs the molecule to a favorable

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location in the membrane where it can then interfere with the propagation of free radicals by both biophysical and biochemical mechanisms^{2, 8, 16, 21}. In the same way, the chemical structure of ATM consistent with an amphiphile that could be expected to interact strongly with the membrane bilayer, as illustrated in Figure 13 and supported by x-ray diffraction analyses.

The antioxidant activity of AML may contribute to its benefit in patients with documented CAD, as recently reported in the Prospective Randomized Evaluation of the Vascular Effects of Norvasc Trial (PREVENT)⁴. Patients randomized to AML experienced a significant reduction in angina (-33%, $p<0.01$), major vascular procedures (-45%, $p<0.001$), and the composite of any major event (-30%, $p>0.01$), as compared to placebo, independent of changes in lipid levels¹. The benefits of AML in CAD have not been observed with other CCBs, including dihydropyridine agents⁵⁻⁷, suggesting that the additional antioxidant properties of AML contribute to direct antiatherosclerotic effects².

Synergistic Antioxidant Effects of Amlodipine with the Hydroxylated Atorvastatin Metabolite in Human LDL: The separate and combined antioxidant effects of AML and the ATM were evaluated in human LDL preparations. The ability of these agents to inhibit LDL peroxidation was assessed in vitro following addition of copper (10 μM) in order to stimulate oxidative damage. The rate of LDL oxidation was consistent with sigmoidal curve kinetics, as described by Esterbauer et al., characterized by an initial lag phase followed by a sharp propagation and final plateau phase (Figure 14)²².

After a 4 h incubation period, the differential effects of these compounds could be clearly observed at a concentration of 3.0 μM . Separately, AML and the ATM inhibited LDL oxidation by 7 and 33%, respectively. When combined, however, these agents produced 73% inhibition. This unexpected *synergistic* effect persisted at the 6 h time point when the agents separately did

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not inhibit peroxidation by more than 11% while the combination reduced TBARS-formation by 32%.

Oxidative modification of lipids associated with low-density lipoproteins (LDL) contributes significantly to inflammatory process associated with atherosclerosis, including foam cell formation, endothelial dysfunction and toxicity, leukocyte and platelet adhesion. The results of these experiments demonstrated a strong and unexpected synergistic antioxidant effect for the combination of AML and the ATM in human LDL, consistent with what was observed in membrane vesicles. The drug combination produced a level of oxy-radical inhibition that substantially exceeded any additive effect. The antioxidant activity of vitamin E was similar to that observed for the ATM when used alone, but was substantially less than what was observed for the drug combination.

LDL particles that have greater resistance to oxidative damage, due to the presence of lipophilic antioxidants, exhibit reduced cytotoxicity, interfere less with endothelium-derived nitric oxide production, and do not contribute to foam cell formation²³. Supplementation with vitamin E has been shown to increase LDL resistance to oxidative modification and reduce endothelial cell cytotoxicity²⁴. In addition, the lipophilic antioxidant probucol has been demonstrated to attenuate the formation of atherosclerotic plaques in cholesterol-fed primates, an effect that correlated with an increased resistance of LDL to oxidative damage²⁵.

The synergistic antioxidant activity observed for this drug combination in LDL and reconstituted lipid membranes suggests that these compounds interact directly with each other to scavenge lipid radicals. It is proposed that the ATM reacts more quickly with lipid radicals (equation 1) than AML, as described in equation 2. If these are the only two pathways available for these the drugs when added together to the system, then their combined effect would only be

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additive. However, the combination of both compounds provides the possibility for a third pathway (equation 3) that is supported by the results of the peroxidation experiments in both LDL and lipid membranes. The combination of pathways 1 and 3 would produce a synergistic effect if it occurs more rapidly than pathway 2. Indeed, semi-empirical calculations suggest that reaction 3 is a favorable, exothermic process with an enthalpy of -40.7 kJ/mol. Thus, the presence of the fast inhibitor (ATM) enables the slower inhibitor (AML) to remove free radicals faster than if reacting with the lipid radicals on its own. The three pathways describing these interactions are as follows, in which "LOO•" represents a lipid radical molecule:

- (1) $\text{LOO}\bullet + \text{ATM} \rightarrow \text{LOOH} + \text{ATM}\bullet$ *FAST*
- (2) $\text{LOO}\bullet + \text{AML} \rightarrow \text{LOOH} + \text{AML}\bullet$ *SLOW*
- (3) $\text{ATM}\bullet + \text{AML} \rightarrow \text{ATM} + \text{AML}\bullet$ *FAST*

As a result of this synergy between AML and the ATM, the recycled metabolite is now made available for additional scavenging of lipid radicals. Overall, differences in the rates of inhibition between AML and the ATM are based, in part, on the calculated enthalpies for these compounds and their respective radicals. As shown in Figures 15 and 16, the calculated enthalpies for the ATM are more favorable than for AML. The more negative the ΔH_f value reported in the Table, the easier to abstract the hydrogen atom from that particular site.

Role of Antioxidants in Reducing Coronary Artery Disease: The results of in vivo investigations have indicated an important role for lipophilic antioxidants in reducing cardiovascular morbidity and mortality, especially coronary artery disease. The rationale for this observation is attributed to the ability of these compounds to interfere with the peroxidation of PUFA associated with LDL and vascular cell membranes. In particular, it has been demonstrated that LDL particles with greater resistance to oxidative damage, due to the presence

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of lipophilic antioxidants, have reduced cytotoxicity, interfere less with endothelium-derived nitric oxide production, and do not contribute to foam cell formation ¹⁵. Supplementation with vitamin E has been shown to increase LDL resistance to oxidative modification, and thus, reduce cytotoxicity toward endothelial cells ²⁶. The lipophilic antioxidant probucol has also been demonstrated to attenuate the formation of atherosclerotic plaques in cholesterol-fed primates, an effect that correlated with an increased resistance of LDL to oxidative damage and not changes in lipid levels ²⁷. In addition, probucol inhibited atheroma formation of lesions in Watanabe hereditary hyperlipidemic (WHHL) rabbits, a well-characterized animal model of atherosclerosis, independent of cholesterol-lowering effects ²⁸. Consistent with these animal studies, a double-blind, randomized clinical investigation has shown that probucol reduced restenosis by 47% in patients with CAD following coronary-artery balloon angioplasty, presumably due to its antioxidant effects ²⁹.

Another pathological process associated with oxidized LDL is an inhibition of the constitutive release of endothelium-derived nitric oxide in the arterial wall ³⁰⁻³². Release of nitric oxide inhibits the abnormal adhesion of leukocytes and platelets to the endothelial surface early during the development of foam cell formation. Nitric oxide is also an important mediator of vasodilation; loss of nitric oxide can lead to vasospasm associated with acute coronary syndromes ³³. The interference of this process by oxidized LDL is attributed to a disruption in secondary messenger systems, including G-protein-dependent nitric oxide release ^{31, 32}. Patients treated with the antioxidant probucol had improved endothelial function, as assessed by nitric oxide production, when compared to control subjects. LDL isolated from these patients showed greater resistance to oxidation in vitro and did not interfere with normal nitric oxide

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production from the endothelium, as compared to control LDL samples³⁴. An important benefit associated with lipophilic antioxidants is the ability to inhibit endothelial dysfunction associated with oxidized LDL, a key early event in atherosclerosis¹⁴.

Thus, a comprehensive review of the available data provides a compelling mechanistic rationale for the use of lipophilic antioxidants to prevent cellular changes associated with coronary artery disease. By increasing the resistance of LDL to oxidative damage, agents with antioxidant activity may effectively interfere with pathologic alterations in the vessel wall during atherogenesis, including foam cell formation, endothelial dysfunction and toxicity, leukocyte and platelet adhesion, and arterial vasospasm, secondary to a loss of normal nitric oxide production. These cellular observations also provide important insights into the results of recent clinical studies demonstrating a benefit associated with antioxidant use by reducing the expression of established coronary artery disease despite an absence of pronounced changes in atherosclerotic lesion development³⁵. Indeed, the results of epidemiological analyses have provided direct evidence for an inverse association between the serum levels of antioxidants, especially vitamin E, and adverse outcomes associated with coronary disease³⁶⁻⁴².

This invention demonstrates that the combination of AML and atorvastatin produced a powerful, synergistic antioxidant effect that could not be effectively reproduced by well-known antioxidants such as α -tocopherol or carvedilol. While previous studies have shown that these agents separately have antioxidant benefit in both lipoprotein and cell membrane preparations^{2, 3, 13, 43}, these findings unexpectedly show that the combination of these agents provide a much greater, synergistic effect, especially under atherosclerotic-like condition in which the separate effects of the compounds are especially reduced.

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Experimental Methods

Dilinoleoyl phosphatidylcholine (DLPC) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL) and stored at -80°C. Amlodipine besylate (AML) was a gift from Pfizer Central Research (Groton, CT) while atorvastatin calcium (AT) and its hydroxylated metabolite (ATM) were provided by Parke Davis (Ann Arbor, MI). Verapamil, captopril and diltiazem were purchased from RBI Biochemicals (Natick, MA). Mevastatin and vitamin E were purchased from Sigma Chemical Co. (St. Louis, MO).

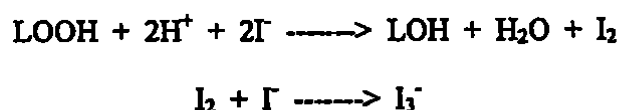
Lipid peroxidation analysis: The separate and combined dose-dependent antioxidant activities of these agents were examined in membranes enriched with PUFA prepared in the absence and presence of cholesterol at a 0.5 sterol to phospholipid mole ratio². The biological lipid (dilinoleoyl phosphatidylcholine) used for these samples was dissolved in HPLC-grade chloroform (25.0 mg/ml). An aliquot of the lipids (1.0 mg) was added to individual glass 13 x 100-mm test tubes and the chloroform was removed under a steady stream of N₂ gas while vortexing. The lipids were shell dried in the absence or presence of drug dissolved in ethanol. Residual solvent was removed under vacuum while the samples were shielded from light. Membrane vesicles were produced by vortexing the dried lipids at room temperature with 1.0 ml HEPES buffered saline (0.5 mM HEPES and 154.0 mM NaCl, pH, 7.2). The final phospholipid concentration was 1.0 mg/ml buffer and the concentrations of drug ranged from 10.0 nM through 10.0 µM.

In the lipid peroxidation experiments, aliquots of membrane vesicles (1.0 mg) were added to separate test tubes. Membrane peroxidation was carried out at 37°C in a shaking water bath. At various time intervals (24, 48, 65, 72h), a small aliquot of lipid sample was removed before

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25 μ l of 5.0 mM ethylenediaminetetracetic acid (EDTA) and 20 μ l of 35.0 mM butylated hydroxytoluene (BHT) was immediately added to the sample to stop the peroxidation reaction.

The extent of membrane lipid peroxidation was measured by the CHOD-Iodide assay as previously described in detail ¹⁷. The quantity of I_3^- was measured from the following reaction in which L represents a phospholipid molecule:



An aliquot of the membrane sample was removed at various time points and then added to 1.0 ml of CHOD color reagent (E.M. Science, Gibbstown, NJ) that includes 20.0 μ M BHT, 24.0 μ M EDTA, and 0.2% Triton-X. The sample was then covered with foil and allowed to incubate for 2 h in the absence of light before measuring the absorbance of the sample at 365 nm ($\epsilon = 2.4 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$). The background sample was run along the test samples in triplicate and contains 76.7 μ l of 0.652 mM HEPES, 20 μ l of 5.0 mM EDTA and 3.3 μ l of DDI water. The extent of lipid peroxidation was measured in triplicate for each drug concentration and compared to control samples that did not contain drug. The statistical significance of these experiments was assessed by the non-paired t-test. Significance was accepted at $p < 0.05$.

In LDL oxidation determination experiments, the separate and combined antioxidant activities of AML and the active ATM were tested in human low-density lipoproteins (LDL), an important target for oxy-radical damage in atherosclerosis. Human LDL preserved with EDTA was obtained from Sigma Chemical Co. (St. Louis, MO). The EDTA content of the LDL samples was removed by gel filtration with PD-10 Sephadex G25-M filtration columns; PBS (nitrogen purged) was used as the eluent. The LDL samples (50 μ g of protein/mL) were preincubated with or without the drugs (3.0 or 6.0 μ M) for 30 min at 37°C. Oxidation of LDL

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was induced by the addition of 10 μ M of CuSO₄ using the colorimetric TBARS method ⁴⁴. The time course of LDL oxidation, measured by TBARS formation, was followed for 6 h at 37°C following addition of copper. LDL oxidation, as determined by the TBARS methods, followed sigmoidal curve kinetics, as described by Esterbauer et al. with an initial lag phase followed by a sharp propagation and final plateau phase ²². The protein content of the LDL was determined using the Pierce Coomassie Protein Plus assay kit (Pierce Chemical) ⁴⁵.

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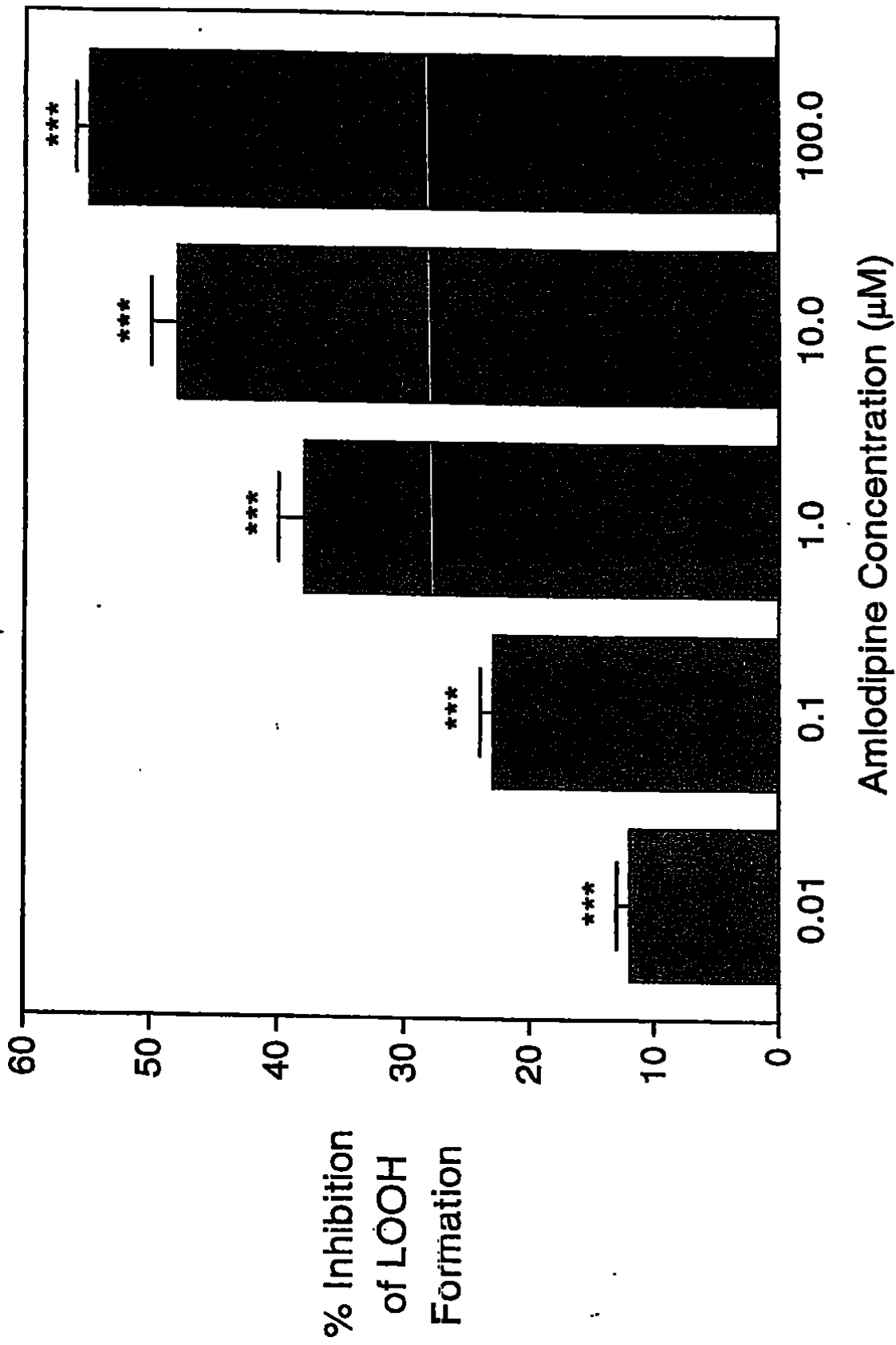
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Abstract

The combination of amlodipine besylate and atorvastatin calcium shows a synergistic antioxidant effect on lipid peroxidation in membrane vesicles enriched with polyunsaturated fatty acids, the key target for free radicals. Inhibition of oxy-radical damage by this drug combination was observed at therapeutic levels and could not be reproduced with other representative CCBs, statins, or even the highly lipophilic antioxidants, vitamin E and carvedilol. This combination therapy can be used to treat cardiovascular disorders, especially coronary artery disease, by reducing the levels of LDL in plasma and improving protection against its oxidation.

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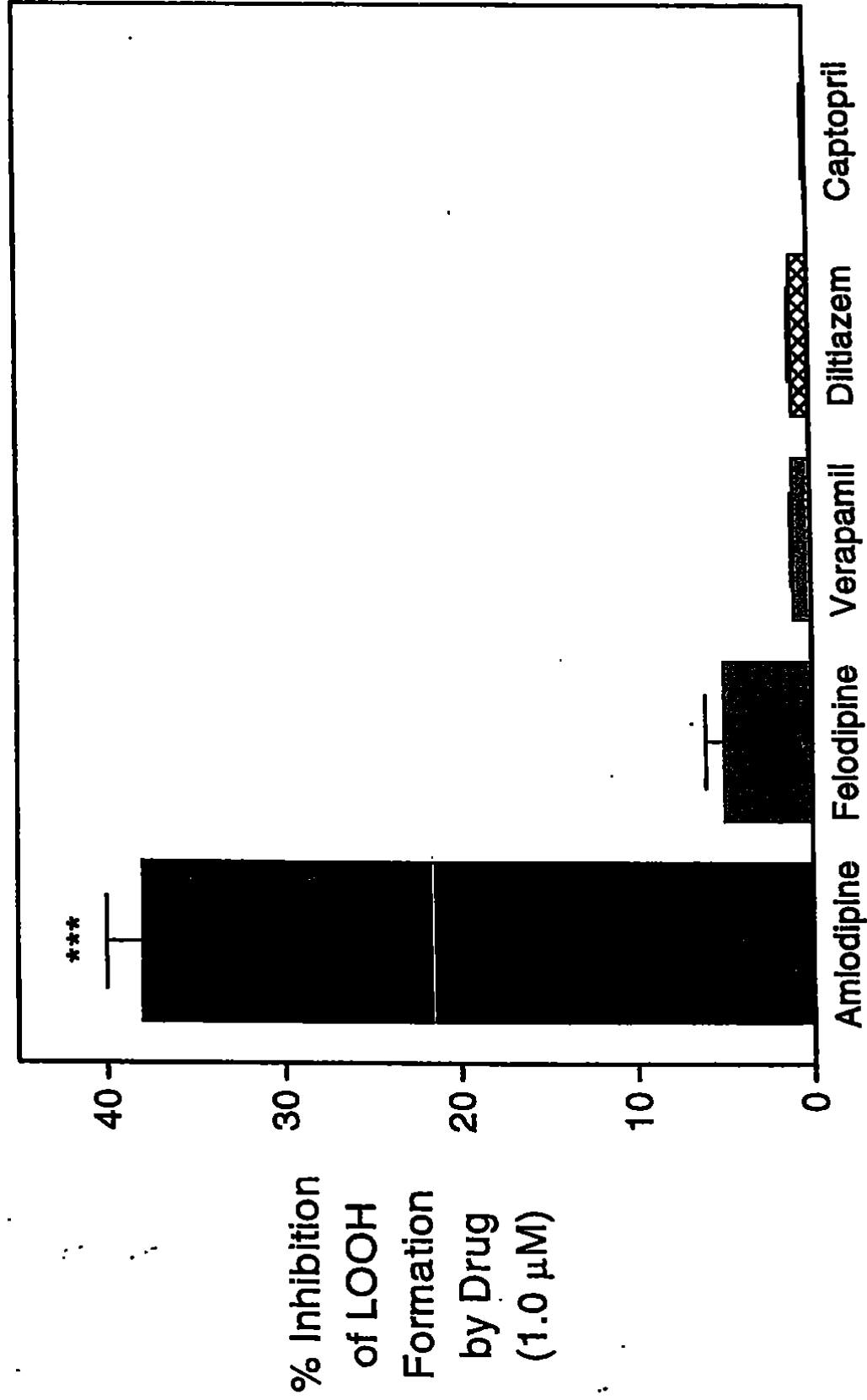


*** $p < 0.001$ versus control
 Values are mean $\pm$ S.D. (n = 3)

Figure 1

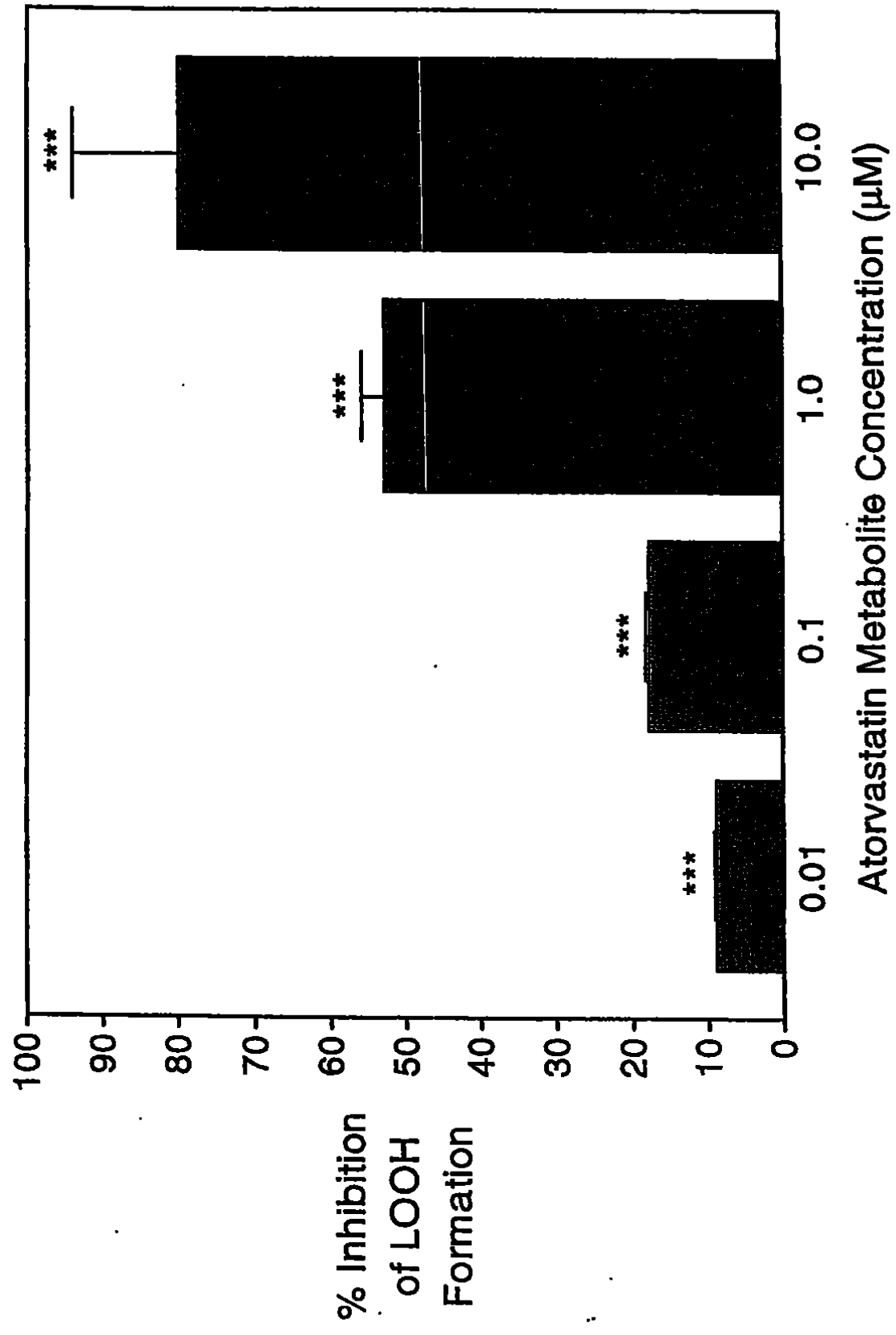
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*** $p < 0.001$ versus control and other drugs
Values are mean $\pm$ S.D. (n = 3)

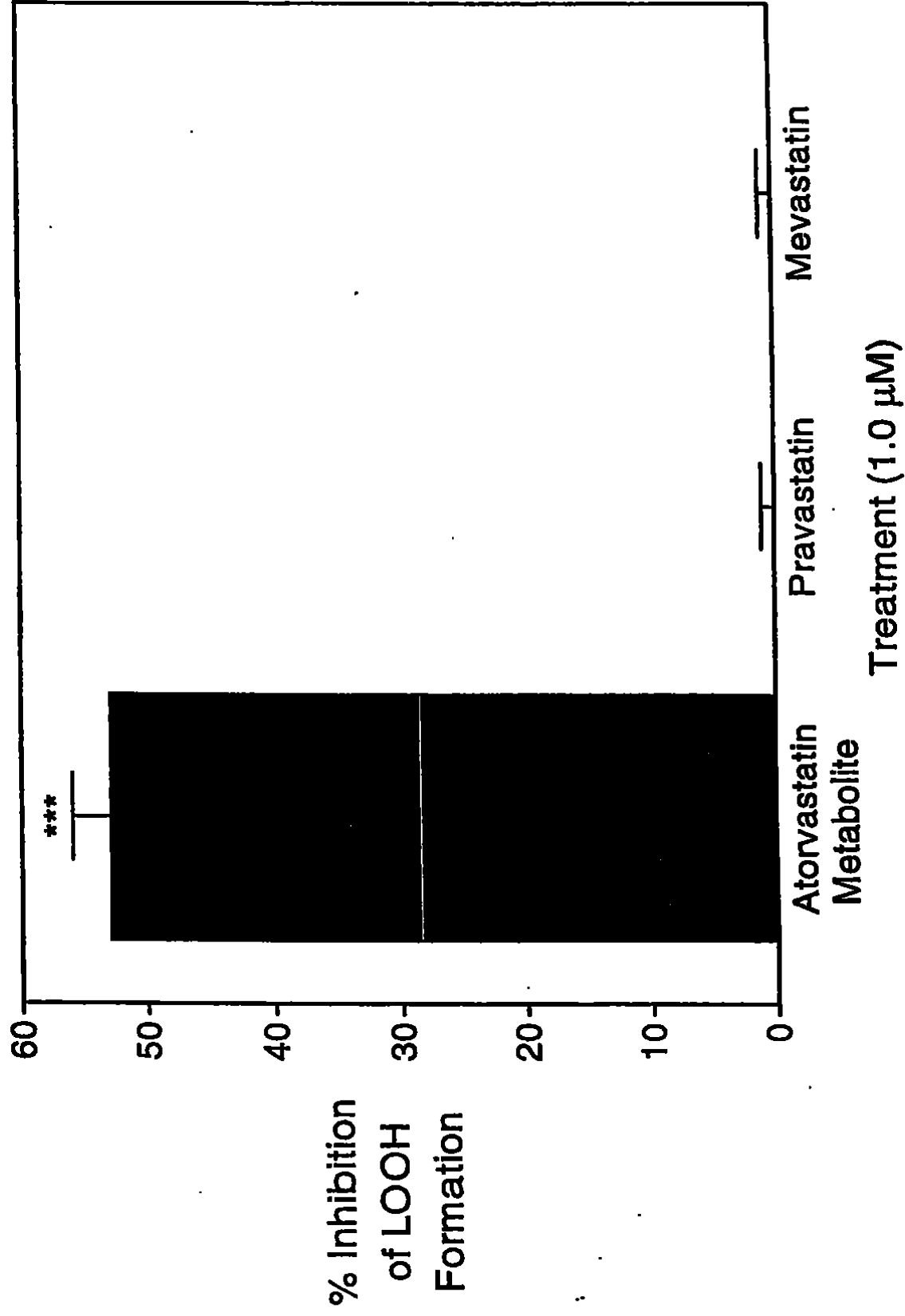
Figure 2



*** $p < 0.001$ versus control
 Values are mean $\pm$ S.D. (n = 3)

Figure 3

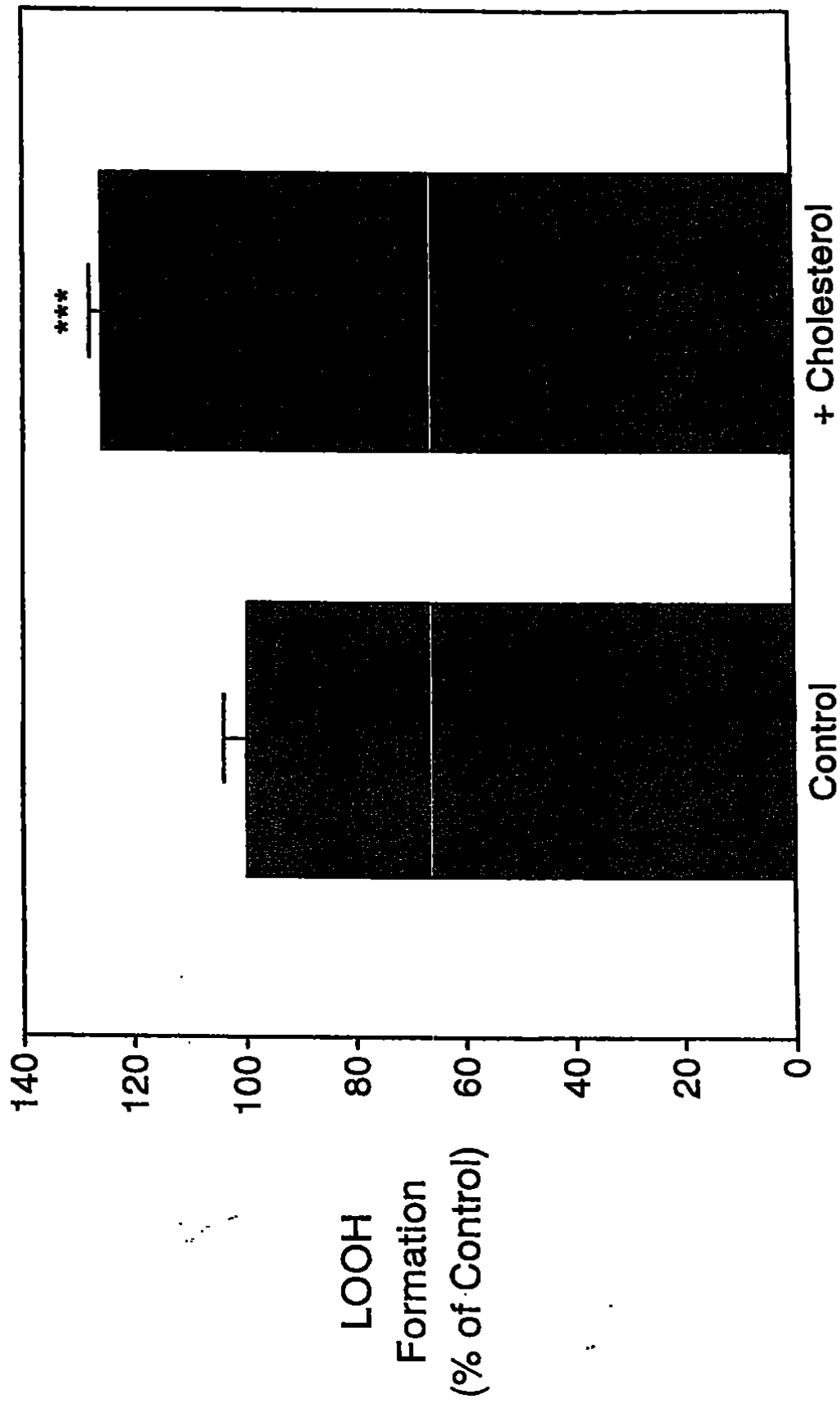
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*** $p < 0.001$ versus control
Values are mean $\pm$ S.D. ($n = 3-6$)

Figure 4

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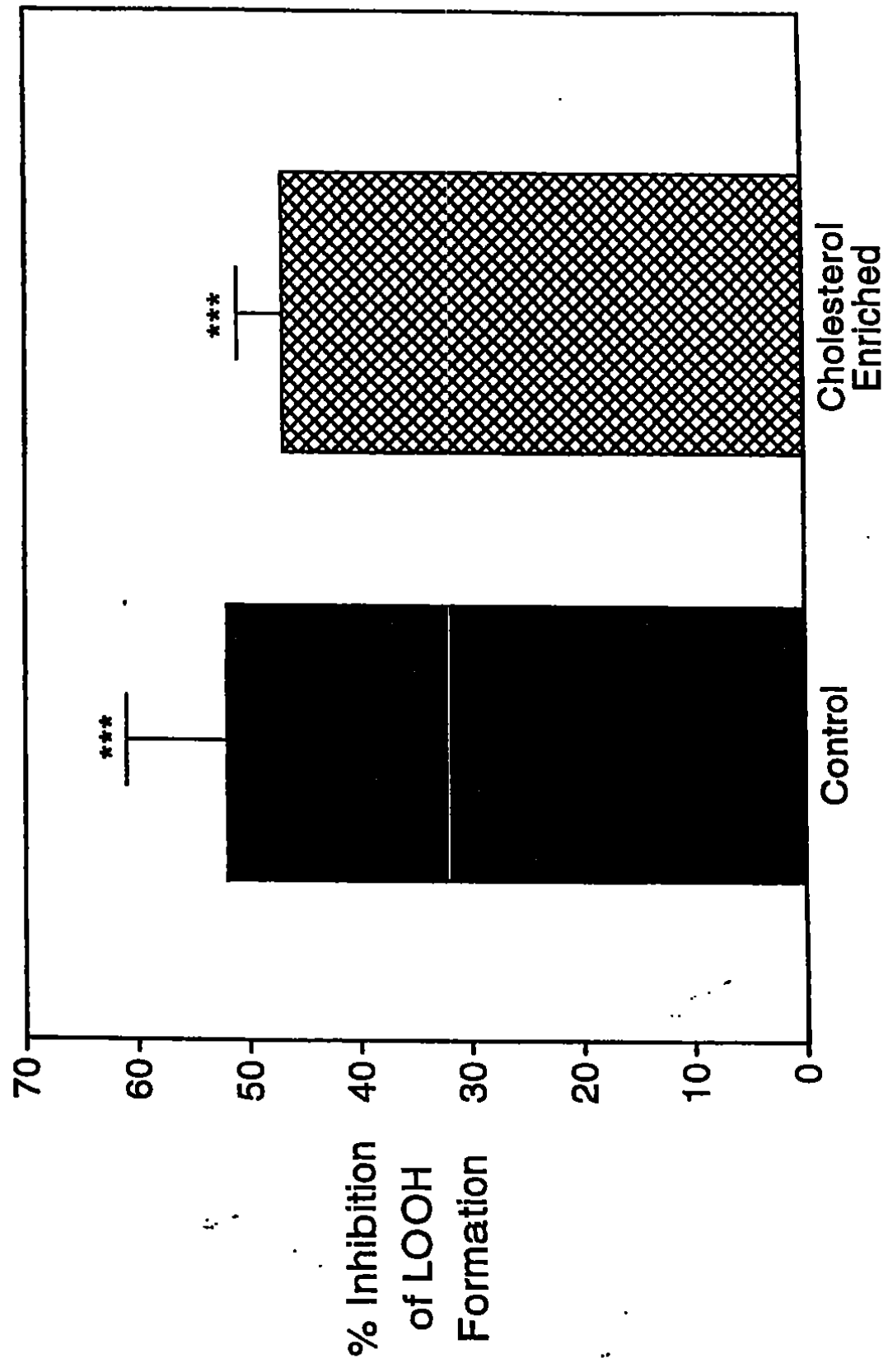
*** $p < 0.001$ versus control
Values are mean $\pm$ S.D. (n = 4)

Figure 5

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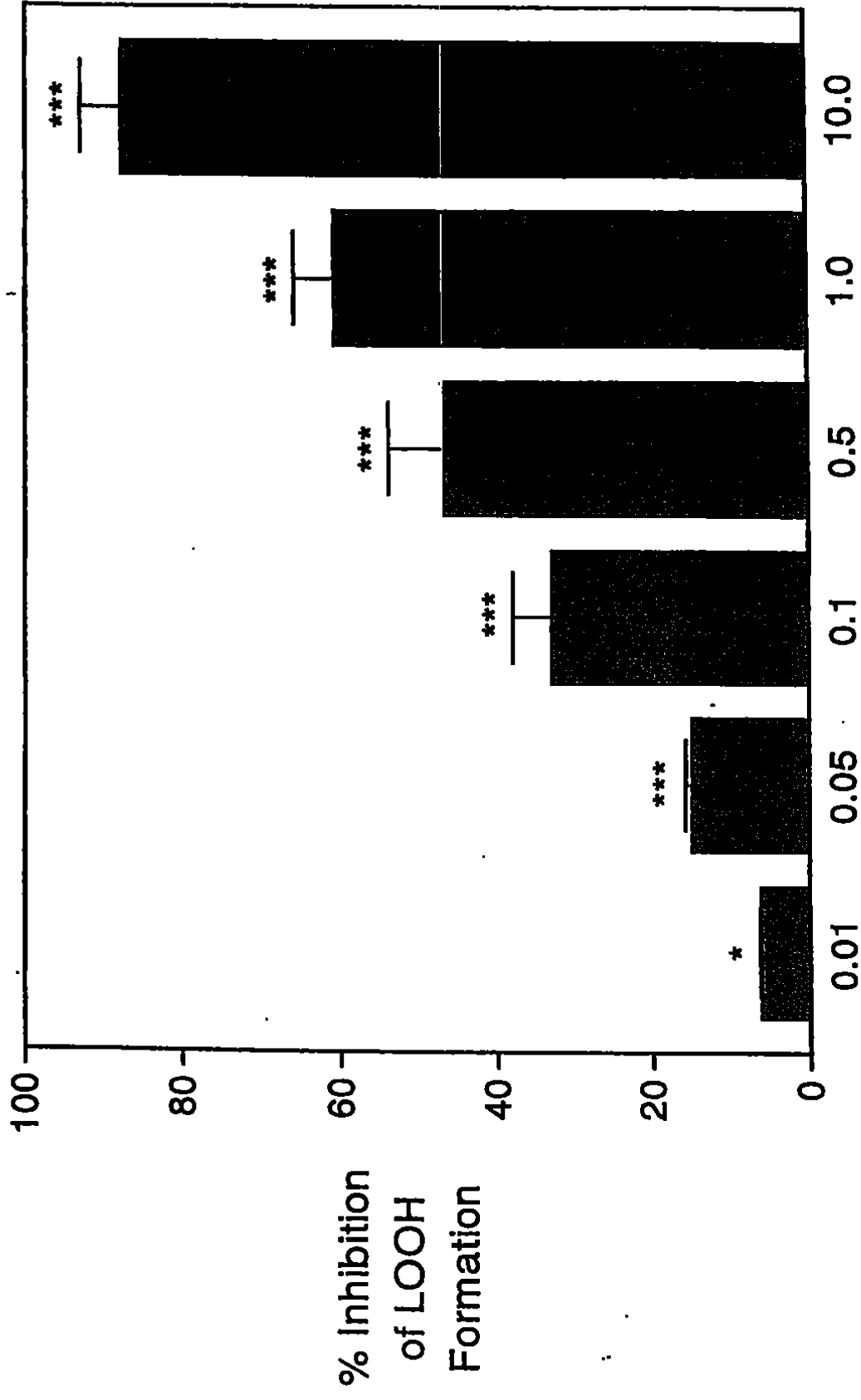


Atorvastatin Metabolite Treatment (1.0 μ M)

*** $p < 0.001$ versus no treatment
Values are mean $\pm$ S.D. (n = 4)

Figure 6

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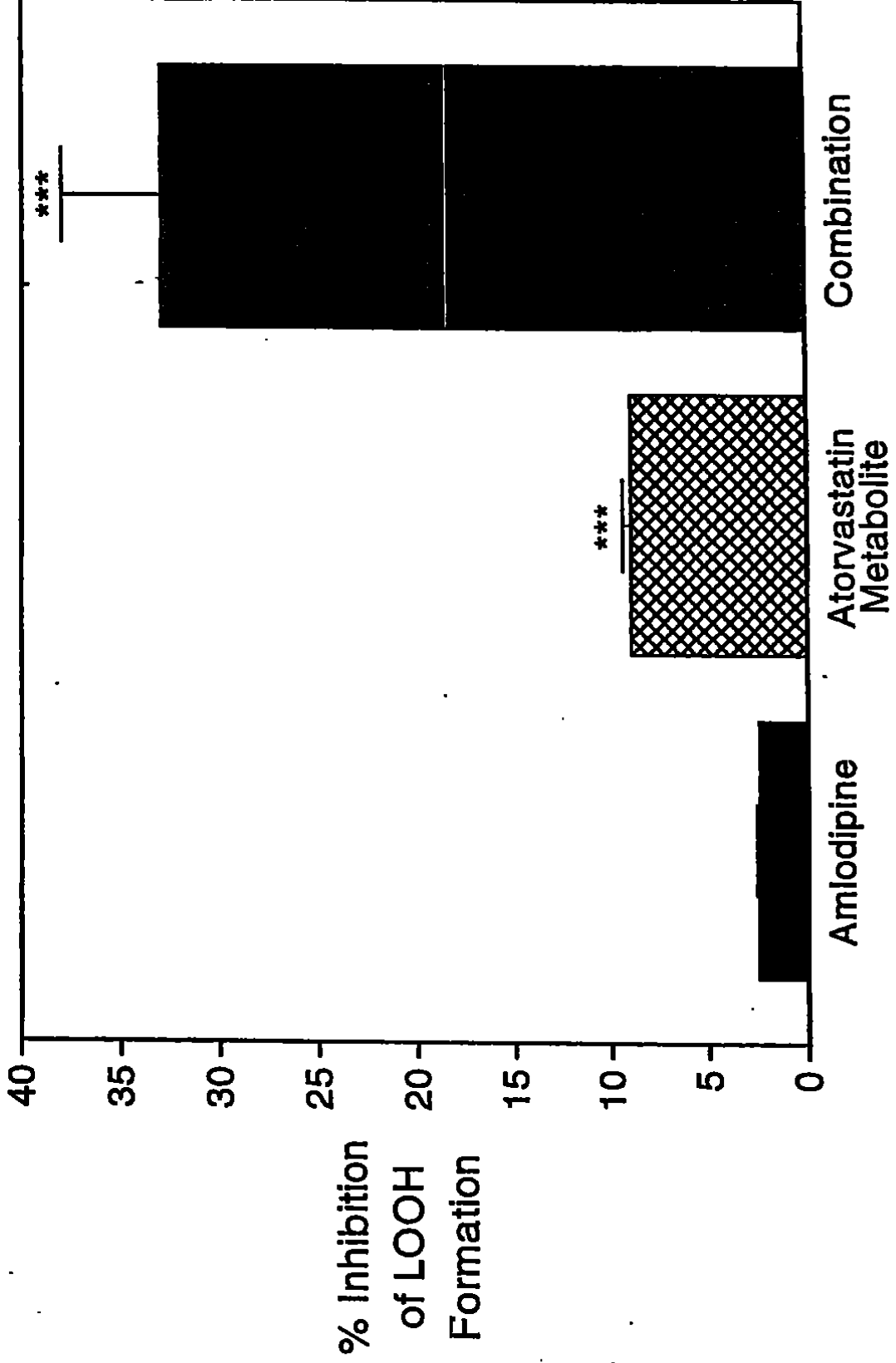


* $p < 0.05$; *** $p < 0.001$ versus control
Values are mean $\pm$ S.D. ($n = 6$)

Figure 7

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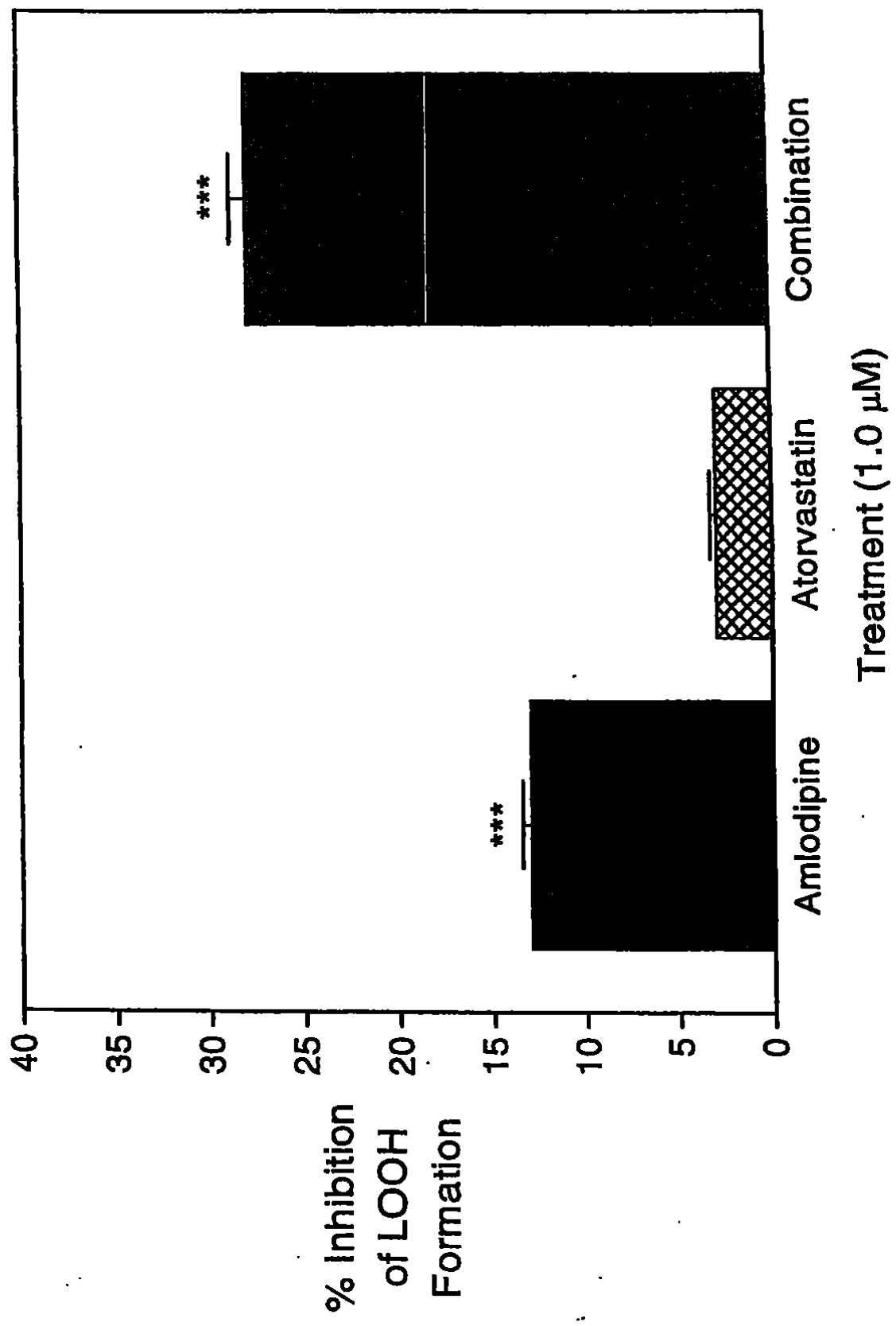
Treatment (100.0 nM)

*** $p < 0.001$ versus control and other tr atm nts
Values are mean $\pm$ S.D. (n = 6)

Figure 8A
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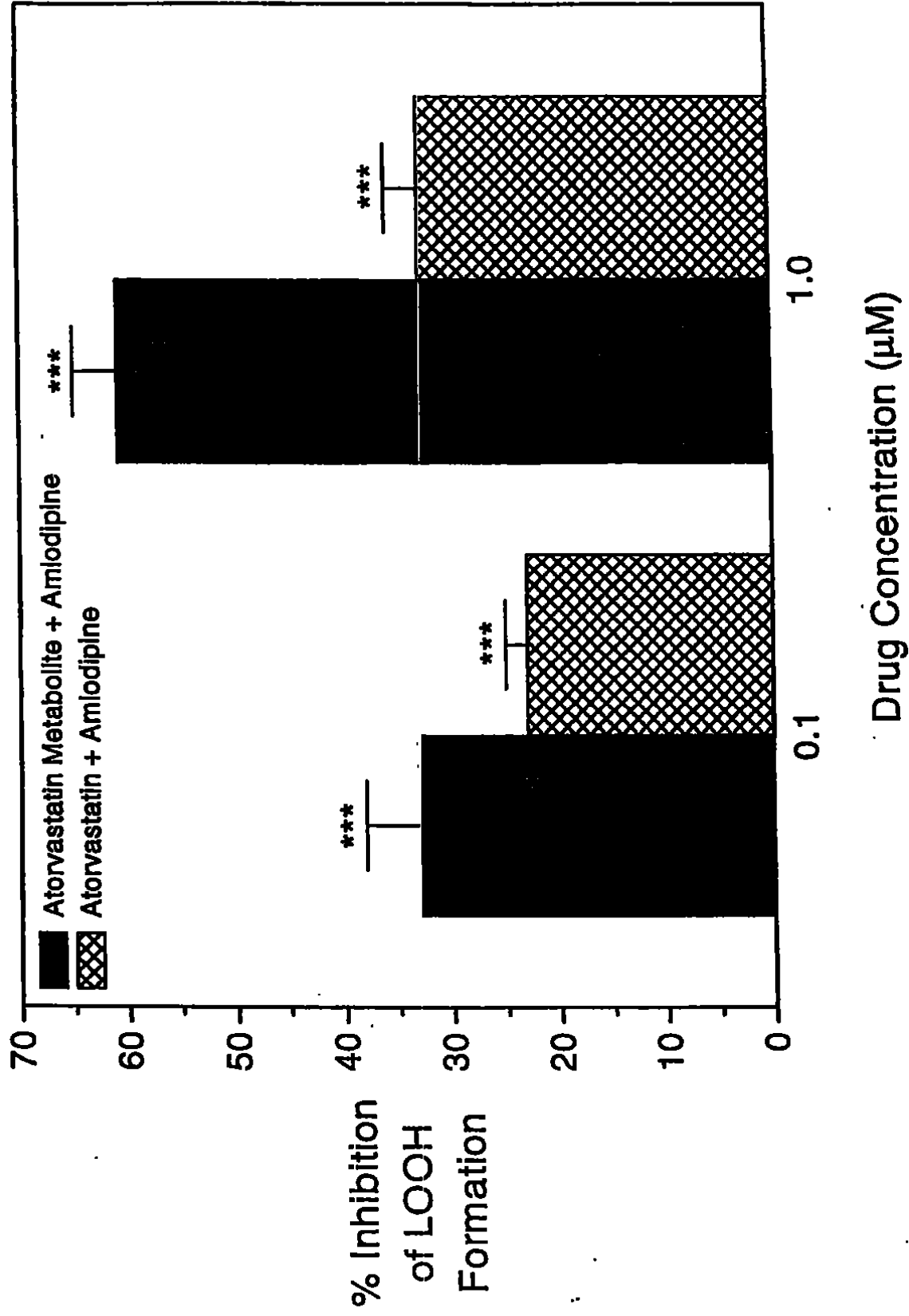
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*** $p < 0.001$ versus control
Values are mean $\pm$ S.D. (n = 6)

Figure 8B

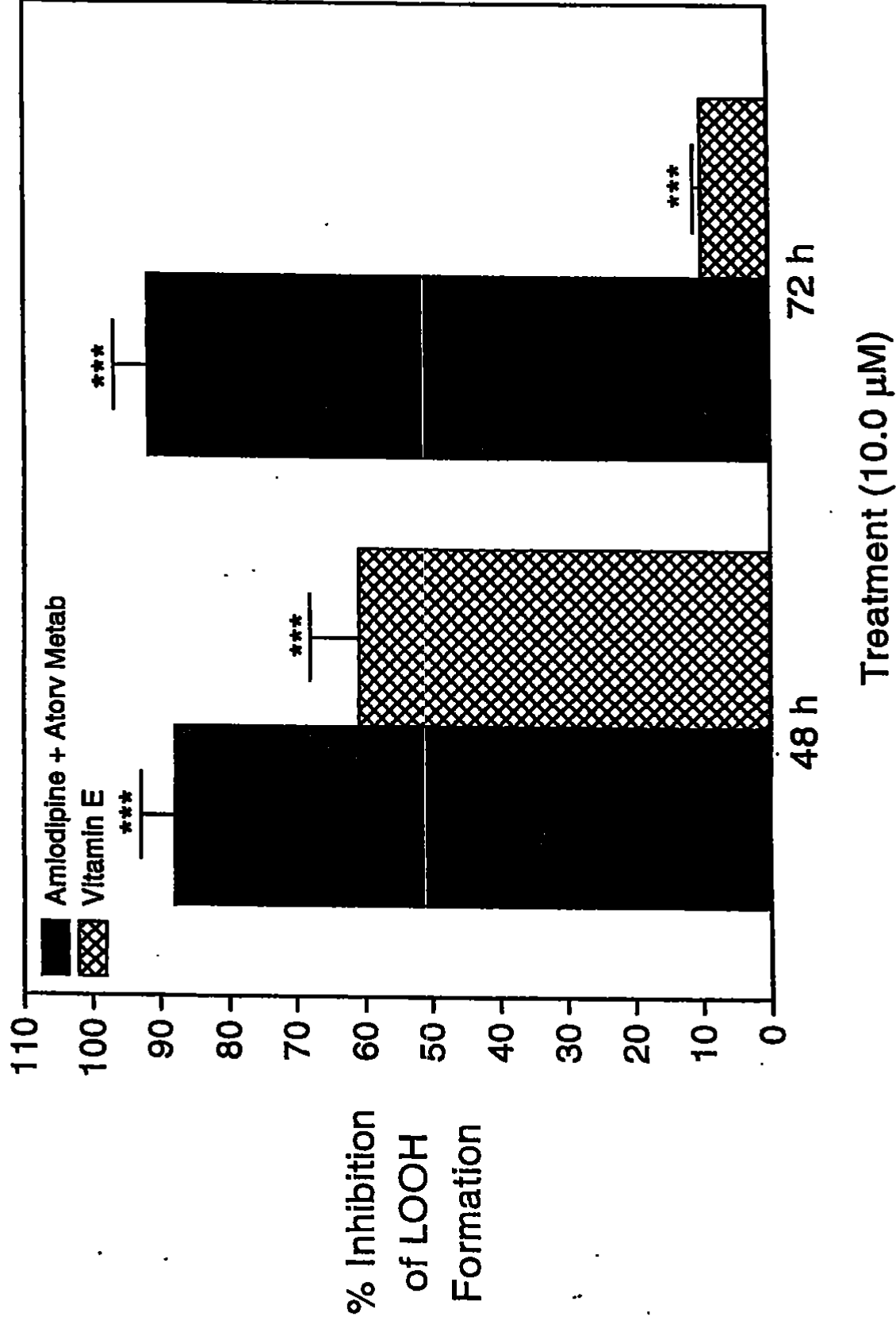
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*** $p < 0.001$ versus control
Values are mean $\pm$ S.D. (n = 5-6)

Figure 9

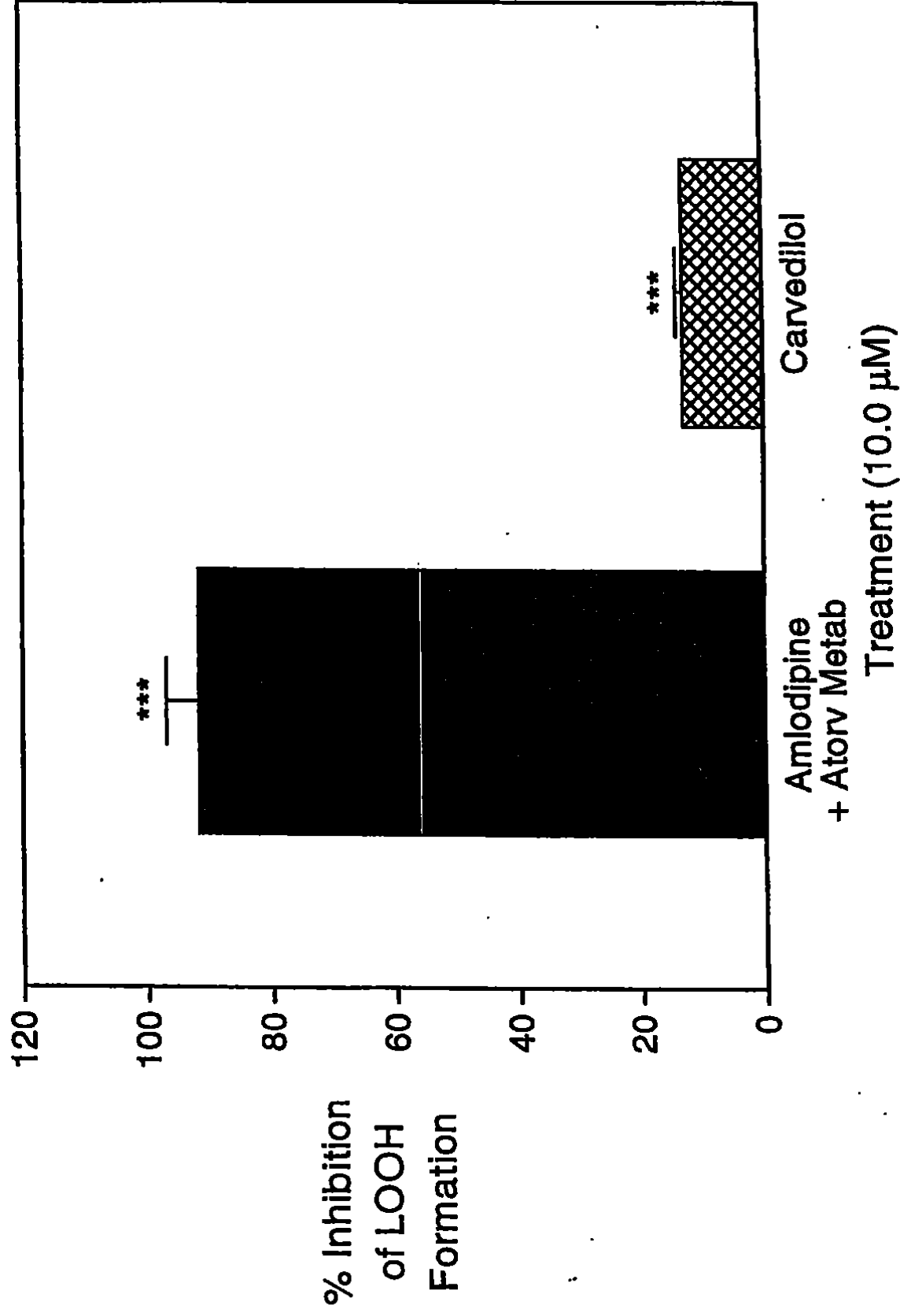
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*** $p < 0.001$ versus control
Values are mean $\pm$ S.D. (n = 6-12)

Figure 10A

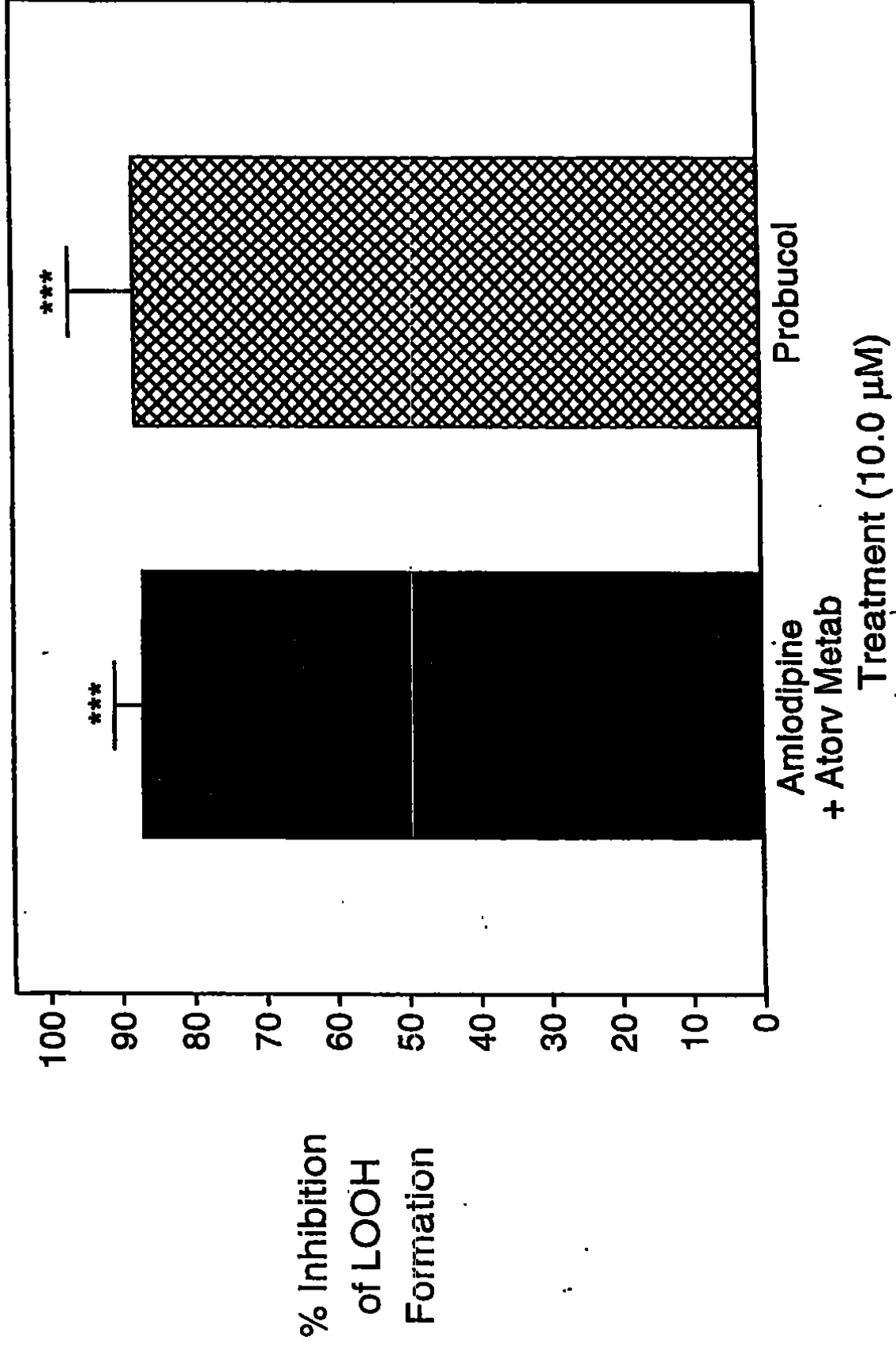
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*** $p < 0.001$ versus control
Values are mean $\pm$ S.D. ($n = 6$)

Figure 10B

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*** $p < 0.001$ versus control
 Values are mean $\pm$ S.D. (n = 6)

Figure 10C

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~~2/2~~

Chemical Antioxidant Mechanisms for Amlodipine: Abstractable Hydrogens

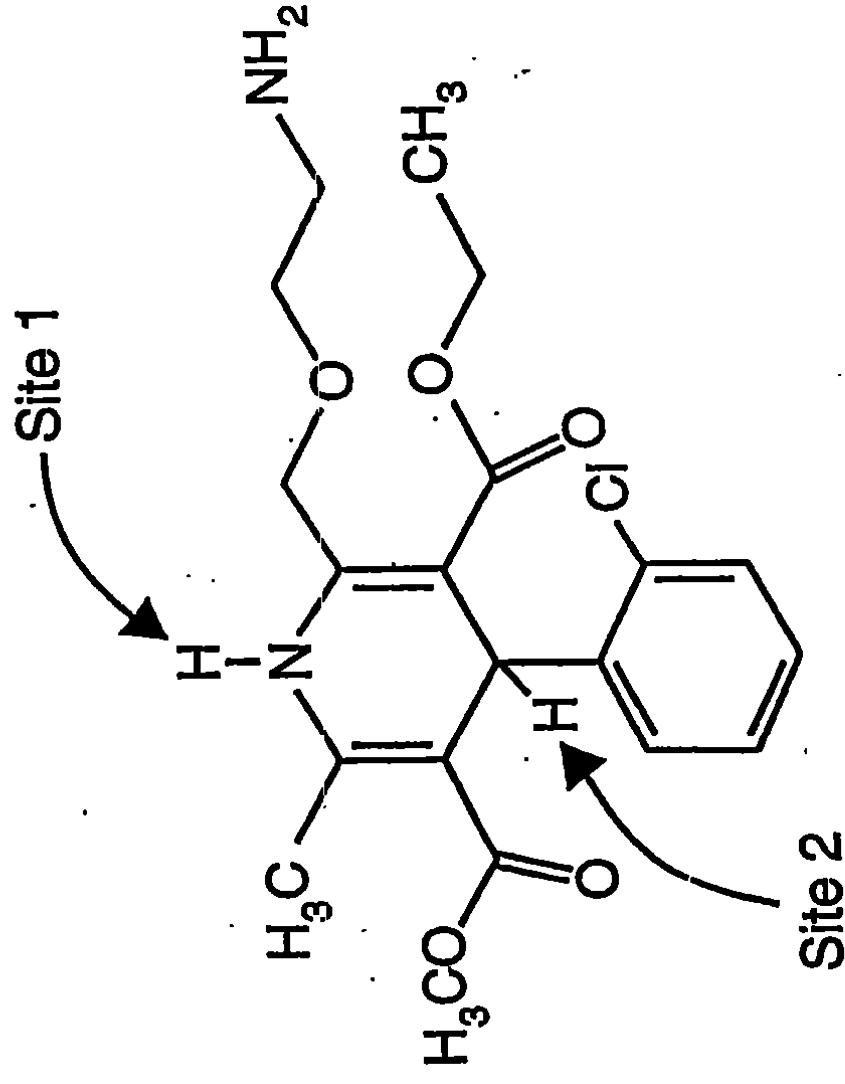


Figure 11 A

Resonance Stabilization of Free Radical at Site 1 of Amlodipine

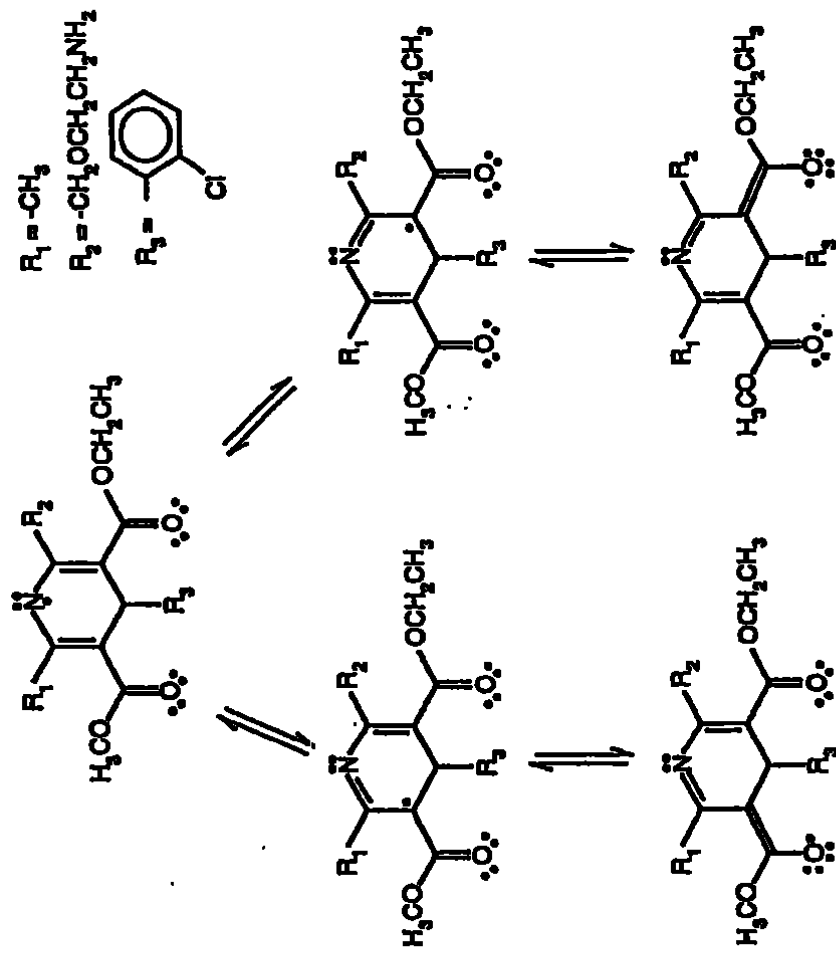


Figure 11 B

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Resonance Stabilization of Free Radical at Site 2 of Amlodipine

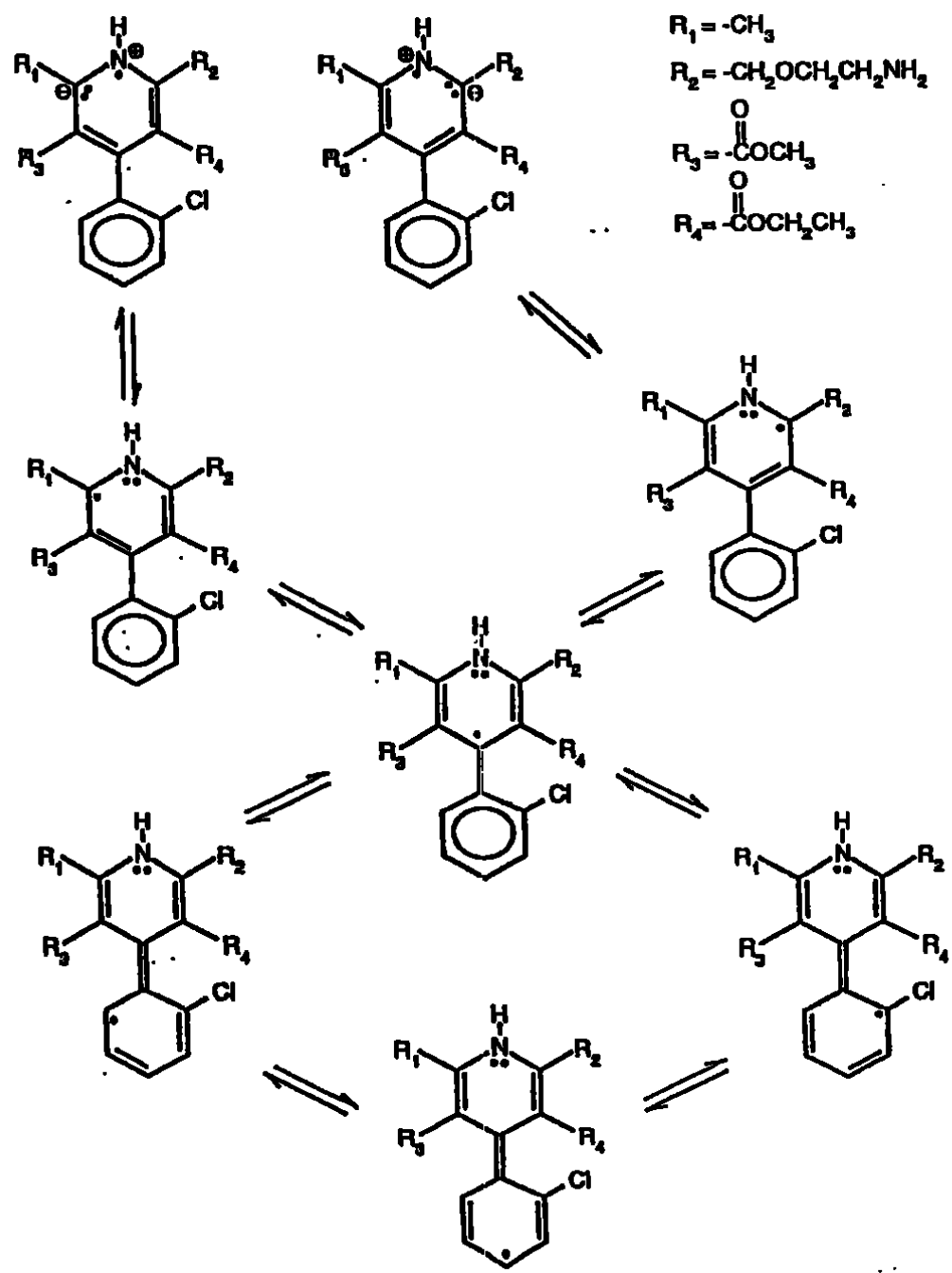


Figure 11C

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Antioxidant Mechanisms for Atorvastatin Metabolite: Abstractable Hydrogens

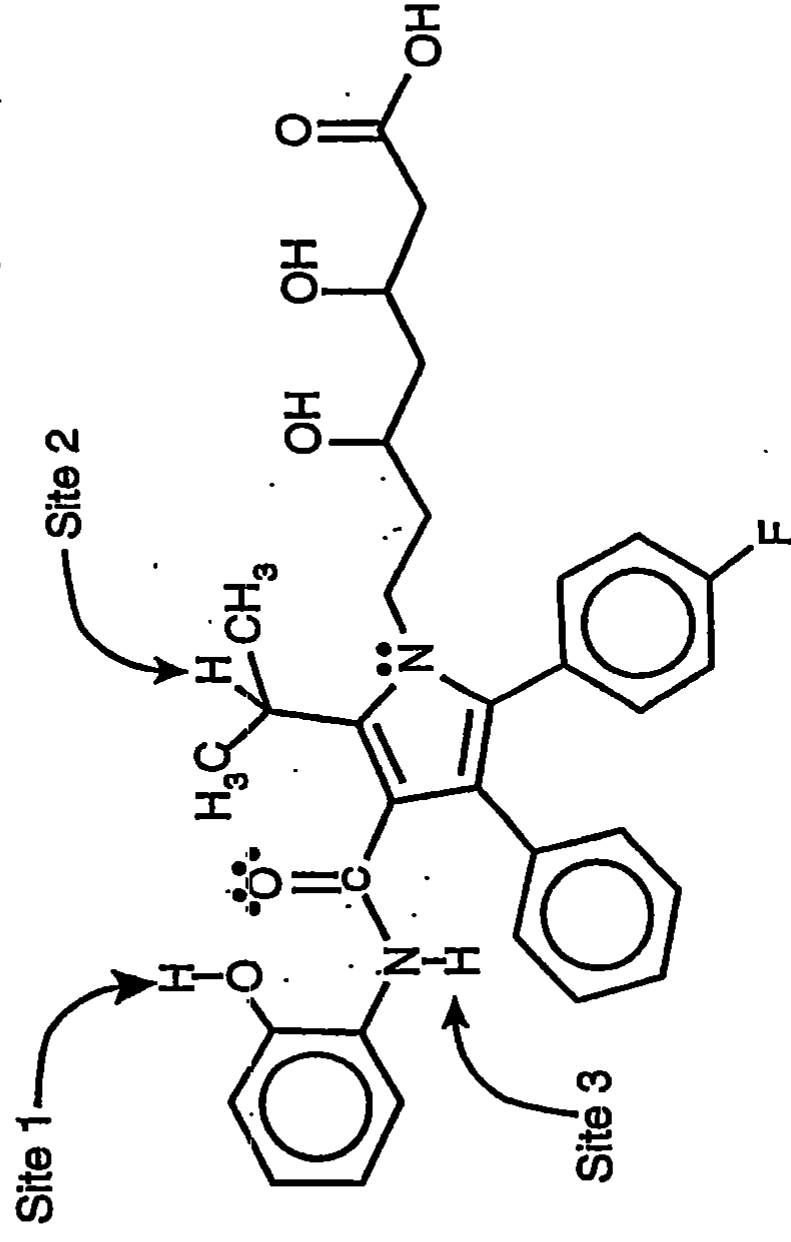


Figure 12A

Antioxidant Mechanisms for Atorvastatin Metabolite: Resonance Stabilization of Free Radical at Site 1

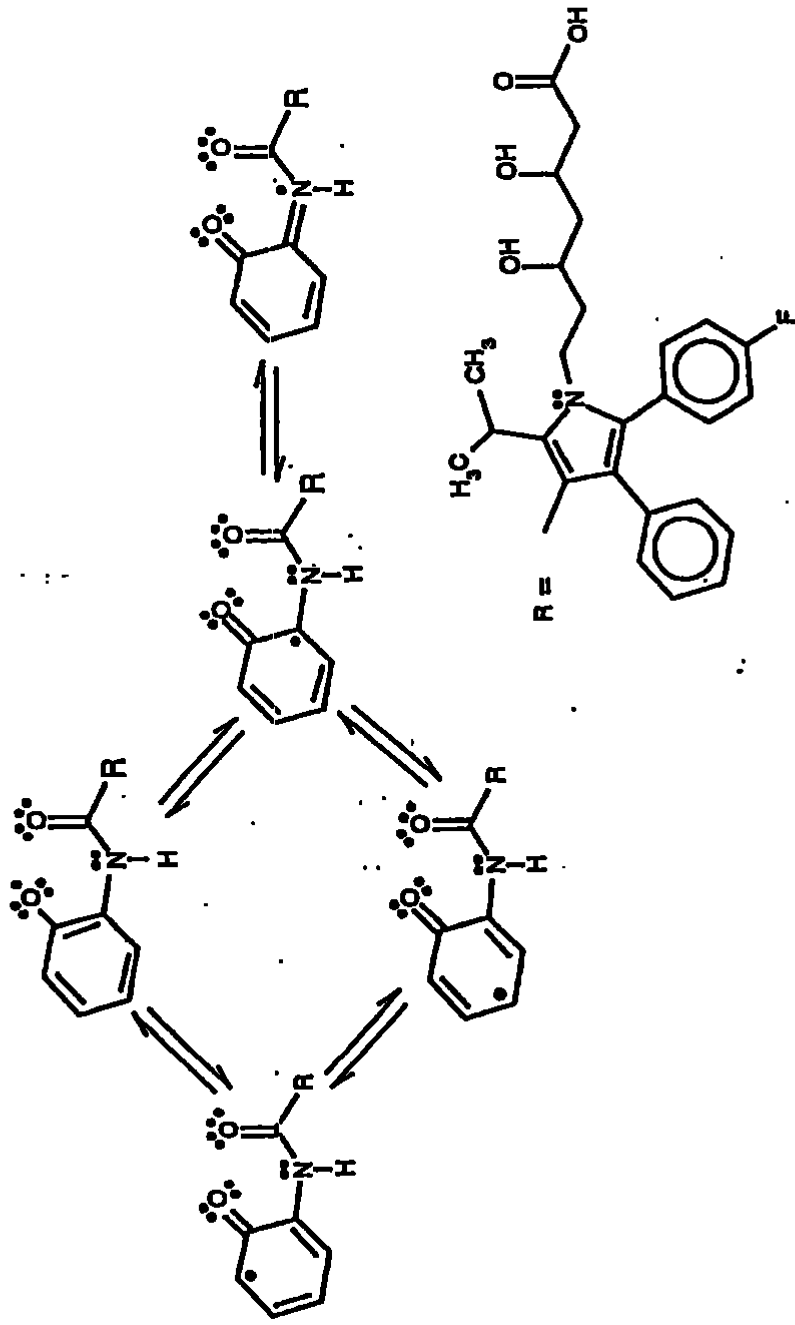


Figure 12B

272

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317

323

Antioxidant Mechanisms for Atorvastatin
Metabolite: Resonance Stabilization of
Free Radical at Site 2

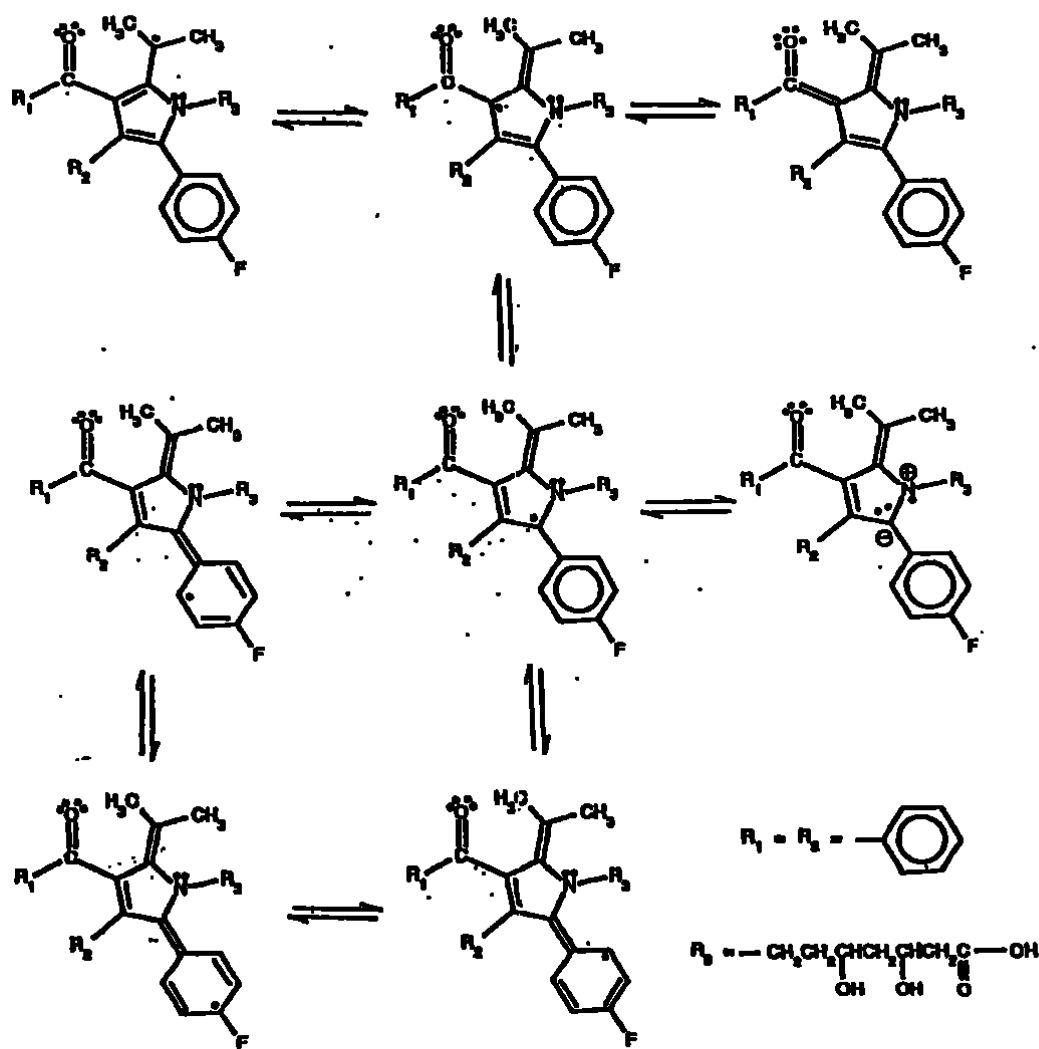


Figure 12C

Antioxidant Mechanisms for Atorvastatin Metabolite: Resonance Stabilization of Free Radical at Site 3

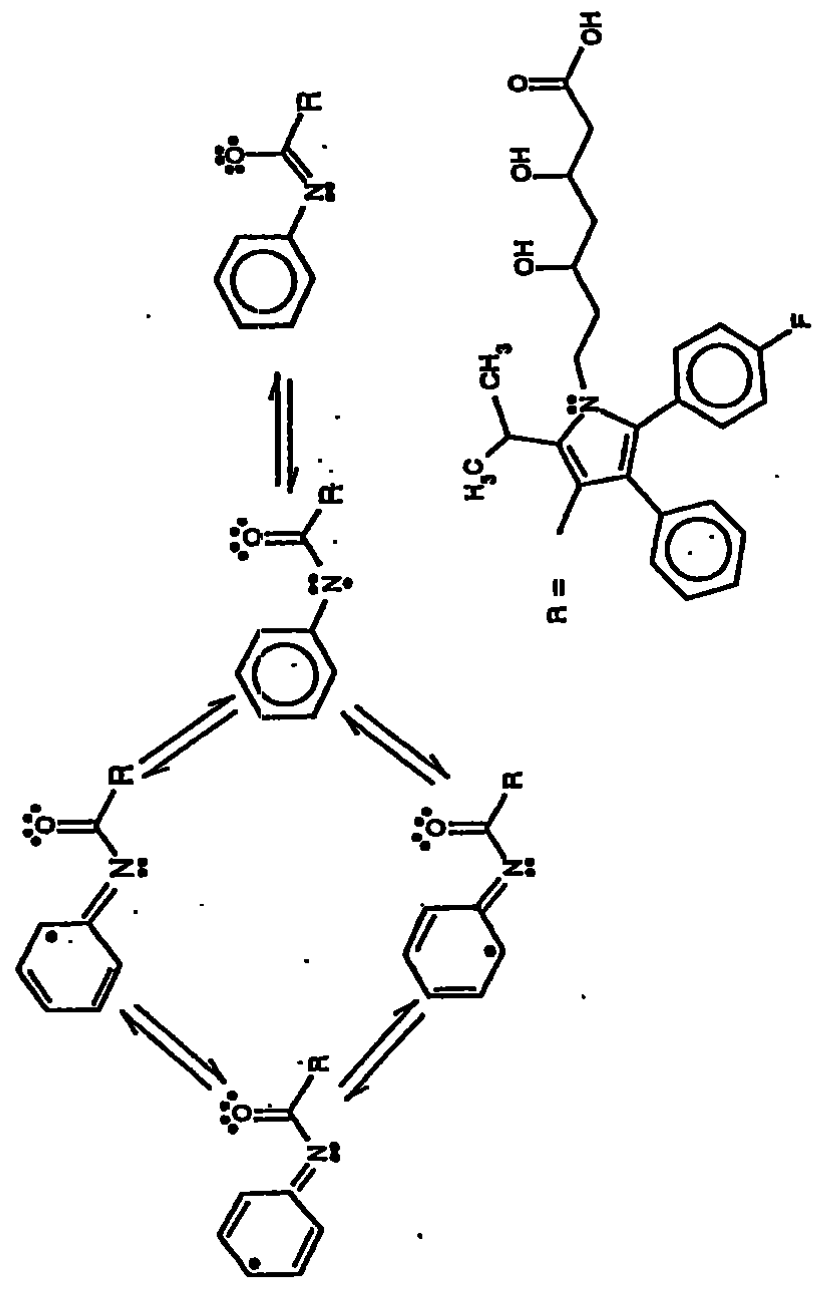


Figure 12D

374

714

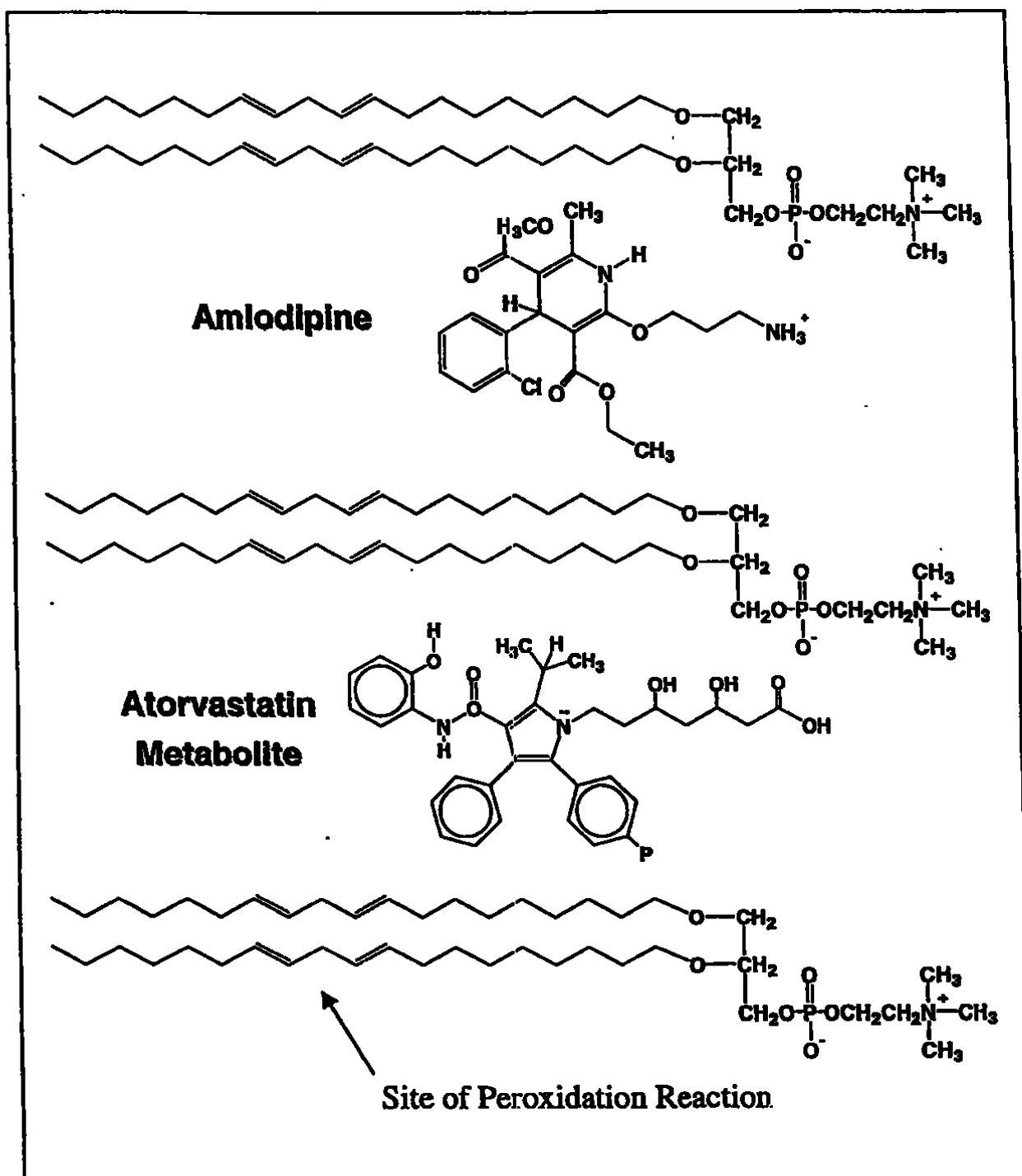


Figure 13

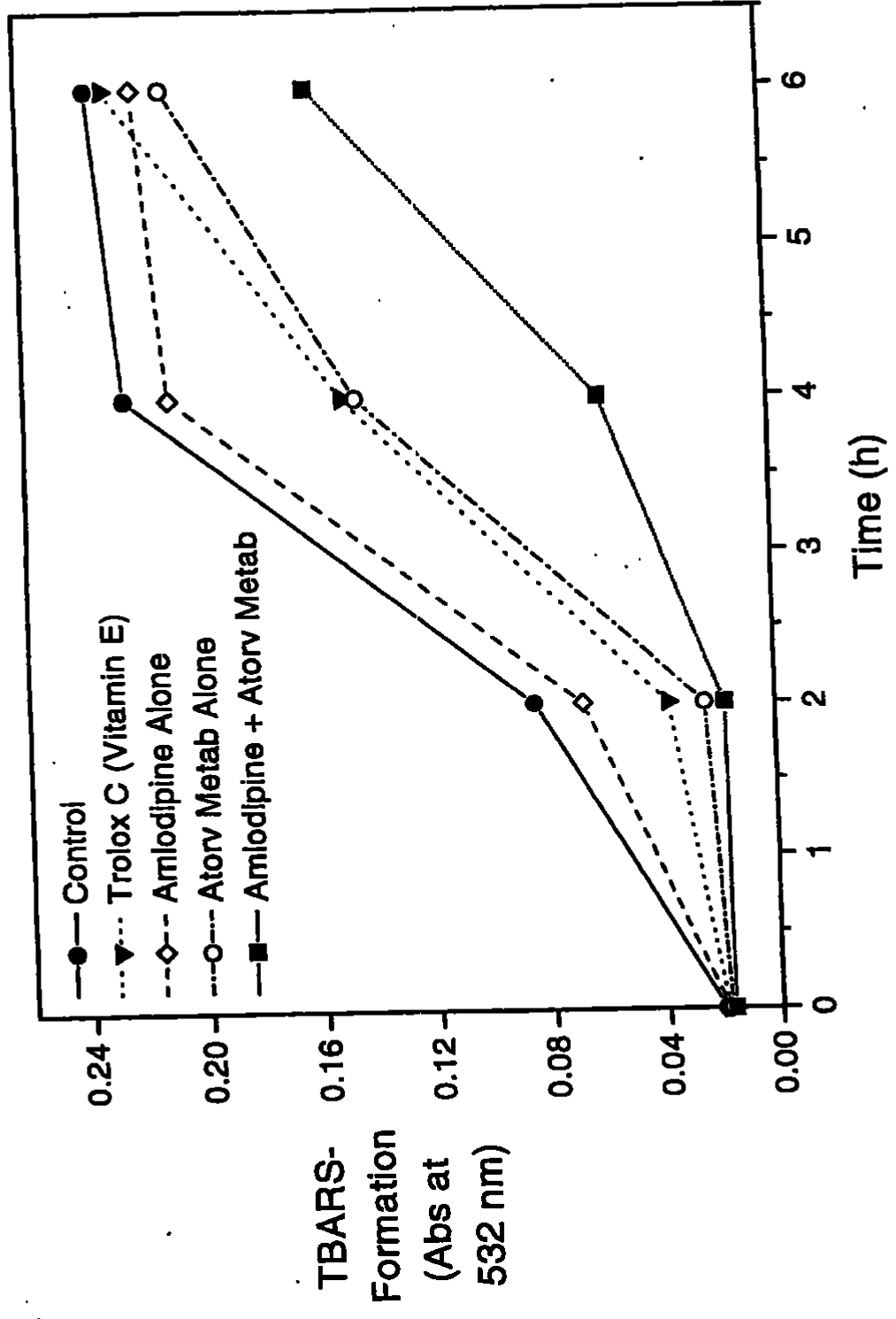


Figure 14

325

211

Figure 15

322
321 2/7

Computed Enthalpies of Formation for Atorvastatin Metabolite and its Radicals

Molecule	ΔH_f (kcal/mol)
Atorvastatin Metabolite	-226.10
Carbon radical	-209.50
Oxygen radical	-199.89
Nitrogen radical	-192.55
Methyl radical	-193.51
Phenyl radical	-176.05
Carbon/Oxygen Diradical	-182.07
Carbon/Nitrogen Diradical	-174.25

*The smaller (i.e., more negative) the ΔH_f value, the easier to abstract the H atom from that site.

60151121.082799

Figure 16

328
322
218

**Computed Enthalpies of Formation for Amlodipine
and its Radicals**

Molecule	ΔH_f (kcal/mol)
Amlodipine	-171.61
Carbon radical	-164.28
Nitrogen radical	-148.27

*The smaller (i.e., more negative) the ΔH_f value, the easier to abstract the H atom from that site.

60151121.082799

2do. EXAMEN DE FORMA PATENTE DE INVENCION

Folio 330

323

Radicación no 00-29421

Concepto Técnico no 521

Clasificación Internacional de Patentes A61K 31/44 // 31/41

Practicado el examen de acuerdo a lo establecido en el Art. 38 de la Decisión 486 se determinó que esta solicitud de Patente de Invención:

- SI ☒ NO ☐ Contiene un resumen de la invención (Art.26-e).
- SI ☒ NO ☐ Contiene el título o nombre de la invención (Art.27-d).
- SI ☒ NO ☐ Contiene la descripción de la invención (Art.26-b).
- SI ☐ NO ☐ NO ES APLICABLE ☒ Contiene los dibujos necesarios para comprender la invención (Art.26-d)
- SI ☒ NO ☐ Contiene una o más reivindicaciones. (Art.26-c).
- SI ☐ NO ☐ NO ES APLICABLE ☒ Han sido desarrollados los productos y/o procedimientos a partir de recursos genéticos o de sus productos derivados de los que cualquiera de los Países Miembros es país de origen. (Art. 26- h).
- SI ☐ NO ☐ NO ES APLICABLE ☒ Los productos y/o procedimientos han sido obtenidos o desarrollados a partir de los conocimientos tradicionales de las comunidades indígenas, afroamericanas, o locales de los países miembros, de acuerdo a lo establecido en la Decisión 391 y sus modificaciones y reglamentaciones vigentes.(Art.26-i).
- SI ☐ NO ☒ La invención se refiere a un producto relativo a un material biológico. (Art.26-J).
- SI ☒ NO ☐ La prioridad coincide con la materia reivindicada.
- SI ☒ NO ☐ CUMPLE LOS REQUISITOS TÉCNICOS

OBSERVACIONES Y OTROS: SI ☒ NO ☐ ver hoja(s) anexa(s)

Se anexa nuevo capítulo reivindicatorio folios 122-149 en donde se describe de las reivindicaciones 6 a 182. y que reemplaza el originalmente presentado.

EXAMINADOR TÉCNICO
202038

Bogotá D. C., 11/02/20 20/02

2020
Bogotá DC

324

00765

Doctor
CARLOS R. OLARTE
Apoderado

REFERENCIA:	Radicación No.	00-29421
	Trámite	002
	Evento	001
	Actuación	416
	Oficio No.	
	Folios	001

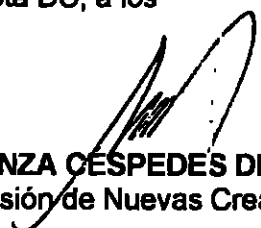
Teniendo en cuenta que la solicitud de privilegio de patente que se tramita bajo el expediente indicado en la referencia, reúne los requisitos de forma establecidos en los artículos 26 y 27 de la Decisión 486 de la Comisión de la Comunidad Andina, y en las demás disposiciones vigentes sobre la materia, se envía el extracto de la solicitud a la Oficina de Comunicaciones para efecto de su publicación en la Gaceta de Propiedad Industrial, conforme lo establece el artículo 40 de la misma Decisión.

No obstante lo anterior, no se tendrá en cuenta la prioridad invocada bajo el número 60/130,659 de fecha abril 23 de 1999 ya que no se presentó de acuerdo con lo solicitado en el folio 107.

Cumplase

Dado en Bogotá DC, a los

23 ABR. 2002


ALIX CARMENZA CESPEDES DE VERGEL
Jefe de la División de Nuevas Creaciones

Bogotá, DC, 04-2002 El extracto de esta solicitud fue publicado en el número 517 de la Gaceta de Propiedad Industrial, de fecha 27-06-2002 El término hábil señalado por la ley expiró el 24-09-2002 y ~~SI~~ NO se presentaron oposiciones por parte de terceros.

202027

Carrera 13 No. 27-00 Pisos 5, 7 y 10
Conmutador: 382 0840
Fax: 382 2695
E-mail: info@sic.gov.co
Bogotá, D C , Colombia