Title: USE OF INHIBITORS OF PP2C FOR TREATING OR PREVENTING ARTERIOSCLEROSIS

Abstract: Activity of serine/threonine protein Phosphatase type 2C (PP2C) is known to be stimulated by certain unsaturated fatty acids and this enzyme dephosphorylates BAD1 thus acting on apoptosis. The invention provides saturated fatty acid (SFA) as new inhibitors for the activity of the PP2C. Therefore, the invention offers a new therapeutic strategy for the treatment and prevention of arteriosclerosis.
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INHIBITORS FOR PP2C

This invention relates to serine-/threonine protein phosphatase type 2C (PP2C) inhibitors comprising at least a derivative of a fatty acid. Furthermore, the invention relates to a pharmaceutical composition comprising respective inhibitors and their use in therapy.

Reversible protein phosphorylation is an ubiquitous strategy used to control many of the intracellular events in eukaryotic cells. It is estimated that more than ten percent of proteins active in a typical mammalian cell are phosphorylated. Kinases catalyze the transfer of high-energy phosphate groups from adenosine triphosphate (ATP) to target proteins on the hydroxyamino acid residues serine, threonine, or tyrosine. Phosphatases, in contrast, remove these phosphate groups.

Extracellular signals including hormones, neurotransmitters, and growth and differentiation factors can activate kinases, which can occur as cell surface receptors or as the activator of the final effector protein, but can also occur along the signal transduction pathway. Cascades of kinases are, as well as kinases sensitive to second messenger molecules. This system allows for the amplification of weak signals (low abundance growth factor molecules, for example), as well as the synthesis of many weak signals into an all-or-nothing response. Phosphatases, then, are essential in determining the extent of phosphorylation in the cell and, together with kinases, regulate key cellular processes such as metabolic enzyme activity, proliferation, cell growth and differentiation, cell adhesion, cell cycle progression and apoptosis.

Apoptosis, or programmed cell death, is involved in fundamental biological processes. It also plays a fundamental role in pathological processes like atherogenesis (Geng et al., 1997; Kockx et al., 2000; Kockx et al., 1998). More than 25 years ago it was proposed that arteriosclerosis arises as a response of the vascular wall to endothelial
injury. Evidence accumulated in the last decade showed that this injury could be due to endothelial apoptosis (Rössig et al., 2001; Choy et al., 2001; Stefanec et al., 2000). Endothelial cell (EC) apoptosis may result in increased permeability of the endothelial monolayer through the loss of EC number. This loss of integrity could facilitate the migration and deposition of lipids, like oxidized LDL, or monocytes in the subintimal space, further damaging vascular development and propagating plaque development. Since apoptotic cells are procoagulant, atherogenesis may also depend on increased coagulation of platelets resulting from EC apoptosis (McDonald et al., 1999). Therefore, enhanced apoptosis of endothelial cells (ECs) and macrophages is an important pathophysiologic mechanism involved in atherogenesis.

Apoptosis is an energy dependent process that may be regulable at several levels. However, identifying the role of the regulators of the apoptotic pathway may lead to a greater understanding as to how cellular interactions contribute to atherogenesis, as well as providing new insights into potential therapeutic targets for the treatment of arteriosclerosis. Reversible phosphorylation is the most common post-translational modification of proteins. By phosphorylation almost all aspects of cellular life from classical metabolic pathways and even cell death are regulated. As mentioned before, many cellular constituents are switched "on" or "off" by phosphorylation and dephosphorylation. These processes are catalyzed by various specific protein kinases and phosphatases.

Protein phosphatases are generally characterized as either serine/threonine or tyrosine specific, based on their preferred phospho-amino acid substrate. However, some phosphatases (DSPs, for dual specificity phosphatases) can act on phosphorylated tyrosine, serine, or threonine residues.


PP2C is a 42 kDa monomer with broad substrate specificity and is dependent on divalent cations (mainly Mn$^{2+}$ or Mg$^{2+}$) for its activity. PP2C proteins share a conserved N-terminal region with an invariant DGH motif, which contains an aspartate residue involved in cation binding (PROSITE PDOC00792).


The list of potential physiological functions proposed for PP2C alpha and PP2C beta comprises a wide range. A growing list of substrates has been described to be specifically dephosphorylated by PP2C alpha and PP2C beta indicating an involvement in various metabolic pathways:

(i) Hydroxymethylglutaryl-CoA reductase (Ball, K.L., Dale, S., Weekes J. and Hardie, D.G. (1994) Biochemical characterization of tow forms of 3-hydroxy-3-


As mentioned before it was recently found that protein phosphatase 2C (PP2C) is implicated in the regulation of apoptosis. It was found that BAD, a proapoptotic member of the Bcl-2 family, is a substrate of PP2C, indicating that PP2C is a responsible factor triggering the pro-apoptotic function of BAD (Klumpp et al., 2003).

BAD (BAD stands for Bcl-2/Bcl-XL-antagonist, causing cell death) represents a point of convergence of several different signal-transduction pathways which are activated by survival factors inhibiting apoptosis in mammalian cells. BAD is a pro-apoptotic protein which binds to the anti-apoptotic proteins Bcl-2 and Bcl-XL (Yang, E., Zha, J., Jockel, J. Bioise, L.H., Thompson, C.B. and Korsemeyer, S.J. (1995) BAD, a heterodimeric partner for Bcl-XL and Bcl-2 displaces Bax and promotes cell death. Cell Vol: 80, 285-
291). This interaction is thought to neutralize the anti-apoptotic effects of Bcl-2/Bcl-XL and may represent one of the mechanisms by which BAD promotes apoptosis. Phosphorylation of BAD results in its interaction with 14-3-3 proteins instead of Bcl-2 or Bcl-XL, leading to liberation of these anti-apoptotic proteins which can then interact with Bax to inhibit apoptosis (Zha, J., Harada, H., Yang, E., Jokel, J. and Korsemeyer, S.J. (1996) Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not Bcl-XL. Cell Vol: 87, 618-628). Two sites on BAD, Ser<112> and Ser<136> have been reported to be phosphorylated in vivo. The dephosphorylation of those sites was shown to be carried out by PP1 and PP2B (Ayllon, V., Martinez-A., C., Garcia, A., Cayla, X. and Rebollo, A. (2000) Protein phosphatase 1 alpha is a ras-activated bad phosphatase that regulates interleukin-2 deprivation-induced apoptosis. EMBO J. Vol: 19, 2237-2246 and Wang, H.G., Pathan, N., Ethell, I.M., Krajewski, S., Yamaguchi, Y., Shibasaki, F., McKeon, F., Bobo, T., Franke, T.F. and Reed, J.C. (1999) Ca<2+>-induced apoptosis through calcineurin dephosphorylation of BAD. Science Vol: 284, 339-343). In addition, PP2A also has also been reported to act on phospho-BAD (Deng, X., Ito, T., Carr, B., Mumby, M. and May, W.S. (1998) Reversible phosphorylation of Bcl2 following interleukin 3 or bryostatin 1 is mediated by direct interaction with protein phosphatase 2A. J. Biol. Chem. Vol: 273, 34157-34163). The major site on BAD phosphorylated by PKA in vitro just recently discovered was Ser<155>. The phosphorylation of Ser<155> triggers the dissociation of BAD from Bcl-2 and Bcl-XL and promotes its interaction with 14-3-3 proteins (Lizcano, J.M., Morrice, N. and Cohen, P. (2000) Regulation of BAD by cAMP-dependent protein kinase is mediated via phosphorylation of a novel site, Ser<155>. Biochem. J. Vol: 349, 547-557).

Previous studies suggested that PP2C is an additional player in the development and progress of apoptosis (see above) and is especially involved in apoptotic processes of endothelial cells. Hence, it appeared as an interesting point to regulate apoptosis and especially to provide a treatment or a prophylaxis for arteriosclerosis. However, until now, there is no such prophylaxis/treatment available as no specific inhibitor for PP2C is available.

Accordingly, it is an object of the present invention to provide an inhibitor for PP2C.

The intention is to provide modulators which can be used in the specific treatment of apoptosis (especially of EC cells) thereby providing a treatment or prevention for cardiovascular diseases, especially arteriosclerosis.

This object is solved according to the present invention by a serine/threonine protein phosphatase type 2C (PP2C) inhibitor, which comprises at least a derivative of a fatty acid of the group consisting of
- fatty acids
- glycerol lipids
- sphingolipids.

Said derivative of a fatty acid has a sufficient lipophilicity to inhibit fatty acid induced activation of PP2C and a free carboxyl group. Apparently the inhibitors according to the present invention are able to bind to PP2C thereby blocking the binding pocket for fatty acids that otherwise activate PP2C such as e.g. oleic acid.

Said derivative of a fatty acid is preferably a fatty acid, more preferably one selected from the group consisting of saturated fatty acids (SFA), unsaturated fatty acids, straight and branched fatty acids.

Branches fatty acids may carry C1 – C5 alkyl groups. Preferred are small alkyl groups such as C1 to C3 alkyl group. Apparently, such branched fatty acids block the binding pocket sterically.

Furthermore, said fatty acid derivative may comprise at least one polyethylene moiety. The polyethylene moiety may be within the fatty acid chain or at the end of it.

According to one embodiment, said fatty acid is a saturated fatty acid (SFA). The saturated fatty acid (SFA) used according to the present invention has preferably a chain length of more than 16 C-atoms. E.g. SFAs can be used wherein the chain length is between C17:0 and C30:0, preferably between C17:0 and C25:0, even more preferred between C17:0 and C23:0. For example, the SFA could be selected from the group consisting of heptadecanoic acid (C17:0), stearic acid (C18:0), arachidic acid (C20:0) and behenic acid (C22:0).

According to a further embodiment said derivative of a fatty acid is either a neutral or a phospholipid. Suitable examples are triglycerides. The triglycerides preferably comprise at least one of the fatty acids mentioned above.

According to a further embodiment, said inhibitory derivative is an ester. Said ester preferably comprises at least one of the fatty acids mentioned above.

The use of a triglyceride or an ester as described offers the opportunity to develop a pharmaceutical which comprises the active inhibitory substance in form of a prodrug. Such prodrugs are metabolized to release the actual active ingredient in the body through enzymatic cleavage.
To provide new protein phosphatase inhibitors and control mechanisms of apoptose satisfies a need in the art by providing compounds that inhibit PP2C which are useful in the diagnosis, prevention, and treatment of disorders caused by apoptosis, including atherogenesis, arteriosclerosis and in the assessment of function of PP2C in vitro and in vivo. As outlined before, despite intensive searches, no inhibitors of PP2C were known in the prior art.

According to a further embodiment said derivative of a fatty acid carries a substituent. Said substituent is preferably a halogen substituent such as a fluorine.

Also provided are the above described inhibitors in form of a salt.

The inhibitors provided with the present invention have several major advantages.

First of all, they are able to selectively inhibit the activation of PP2C by certain activating unsaturated fatty acids like oleic acid. Such activators of PP2C usually have to fulfill special structural requirements as they have to be lipophilic, oxidizable, acidic, and must be cis-configurated (Klumpp et al., 1998). The results of the present experiments on activation and on specific inhibition by the inhibitors according to the invention can be summarised as follows:

The essential chemical and structural features of compounds essential for activation of PP2Cbeta are as follows:

(i) at least one double bond at a special position;
(ii) a minimum chain length of 15 C-atoms;
(iii) a free negatively charged group and
(iv) cis-configuration.

Selected compounds fulfilling these requirements are capable of stimulating PP2Cbeta activity and simultaneously causing apoptosis in ECs. In addition it was demonstrated that BAD, a proapoptotic member of the Bcl-2 family, is a substrate of PP2Cbeta, indicating that PP2Cbeta can trigger the pro-apoptotic function of BAD. In its dephosphorylated state, BAD promotes apoptosis by heterodimerization with the antiapoptotic oncogenes Bcl-2 and Bcl-xL. Immucytochemistry revealed that PP2C and its substrate BAD are co-localized within the cytosol of HUVECs. Further the co-localization of these two proteins in macrophages was demonstrated (Fig. 1).

Extensive studies with a variety of fatty acids further demonstrated a striking correlation between activation of PP2C and induction of apoptosis in ECs. Also in macrophages the described correlation was verified: SFAs (C14:0 – C22:0), trans fatty acids like
eladic acid (18:1 trans-Δ⁹) or methylesterderivatives like oleic acid methylester (18:1 cis-Δ⁹ ester) do not fulfill the structural chemical requirements for PP2C-activation and could also not damage cultured macrophages (Fig. 2 and 6).

However, the inventors found that e.g. SFAs which are not able to activate PP2C in vitro (Fig. 3) were able to inhibit the oleic acid-induced activation of PP2C and thus act as specific inhibitors of fatty acid mediated PP2C activation. These SFAs have a free carboxyl group and are lipophil. Apparently, they displace oleic acid from the active center of the PP2C phosphatase without activating the enzyme and consequently avoid the oleic acid-induced activation by blocking the binding site necessary for activation.

Saturated fatty acids (C14:0 – C22:0) carrying a free carboxyl group and being lipophil thus comprise one preferred embodiment according to the present invention. However, as described above, also other derivatives of fatty acids can act as inhibitors of PP2C. Their characteristics were described above in detail.

Saturated fatty acids (SFA) do not contain double bonds or other functional groups along the lipophilic chain part interacting with the PP2C molecule. However, also unpolar substituents such as the alkylgroup may be present in the molecule (see above).

The analysed mechanism of specific fatty acid activation and inhibition of PP2C in EC cells enables a targeted therapy approach for treatment and prophylaxis of atherogenesis and even arteriosclerosis due to the following reasons:

The vascular endothelium is incessantly exposed to free fatty acids (FFAs) because the lipoprotein lipase (LPL) that is associated with the luminal surface of ECs hydrolyzes triglycerides of circulating lipoproteins (Stins et al., 1992). It was demonstrated that physiologic fatty acids from human lipoproteins activate PP2C in vitro and induce apoptosis in cultured human umbilical endothelial cells (HUVECs) (Hufnagel et al., 2005). These data suggest PP2C as an additional player in the complex system of atherogenesis/arteriosclerosis. RNA interference (RNAi) mechanisms were employed to demonstrate the causal relationship between fatty acid-induced activation of PP2C and induction of apoptosis by fatty acids in ECs (Schwarz et al., 2006).

The obtained results provide evidence that the phosphatase PP2C in fact causes oleic acid-induced apoptosis in ECs. Therefore, the inhibition of FFAs-mediated PP2C activation is a new therapeutic strategy to prevent the loss of endothelial integrity or rather atherogenesis and/or to treat arteriosclerosis. This therapy strategy is made available by the present invention as inhibitors of PP2C are provided that specifically
inhibit the FFA mediated activation of PP2C. Such a selective inhibition is favourable as an unspecific inhibition of apoptotic processes might lead to uncontrollable side-effects.

Another majority of apoptotic cells in atherosclerotic lesions are macrophages. In late lesional events, in which clearance of apoptotic cells is probably meanly, apoptosis leads to secondary necrosis, and necrosis in turn almost certainly promotes plaque destabilization or rupture and this process can ultimately induce vascular obliteration (Tabas, 2004; Ball et al., 1995; Libby et al., 1996). Macrophages produce a variety of biologically active products including proteases, growth factors, apo E (Nathan, 1987; Kayden et al., 1985) and they are also able to secrete active LPL (Mahoney et al., 1982; Khoo et al., 1981). LPL-mediated hydrolysis of triglycerides plays not only a role in the uptake of lipids in ECs but also in macrophages (Floren et al., 1981; Lindquist et al., 1983).

Like ECs, macrophages in atherosclerotic plaque are also exposed to high levels of fat. The liberated FFAs by LPL could theoretically activate PP2C within the cytosol, thus acting on apoptosis and this process finally lead to plaque destabilization. As already demonstrated in ECs, immunocytochemistry revealed PP2C and its substrate BAD within the cytosol of macrophages (please refer to Fig. 1). Furthermore it could be observed that in macrophages the same striking correlation between PP2C-activation and induction of apoptosis like in ECs (please refer to Fig. 2).

The present invention provides inhibitors of FFA and especially oleic acid-induced activation of PP2C. It was demonstrated by experiments that the inhibitors according to the present invention can protect both cell types (EC and macrophages) from the damaging effect of FFAs as exemplified by oleic acid.

According to one embodiment of the present invention the inhibitors are SFAs with a sufficient lipophilicity to bind to and to inhibit activation of PP2C by FFAs. Preferably, they have a chain length longer than 16 C-atoms. It was shown that they are able to inhibit the oleic acid-induced activation of PP2C in vitro. Furthermore, it could also be exhibited that these SFAs abolish the damaging effect of oleic acid in cultured HUVECs and macrophages and thus prove their therapeutic potential. These results provide a targeted inhibition of fatty acid-induced apoptosis in cells such as EC and macrophages and hence provide a new strategy to prevent atherogenesis and to treat/prevent arteriosclerosis.

Saturated fatty acids form straight chains and, as a result, can be packed together very tightly, allowing living organisms to store chemical energy very densely. The fatty tissues
of animals contain large amounts of long-chain saturated fatty acids. In IUPAC nomenclature, fatty acids have an -oic acid suffix.

For example, the effect of SFAs with different chain length (C14:0 – C22:0) in combination with oleic acid on PP2C activity was tested in the experiments described below.

Surprisingly, SFAs with an insufficient lipophilicity e.g. with a chain length shorter than 16 C-atoms, like e.g. myristic acid (C14:0), pentadecanoic acid (C15:0), and palmitic acid (C16:0), could not influence the oleic acid-induced activation of PP2C, wherein SFAs with a chain length longer than 16 C-atoms, like e.g. heptadecanoic acid (C17:0), stearic acid (C18:0), arachidic acid (C20:0), and behenic acid (C22:0), could do so by inhibiting the activation of PP2C (Fig. 4).

Therefore, compounds with a sufficient lipophilicity allowing interaction with the binding pocket of PP2C (e.g. molecules having a chain length longer than 16 C-atoms) and with a free carboxyl group are able to interact with the binding pocket of PP2C wherein the activating FFAs bind. However, these molecules according to the present invention cause no activation of the PP2C enzyme. However, they avoid the binding of oleic acid (as an example of an activated FFA), thus inhibiting the oleic acid-induced activation by preventing binding of these activators. These molecules thus basically antagonise the activators.

Exposure of HUVECs and macrophages to myristic acid (C14:0), pentadecanoic acid (C15:0) and palmitic acid (C16:0) in combination with oleic acid caused apoptosis. In contrast, treatment with e.g. heptadecanoic acid (C17:0) or stearic acid (C18:0) in combination with oleic acid in an equimolar (Fig. 7 and 8) or higher concentration (data not shown) abolished the oleic acid-induced cell death. These data proves the inhibitory effect of the molecules according to the present invention.

Double staining with the DNA fluorochrome Hoechst 33258 and Nile blue revealed that the different SFAs and oleic acid were similarly taken up by HUVECs and preferably heptadecanoic acid (C17:0) and stearic acid (C18:0) could slow down the damaging effect of oleic acid (Fig. 9). This could be a competitive displacement from oleic acid from the respective center of PP2C, which slows down the oleic-induced programmed cell death of ECs and macrophages. The striking correlation observed between inhibition of oleic acid-induced activation of PP2C in vitro and inhibition of oleic acid-induced apoptosis in both described cell types points to a hitherto unknown mechanism of inhibition of the fatty acid-induced activation of PP2C.
In conclusion, apoptosis of ECs and macrophages mediated by PP2C activation is a crucial step in atherogenesis/arteriosclerosis and plaque destabilisation. Unsaturated physiologic FFAs like oleic acid increased PP2C activity dramatically, thus acting on apoptosis by dephosphorylation of the proapoptotic Bcl-2-protein BAD in various cell types (Hufnagel et al., 2005; Schwarz et al., 2006; Zhu et al., 2005). Since most of the fatty acids are transported by lipoproteins and have to pass the endothelium to be absorbed by the body, this mechanism of fatty acid-induced apoptosis should be a crucial factor for atherogenesis in organs with high energy consumption like the heart and hence for the development arteriosclerosis.

This could be an explanation why the coronary arteries are mostly involved in arteriosclerosis and why patients with diabetes mellitus exhibit macroangiopathies. Further this hypothesis could explain why lipid loaded macrophages in atherosclerotic plaque undergo apoptosis and finally induce plaque instability and rupture.

Therefore, inhibition of the fatty acid-induced mediated activation of PP2C by the inhibitors of the present invention is a new and promising therapeutic strategy for preventing apoptosis in EC and/or macrophage cells. The experimental results demonstrate that it is possible to specifically inhibit the fatty acid-induced apoptosis of ECs and macrophages with the inhibitors according to the invention and provide new insights to avoid the fatty acid-induced progression of atherogenesis/arteriosclerosis.

Furthermore, a pharmaceutical composition is provided comprising at least one inhibitor as described above as a physiologically active substance and a pharmaceutically acceptable carrier, wherein said inhibitor possesses sufficient lipophilicity to bind to and inhibit PP2C in particular inhibiting the FFA induced activation.

Also part of the present invention is the use of an inhibitor according to the present invention for the manufacture of a medicament for the treatment or prevention of fatty acid induced apoptosis in cells, wherein said inhibitor has a sufficient lipophilicity to inhibit fatty acid induced activation of PP2C and comprises a free carboxyl group.

The inhibitors according to the present invention may be used for the manufacture of a medicament for the treatment or prevention of cardiovascular diseases, especially for the treatment or prevention of atherogenesis and/or arteriosclerosis, wherein said inhibitor has a sufficient lipophilicity to inhibit fatty acid induced activation of PP2C and comprises a free carboxyl group.

The following figs. illustrate exemplary results of preferred embodiments according to the present invention:
Fig. 1 shows a Western blots for detection of PP2C and BAD in macrophages. The localization of PP2Cbeta and BAD in macrophages is shown. (A) shows Western blots for detection of PP2Cbeta and BAD. Macrophage proteins (30 µg/lane) were electrophoresed on 15% SDS-polyacrylamide gels and after transfer to nitrocellulose immunoblotted with an antibody specific for PP2Cbeta diluted 1:1000 (A, left panel) or with an antibody against BAD diluted 1:1000 (A, right panel). (B-E) Depicted are fluorescence laser scanning photographs showing subcellular co-localization of PP2Cbeta and BAD. Macrophages cell culture and immunocytochemistry were performed as described in Section 2. (B) Incubation with the antibodies against PP2Cbeta (1:100), and (C) directed against BAD (1:350). (D) Merge. (E) Control omitting primary antibodies against PP2Cbeta and BAD.

Fig. 2: shows two bar graphs, wherein in item (A) the effect of SFAs on PP2Cbeta activity and in item (B) the effect of oleic acid in combination with different SFAs on PP2Cbeta activity are shown. (A) shows the effect of SFAs on PP2Cbeta activity. Dephosphorylation of \[^{32}P\]casein by PP2C was measured upon addition of various fatty acids. Oleic acid stimulated the activity 9-fold, wherein SFAs with different chain lengths failed to activate the enzyme. Fatty acids added were oleic acid (18:1 cis-\(\Delta_9\)), myristic acid (14:0), pentadecanoic acid (15:0), palmitic acid (16:0), heptadecanoic acid (17:0), stearic acid (18:0), arachidic acid (20:0) and behenic acid (22:0). Dephosphorylation of \[^{32}P\]casein was determined with 98 ng PP2Cbeta per incubation. Activity in the absence of fatty acids (1) was 6.8 nmol P/min/mg. (B) shows the effect of oleic acid in combination with different SFAs on PP2Cbeta activity. Phosphatase activity was measured with oleic acid and oleic acid in combination with different SFAs. Short chain SFAs (<17:0) could not affect the oleic acid-induced activation of PP2C, wherein SFAs with a chain length longer than 16 C-atoms could inhibit activity. Dephosphorylation of \[^{32}P\]casein was determined with 98 ng PP2Cbeta per incubation. Activity in the absence of fatty acids (1) was 6.8 nmol P/min/mg.

Fig. 3: shows two bar graphs, wherein in item (A) the effect of SFAs with different chain length on ECs and in item (B) the effect of different SFAs (14:0 – 18:0) on macrophages are shown. (A) illustrates the effect of SFAs with different chain length on ECs. HUVECs were incubated with 200 µM of different SFAs. Control cells were treated with vehicle only (0.2% DMSO). Apoptotic damage was revealed by Hoechst 33258 staining as described before. Apoptotic cells are expressed as percentage of total number of cells \(\pm\) S.D. **p<0.001, compared with vehicle-treated control. (B) demonstrates the effect of different SFAs (14:0 – 18:0) on macrophages. Cells were incubated with myristic acid (14:0), pentadecanoic acid (15:0), palmitic acid (16:0), heptadecanoic acid (17:0) and stearic acid (18:0) at a concentration of 200 µM. Morphology was analyzed under a fluorescence microscope after nuclear staining with
Hoechst 33258. The cells showing fragmented nuclei and condensed chromatin were counted as apoptotic cells. Apoptotic cells are expressed as a percentage of total number of cells ± S.D. ***p<0.001.

Fig. 4 shows two bar graphs, wherein in item (A) effect of heptadecanoic acid and stearic acid in view of the protection of ECs from oleic acid-induced apoptosis an in item (B) the effect of heptadecanoic acid and stearic acid in the protection of macrophages from oleic acid-induced apoptosis are shown. (A) depicts that heptadecanoic acid and stearic acid protect ECs from oleic acid-induced apoptosis. Double treatment of HUVECs with oleic acid (18:1 cis-Δ9) and different short chain SFAs (myristic acid (14:0), pentadecanoic acid (15:0), palmitic acid (C16:0)) could not abolish the toxic effect of oleic acid, wherein heptadecanoic acid (17:0) and stearic acid (18:0) could slow down the oleic acid-induced apoptosis of ECs. The percentage of apoptotic cells was determined by nuclear staining with Hoechst 33258. Control cells received vehicle only (0.4% DMSO). Apoptotic cells are expressed as percentage of total number of cells ± S.D. ***p<0.001, compared with vehicle-treated control. (B) Heptadecanoic acid and stearic acid protect macrophages from oleic acid-induced apoptosis. Double treatment of macrophages with oleic acid (18:1 cis-Δ9) and different short chain SFAs (myristic acid (14:0), pentadecanoic acid (15:0), palmitic acid (16:0)) could not abolish the toxic effect of oleic acid, wherein heptadecanoic acid (17:0) and stearic acid (18:0) could inhibit the oleic acid-induced apoptosis of ECs. The percentage of apoptotic cells was determined by nuclear staining with Hoechst 33258. Control cells received vehicle only (0.4% DMSO). Apoptotic cells are expressed as percentage of total number of cells ± S.D. ***p<0.001, compared with vehicle-treated control.

Fig. 5 shows a Nile blue staining of the cells, wherein the uptake of SFAs and with or without oleic acid is demonstrated. ECs were incubated with vehicle (0.4% DMSO) oleic acid (18:1), myristic acid (14:0), pentadecanoic acid (15:0), palmitic acid (16:0), heptadecanoic acid (17:0) and stearic acid (18:0) for 6 h and 24 h at a concentration of 200 μM, respectively. Double treatment with oleic acid revealed, that myristic acid, pentadecanoic acid (15:0), palmitic acid (16:0) are not able to protect HUVECs from oleic acid-induced apoptosis, cognisable on the apoptotic morphology of ECs. In contrast, heptadecanoic acid (17:0) and stearic acid (18:0) slow down the toxicity of oleic acid and protect ECs. Apoptotic damage was revealed by Hoechst 33258 staining as described in Section 2.

Fig. 6 shows further examples of inhibitors.
Examples
The concept of the present invention was proven inter alia by the following experiments:

1. Materials and Methods

1.1 Materials, reagents and equipment
HUVECs and cell growth medium containing 2% heat-inactivated fetal bovine serum, 0.1 ng/ml epidermal growth factor, 1 μg/ml hydrocortisone, 1 ng/ml fibroblast factor and antibiotics were purchased from Promocell (Heidelberg, Germany);

THP-1 cells were obtained as a gift from Institut für Atheroskleroseforschung Münster, Germany; THP-1 monocytes cell medium containing RPMI-1640, FSC 10%, penicillin 100U/ml, streptomycin 100 μg/ml, L-glutamine 2 mM, sodiumpyruvate 100 mM, phorbol-12-myristat-13-acetat (PMA) and mercaptoethanol were received from Sigma Aldrich (Taufkirchen, Germany).

Hoechst 33258, Nile blue and fatty acids (myristic acid, pentadecanoic acid, palmitic acid, heptadecanoic acid, stearic acid, arachidic acid and behenic acid), bovine serum albumin (BSA) were obtained from Sigma Aldrich (Taufkirchen, Germany). Nile blue staining was performed with a confocal laser scanning microscope Zeiss LM510 (Jena, Germany) and analysis of apoptotic cells was accomplished with the fluorescent microscope Axiovert 100 Zeiss (Jena, Germany).

ECL (enhanced chemoluminescence) Western blotting detection reagents and secondary antibody rabbit IgG, peroxidase linked, from donkey, were from Amersharm Pharmacia Biotech (Freiburg, Germany). Antibodies against BAD were purchased from New England Biolabs GmbH (Frankfurt, Germany) and recombinant antibody against PP2Cbeta was prepared as described (Klumpp et al., 2000). Biotinylated secondary anti rabbit antibody and secondary Texas-red anti mouse antibody were obtained from Alexis (Grünberg, Germany).

1.2. Measurement of PP2Cbeta activity
Activity of PP2Cbeta was measured using [32P]-labelled casein as a substrate (Mc Gwoan et al. 1988, Klumpp et al., 1998). PP2Cbeta assays contained 30 mM Tris-HCl, pH 7.0, 0.1% 2-mercaptoethanol, 0.6 mg/ml BSA, 1 μM [32P] casein (5 x 10^4 cpm), and 10-100 ng PP2Cbeta in a final volume of 30 μl. PP2Cbeta is characterized by its dependence on divalent cations for activity. If not otherwise indicated, 1 mM magnesium acetate was present. Reactions were terminated after 10 min at 37°C adding trichloroacetic acid, the samples were centrifuged, and the supernatant was
analyzed for $[^{32}P]$-phosphate content. For determination of PP2Cbeta activity in HUVECs (5-10 g protein per assay), 100 nM okadaic acid was added to inhibit phosphatases type-1 and type-2A. Assays were additionally run in the absence and in the presence of magnesium ions (1 mM EDTA vs. 1 mM Mg$^{2+}$) to verify identity of PP2Cbeta.

1.3. Human umbilical vein endothelial cell and macrophages cell cultures
HUVECs were cultured in endothelial cell growth medium to 90% confluence. In all experiments cells were used at passages 3-6 and morphology was controlled prior to treatment. The cells were seeded at a density of 500000 cells per 9.6 cm$^2$ dishes. THP-1 monocytes were cultured in suspension at a density of 4 million cells/5 ml for four days. For differentiation to macrophages, monocytes received 100 ng/ml phorbol-12-myristic-13-acetate and 50 μM 2-mercaptoethanol (Dory et al., 1993). After 72 h culture, monocytes differentiated to macrophages and became adherent. Experiments were only accomplished with adherent and confluent macrophages (density > 1 million cells/9.6 cm$^2$ dishes). Both cell types were cultured at 37°C in an incubator supplemented with 5% CO$_2$ and 95% air.

1.4. Fatty acid treatments of HUVECs and macrophages
After the replacement of culture medium, HUVECs and macrophages were incubated for an additional 48 h. Cell cultures were then treated for 24h with oleic acid (200 μM) to induce apoptosis, with different concentrations of c) or with combinations of both. The FFAs were dissolved in dimethylsulfoxid (DMSO), and DMSO was used as control. The final concentration of DMSO in the culture medium did not exceed 0.5%.

1.5. Nuclear staining with Hoechst and Nile blue
Fatty acid-treated cells were washed with phosphate buffered saline (PBS), fixed for 30 min with paraformaldehyde and then incubated for 30 min with the DNA fluorochrome Hoechst 33258 (10 μg/ml) in methanol at room temperature in the dark. After washing with PBS, the nuclear morphology of HUVECs and macrophages was analyzed under a fluorescent microscope at an excitation wavelength of 350 nm and an emission wavelength of 450 nm. Cells showing shrunken or fragmented nuclei or chromatin condensation were counted as apoptotic cells. All values were expressed as means ± S.D.

Nile blue was used to reveal uptake of FFAs in ECs. After fixation with paraformaldehyde for 30 min, HUVECs were washed with PBS, stained with Nile blue solution (10 μg/ml) for 2 h and analyzed under a confocal laser scanning microscope at a wavelength of 488/525 nm.
1.6. **Protein determination**
The concentration of proteins was determined by BCA- or Lowry-assay (Lowry et al., 1951) using BSA as a standard.

1.7. **Western blots and immunocytochemistry**
BAD and PP2Cbeta were analyzed on 15% SDS-PAGE and transferred to nitrocellulose membranes. Blots were blocked for 1 h at room temperature with 5% non-fat milk powder in TBS-T (Tris buffered saline with Tween) (10 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1% Tween 20), incubated overnight at 4°C in TBS-T containing 5% BSA and primary antibody (anti-BAD and anti-PP2Cbeta 1:1000, respectively), followed by incubation in 5% non-fat milk powder in TBS-T for 1 h with peroxidase-conjugated anti-rabbit IgG (1:2500). Blots were developed with ECL reagent.

For immunocytochemistry, macrophages were fixed with paraformaldehyde for 30 min at room temperature. Cell membrane was permeabilized by incubation with 0.2% Triton X-100 for 5 min. Unspecific binding was blocked by 5% goat serum in PBS for 30 min. Cells were washed three times with PBS and subsequently incubated with polyclonal anti-PP2Cbeta antibody (1:100) overnight at 4°C. Macrophages were washed with PBS, exposed to a biotinylated secondary antibody (1:200) for 1 h at room temperature and then incubated with fluorescein isothiocyanate Avidin for 1 h in darkness. For the detection of BAD, the cells were blocked with 5% horse serum in PBS and incubated with BAD-antibody (1:200) at 4°C overnight. After washing with PBS, cells were exposed to Texas Red anti-mouse secondary antibody (1:200) for 1 h in dark. Controls were performed by omitting the primary antibody.

1.8. **Statistics**
All values are given as means ± S.D.. One way analysis of variance (ANOVA) followed by Scheffé's test was applied. PP2Cbeta-assays were run in duplicate, respectively, and independently repeated at least twice.

2. **Results**

2.1. **Co-localization of PP2Cbeta and BAD in macrophages**
The phosphorylation status of BAD is crucial to life and death (Zha et al., 1996). PP2Cbeta activated by unsaturated fatty acids is one of the phosphatases that acts on BAD (Klumpp et al., 2003). In previous work, we could demonstrate the co-localization of these two proteins in HUVECs (Hufnagel et al., 2005). Therefore, we first set out to search for PP2Cbeta and BAD in macrophages.
An antibody specific for PP2Cbeta and BAD showed the presence of these proteins also in macrophages (Fig. 1A). More detailed studies using immunocytochemistry localized PP2Cbeta and BAD within the cytosol of macrophages (Fig. 1B; 1C). Like in ECs, the demonstrated co-localization can be interpreted as a prerequisite for molecular interactions among PP2Cbeta and BAD in macrophages.

2.2. Effects of oleic acid and derivatives in macrophages

Oleic acid (18:1 cis-Δ^9) stimulates the activity of PP2Cbeta at least 10-fold, wherein the trans- and methylester derivatives do not (Klumpp et al., 1998). Overall, the structural requirements for activation of PP2Cbeta strikingly correlate with the induction of apoptosis in endothelial cells (Hufnagel et al., 2005). To evaluate this relation to macrophages, they were treated with 18:1 compounds and tested for survival. Exposure of macrophages to oleic acid (250 μM) for 24 h caused cell death (Fig. 2). In contrast, the corresponding trans derivative and the oleic acid methylester both were not harmful when administered in the same concentration range as oleic acid (Fig. 2).

2.3. Saturated fatty acids with a particular chain length inhibit the oleic acid-induced activation of PP2Cbeta in vitro.

In previous work we set out to search for regulatory components of PP2C (Klumpp et al., 1998). We found out that the activity of PP2C increases at a physiological concentration of 0.5-1 mM free Mg^{2+} after the addition of certain unsaturated fatty acids, like oleic acid, 10-12 fold. The result of our studies demonstrated that activators of PP2C must fulfill special structural requirements, as a minimum chain length of 15 C-atoms, cis-configuration and a free carboxyl group. For example SFAs are incapable of stimulating the activity of PP2C (Klumpp et al., 1998).

For instance, specific inhibitors of PP2C are not available and targeting subunits are unknown. To further study the regulation of PP2C, we wanted to elucidate if SFAs with different chain length are able to abolish the oleic acid-induced activation of PP2C. Treatment of PP2C with SFAs with chain lengths from C-14:0 until C-22:0 showed no activation of the enzyme (Fig.3). Double-treatment of PP2C with oleic acid and SFAs showed interesting new results: SFAs with a chain length shorter than 16 C-atoms, like myristic acid (C14:0), pentadecanoic acid (C15:0) and palmitic acid (C16:0), were not able to inhibit the activation-capacity of oleic acid, wherein SFAs with a chain length of more than 16 C-atoms, like heptadecanoic acid (C17:0), stearic acid (C18:0), arachidic acid (C20:0) and behenic acid (C22:0) inhibited the oleic acid induced activation of PP2C (Fig. 4) in vitro.
2.4. Effects of saturated fatty acids on oleic acid-induced apoptosis in HUVECs and macrophages

In further experiments we wanted to analyze if these "long-chain" SFAs are also able to abolish the oleic acid-induced apoptosis in ECs (Hufnagel et al., 2005) and macrophages. First of all, the different cell types were treated with different SFAs (C14:0- C22:0). Overall, the structural requirements for activation of PP2C correlated strikingly with the induction of apoptosis in ECs and macrophages, and the tested SFAs were not able to influence the viability of both cell types (Fig. 5 and 6). In parallel to the described in vitro results, we attempted in the next experimental step to elucidate how double-treatment of ECs and macrophages with SFAs and oleic acid influences the oleic acid-induced apoptosis. Myristic acid (C14:0), pentadecanoic acid (C15:0) and palmitic acid (C16:0) were not able to abolish the toxic affect of oleic acid, wherein heptadecanoic acid (C17:0) and stearic acid (C18:0) significantly inhibited the oleic acid-induced apoptosis in both cell types and are therefore protective (Fig. 7 and 8). Arachidic acid (C20:0) and behenic acid (C22:0) were almost insoluble in the endothelial cell growth medium and therefore could not be tested.

2.5. Fatty acid-uptake by HUVECs

To demonstrate the fatty acid-uptake, HUVECs were treated with Nile blue, a dye to explore the fate of lipids and to possibly perceive the location within ECs. Control cells received 0,5% DMSO as control for lipid solvent and subsequent staining resulted in a weak fluorescence reflecting endogenous lipids. In contrast, addition of myristic acid (C14:0), pentadecanoic acid (C15:0), palmitic acid (C16:0), heptadecanoic acid (C17:0) and stearic acid (C18:0) resulted in a bright fluorescence. Double treatment of both cell types with the same lipids and oleic acid yielded only cell debris in the presence of SFAs with a chain length from C14:0 up to C16:0. Heptadecanoic acid and stearic acid protect the ECs and could slow down the oleic acid-induced apoptosis (Fig. 9).

Finally we could demonstrate that inhibition of oleic acid-induced stimulation of PP2C activity by SFAs with a chain length longer than 16 C-atoms correlated with the inhibition of oleic acid-induced apoptosis in HUVECs and macrophages.
Claims

1. A serine/threonine protein phosphatase type 2C (PP2C) inhibitor which comprises at least one derivative of a fatty acid of the group consisting of
   - fatty acids
   - glycerol lipids
   - sphingolipids

2. Inhibitor according to claim 1, wherein said derivative of a fatty acid has a sufficient lipophilicity to inhibit fatty acid induced activation of PP2C and comprises a free carboxyl group.

3. Inhibitor according to claim 1, wherein said derivative of a fatty acid is a fatty acid, preferably one selected from the group consisting of saturated fatty acids (SFA), unsaturated fatty acids, straight and branched fatty acids.

4. Inhibitor according to claims 1 to 3, comprising at least one polyethylene moiety.

5. Inhibitor according to claim 3, wherein said fatty acid is a saturated fatty acid (SFA).

6. Inhibitor according to claim 5, wherein the saturated fatty acid has a chain length of more than 16 C-atoms.

7. Inhibitor according to claim 5 or 6, wherein the SFA has a chain length of C17:0 to C30:0, preferably C17: to C25:0.

8. Inhibitor according to claim 5 or 6, wherein it is selected from the group consisting of heptadecanoic acid, stearic acid, arachidic acid and behenic acid.

9. Inhibitor according to claim 1 or 2, wherein said derivative of a fatty acid is either a neutral or a phospholipid.

10. Inhibitor according to claim 9, which is a triglyceride.

11. Inhibitor according to claim 9, wherein the triglyceride comprises at least one of the fatty acids mentioned in the above claims.
12. Inhibitor according to at least one of the above claims 1 to 11, wherein said derivative of a fatty acid is substituted preferably with a halogen substituent.

13. Inhibitor according to at least one of the above claims which is present in form of a salt or an ester.

14. A pharmaceutical composition comprising at least one inhibitor according to at least one of the claims 1 to 13 as a physiologically active substance or a prodrug thereof and a pharmaceutically acceptable carrier, wherein said inhibitor has a sufficient lipophilicity to bind to and inhibit PP2C.

15. The pharmaceutical composition according to claim 14, wherein the inhibitor is present in form of a prodrug which releases the physiologically active substance upon conversion in the body.

16. The pharmaceutical composition according to claim 15, wherein said prodrug is a triglyceride or an ester and wherein said triglyceride or said ester comprises a fatty acid according to at least one of the claims 3 to 8.

17. Use of an inhibitor according to at least one of the claims 1 to 13 for the manufacture of a medicament for the treatment or prevention of fatty acid induced apoptosis in cells, wherein said inhibitor has a sufficient lipophilicity to inhibit fatty acid induced activation of PP2C and comprises a free carboxyl group.

18. Use according to claim 17, wherein said inhibitor is used for the manufacture of a medicament for the treatment or prevention of cardiovascular diseases, especially for the treatment or prevention of atherogenesis and/or arteriosclerosis, wherein said inhibitor has a sufficient lipophilicity to inhibit fatty acid induced activation of PP2C and comprises a free carboxyl group.

19. Use of a composition comprising according to any of claims 14 to 16 for the manufacture of a medicament for the treatment or prevention of cardiovascular diseases, especially for the treatment or prevention of atherogenesis and/or arteriosclerosis.

20. Use of the inhibitor according to any of claims 1 to 13 in an in vitro screening with PP2C and for testing apoptosis.

21. Use of the inhibitor according to claims 20 for testing the inhibition of the oleic acid-induced activation of PP2C in an in vitro screening.
22. Use of a compound according to any of claims 1 to 13 as an inhibitor for PP2C to inhibit fatty acid induced activation of PP2C.
Figure 2

(A)

![Bar chart showing PP2Cβ activity (%) for different fatty acids (500 μM).]

(B)

![Bar chart showing PP2Cβ activity (%) for different fatty acids (500 μM) with oleic acid 500 μM represented as a line.]

Oleic acid 500 μM
Figure 3

(A)

![Bar chart showing percentage of Apoptotic ECs (%) for different SFAs (200 μM).]

(B)

![Bar chart showing percentage of Apoptotic Macrophages (%) for different SFAs (250 μM).]
Figure 5

Oleic acid (18:1)

Control

Myristic acid (14:0)  Myristic acid (14:0) + Oleic acid (18:1)

Pentadecanoic acid (15:0)  Pentadecanoic acid (15:0) + Oleic acid (18:1)

Palmitic acid (16:0)  Palmitic acid (16:0) + Oleic acid

Heptadecanoic acid (17:0)  Heptadecanoic acid (17:0) + Oleic acid (18:1)

Stearic acid (18:0)  Stearic acid (18:0) + Oleic acid (18:1)
Figure 6

\[
\text{cis-Ölsäure}
\]

\[
\begin{align*}
\text{P}(\text{O})(\text{OH})_2 \\
\text{P}(\text{O})(\text{OH})_2
\end{align*}
\]

\[
\text{R} \\
(\text{z.B. } R = \text{CF}_3, \text{C}_2\text{F}_5, \text{CF}_2\text{P(O)(OEt)}_2, \\
\text{C(O)OEt})
\]

\[
\begin{align*}
\text{F} & \text{ F} \\
\text{X} & \text{O, NH, CF}_2
\end{align*}
\]

\[
\begin{align*}
\text{X} & \text{O, NH, CF}_2 \\
\text{R} & \text{CH}_3, \text{CF}_3
\end{align*}
\]
Molecular Formula = C_{37}H_{68}O_{12}P_{2}
Formula Weight = 766.876
Composition = C(57.95%) H(8.94%) O(25.04%) P(8.08%)

Composition = C(50.46%) H(8.94%) O(26.14%) P(14.46%)
Formula Weight = 428.438
Molecular Formula = C_{16}H_{38}O_{7}P_{2}

Composition = C(48.24%) H(8.10%) O(28.11%) P(15.55%)
Formula Weight = 398.369
Molecular Formula = C_{16}H_{32}O_{7}P_{2}
### A. CLASSIFICATION OF SUBJECT MATTER

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According to International Patent Classification (IPC) or to both national classification and IPC

### B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, MEDLINE, EMBASE, CHEM ABS Data

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>EP 1 374 862 A (EUCRO EUROP CONTRACT RES GMBH [DE]) 2 January 2004 (2004-01-02) column 6, paragraph 23 column 8, paragraphs 32,33 columns 8-9, paragraph 35 column 12, paragraph 52 - column 13, paragraph 53 column 16, paragraphs 72,73 column 19, paragraph 89 claims 1,6,7,12,15,19-24,42</td>
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Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:

**A** document defining the general state of the art which is not considered to be of particular relevance

**E** earlier document but published on or after the international filing date

**L** document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

**O** document referring to an oral disclosure, use, exhibition or other means

**P** document published prior to the international filing date but later than the priority date claimed

**“I”** later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

**“X”** document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

**“Y”** document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

**“S”** document member of the same patent family

Date of the actual completion of the international search: 27 September 2007

Date of mailing of the international search report: 09/10/2007

Name and mailing address of the ISA:

European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl
Fac. (+31-70) 340-3016

Authorized officer: Cielien, Elsie
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<td>KAAP S ET AL: &quot;Apoptosis by 6-O-palmitoyl-L-ascorbic acid coincides with JNK-phosphorylation and inhibition of Mg&lt;2+&gt;-dependent phosphatase activity&quot; BIOCHEMICAL PHARMACOLOGY 01 MAR 2004 UNITED STATES, vol. 67, no. 5, 1 March 2004 (2004-03-01), pages 919-926, XP002452316 abstract figure 1 page 925, column 1, paragraph 3 - column 2, paragraph 2</td>
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<td>BAUDOIN EMMANUEL ET AL: &quot;Unsaturated fatty acids inhibit MP2C, a protein phosphatase 2C involved in the wound-induced MAP kinase pathway regulation&quot; PLANT JOURNAL, vol. 20, no. 3, November 1999 (1999-11), pages 343-348, XP002452317 abstract page 344, column 1, paragraph 5 - column 2, paragraph 1 page 344, column 2, paragraph 3 page 345, column 1, paragraph 3 - column 2, paragraph 1 table 2</td>
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<td>WO 99/00116 A (YISSUM RES DEV CO [IL]) 7 January 1999 (1999-01-07) page 5, lines 1-13 claims 1, 6, 12, 15</td>
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<td>GB 1 088 692 A (MICHEL MARIE ANDRE GUERBET) 25 October 1967 (1967-10-25) page 1, lines 9-12 page 2, lines 88-108 claims 1, 8</td>
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<td>MÁS R: &quot;D-003: Antiplatelet therapy treatment of lipoprotein disorders&quot; DRUGS OF THE FUTURE 2004 SPAIN, vol. 29, no. 8, 2004, pages 773-786, XP008083359 ISSN: 0377-8282 abstract page 774, column 2, paragraphs 4,5 page 783, column 2, last paragraph</td>
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INTERNATIONAL SEARCH REPORT

Box II  Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. X Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
   Although claim 22 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. □ Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. □ Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III  Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. □ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. □ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. □ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. □ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

□ The additional search fees were accompanied by the applicant’s protest.

□ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (January 2004)
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