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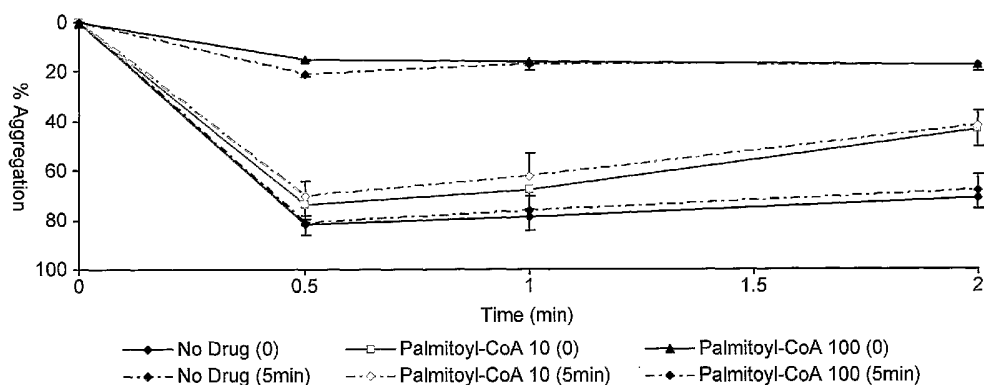
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(54) Title: MODULATION OF BLOOD CLOTTING



(57) Abstract: The invention provides a platelet aggregation modulator comprising coenzyme A or a derivative, mimetic, analogue, agonist, or antagonist thereof. In a preferred embodiment, the coenzyme A derivative comprises an acyl-coenzyme A, for example acetyl- or palmitoyl-coenzyme A. The platelet aggregation modulator binds at one or both of the P2Y₁ and P2Y₁₂ receptors on the platelet. The invention further provides a method of treating clotting disorders comprising the administration of a platelet aggregation modulator according to the invention.

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MODULATION OF BLOOD CLOTTING

This invention relates to modulatory agents for blood clotting. More particularly, the present invention relates to agents which modulate blood clotting by modulating the aggregation of platelets.

Blood clotting is the conversion of blood from a free-flowing liquid to a semi-solid gel. One of the essential elements for blood clotting is the aggregation, or clumping together, of platelets leading to the formation of a thrombus or haemostatic plug.

The ability to be able to modulate platelet aggregation is important since it is necessary to promote platelet aggregation in patients having clotting impaired bleeding disorders, as well as to inhibit platelet aggregation in the prevention of thrombotic disorders. For example, it is known that administration of adenosine diphosphate (ADP) will promote platelet aggregation and therefore clotting, and it is common practice to administer aspirin for the prevention of blood clotting to prevent thrombosis and associated thrombotic events such as myocardial infarction, ischaemic stroke, fissure of atherosclerotic plaques, occlusion of coronary, pulmonary or cerebral arteries, embolism, or ischaemic vascular disease.

Before platelets aggregate they are transformed from the inactive to the active state (known as platelet activation). Platelet activator substances influence platelets through special receptors on the platelet surface. Binding of the appropriate substrate to the receptors begins platelet activation. For example, occupation of ADP receptors by ADP, collagen receptors by collagen or thrombin receptors by thrombin produce changes within the platelet which lead to transformation of specific proteins, glycoproteins, into a form that binds fibrinogen. Once platelets are activated, fibrinogen becomes the "glue" that holds the aggregating platelets together. A single fibrinogen molecule forms a bridge between a glycoprotein complex on one platelet and a glycoprotein complex on another platelet. It is estimated that there are about 50,000 glycoprotein complexes on the surface of a platelet to which fibrinogen can bind. It is known that release of stored ADP amplifies this process, through the

occupation of ADP receptors and that the presence of thromboxane A₂ (TXA₂) further amplifies this process via an interaction with TXA₂ receptors.

It is also known that drugs which reduce the production of TXA₂, such as aspirin, reduce the amplification of platelet aggregation brought about by TXA₂; TXA₂ is produced in the platelets by the enzyme cyclooxygenase-1 (COX-1). Aspirin inhibits TXA₂ production by inhibiting COX-1, however, aspirin also inhibits another cyclooxygenase COX-2, and inhibits the production and/or action of COX-1 in other cells and tissues including the gastric mucosa where side effects are often seen in people taking aspirin for the prevention of thrombotic events.

Other receptors on the platelet which are implicated or involved in aggregation are known. For example, the drug, MRS2179, is known to inhibit platelet aggregation since it functions as a selective antagonist at the P2Y₁ receptor, one of the receptors known to be associated with ADP induced platelet aggregation.

However, the P2Y₁ receptor is ubiquitous in that it is found throughout the body and hence there may be widespread contraindications for therapies involving the modulation of the P2Y₁ receptor.

The main ADP receptors on platelets are P2Y₁ and P2Y₁₂ and both need to be occupied for a full platelet aggregation response. Occupation of P2Y₁ initiates the response and P2Y₁₂ sustains the response. Antagonists that act at either of these receptors are actual or potential antithrombotic agents. Currently the anti-thrombotic focus has mainly been on development of P2Y₁₂ antagonists such as clopidogrel and cangrelor. However, antagonists that act at the P2Y₁ receptor could also be effective antithrombotic agents. Also, a combination of antagonists that act at both of these receptors could be beneficial; Nylander *et al* (Br J Pharmacol 2004; 142:1325-1331) have reported a synergistic action between inhibitors of P2Y₁ and P2Y₁₂ receptors. Recently the present inventors have studied a range of compounds for their ability to inhibit ADP-induced platelet responses.

Lin *et al* (Biochim Biophys Acta 1976; 428:45-55) and Lascu *et al* (Biochem Biophys Res Commun 1988; 156:1020-5) described inhibition of platelet

aggregation by some acyl-coenzyme A thioesters including (in the case of *Lin et al*) palmityl-coenzyme A. However the studies performed were inappropriate for the determination of a physiological response. Platelets were studied after separation from blood cells and/or plasma and in the presence of non-physiological concentrations of divalent cations. Further, any selectivity for inhibition of aggregation induced by ADP was not apparent, and mechanisms of action were not defined.

The present inventors have determined, under new experimental conditions which are as close to physiological conditions as possible, that derivatives of coenzyme A (CoA) will inhibit the aggregation of platelets. Moreover, the present inventors have determined that CoA and its derivatives act as antagonists at more than one platelet receptor, namely the P2Y₁ and the P2Y₁₂ receptors. To advantage, the P2Y₁₂ receptor is not thought to be found in tissues other than cells in blood and as such any contraindications of modulating platelet aggregation by the agonistic or antagonistic action of CoA derivatives are lessened.

Accordingly, the present invention provides a platelet aggregation modulator comprising coenzyme A, a substituted coenzyme A, or a derivative, mimetic, analogue, agonist, or antagonist thereof.

The coenzyme A, substituted coenzyme A or its derivative, mimetic, analogue, agonist, or antagonist may be natural or synthetic. Preferably, the CoA is natural as this would lessen the possibility of adverse affects, since CoA is endogenous to the human or animal body. However, synthetically produced CoA derivatives or mimetics and the like may be modified to attenuate any unwanted contraindications.

In the description which follows the term "the CoA" or "a CoA" is intended to define natural or synthetic coenzyme A, which may be substituted, and derivatives, mimetics, analogues, agonists, or antagonists thereof, unless another compound is particularly or specifically described.

The modulation may be augmentory (up regulatory) or inhibitory (down regulatory). Preferably, the CoA is used to inhibit, platelet aggregation as this is the more commonly encountered need in medicine. In other words it is more

common that it is desired to prevent thrombosis and/or thrombotic events than to promote thrombosis since impaired clotting disorders are more rarely encountered.

Preferably, the platelet aggregation modulator binds at least one of the P2Y₁ and the P2Y₁₂ receptors. More preferably, the platelet aggregation modulator binds at both the P2Y₁ and the P2Y₁₂ receptors on the platelet. The present inventors have found that binding at either receptor site will inhibit platelet aggregation. The present inventors have also found that the use of two compounds acting at the P2Y₁ and the P2Y₁₂ receptors respectively, inhibits platelet aggregation to a greater extent than either agent alone; it is therefore believed that use of a single agent which binds to both the P2Y₁ and the P2Y₁₂ receptors will have at least an additive effect, if not a synergistic effect on the modulation of platelet aggregation.

In a preferred embodiment, the co-enzyme comprises a substituent moiety which is C₁-C₂₀ acyl. It has been found that increasing the length of the acyl group up to about 18 carbon atoms increases the degree of inhibition of platelet aggregation induced by ADP, collagen, TRAP, ADP and TRAP or ADP and serotonin (see Tables 1 and 2) More preferably, the moiety is C₂-C₁₈.

Preferably, the acyl moiety is saturated since it has shown that the presence of double bonds in the acyl groups reduces the degree of inhibition of platelet aggregation compared with the parent saturated derivative for example stearoyl- (18:0) compared with oleoyl- (18:1) compared with linoleoyl- coenzyme A (18:2) or arachidoyl- (20:0) compared with arachidonyl- coenzyme A (20:4): see Tables 1 and 3 and Figure 9. Preferably, the coenzyme A is selected from the group including acetyl-, butenoyl-, hexanoyl-, octanoyl-, decanoyl-, lauroyl-, myristoyl-, palmitoyl-, palmitoleoyl-, heptadecanoyl-, steroyl-, and arachidoyl- coenzyme A. The acyl-coenzyme A is preferably natural acyl-coenzyme A or a derivative, mimetic, analogue, agonist, or antagonist thereof.

TABLE 1

CoA derivatives	No. C in acyl group	No. C=C in acyl grp	ADP (μ M)				Collagen (4 μ g/ml)			
			3		10		- aspirin		+ aspirin	
			1 min	4 min	1 min	4 min	2 min	4 min	2 min	4 min
Vehicle alone			92 $\pm$ 1	78 $\pm$ 5	94 $\pm$ 1	89 $\pm$ 1	91 $\pm$ 1	88 $\pm$ 1	68 $\pm$ 6	75 $\pm$ 2
CoA	0	0	88 $\pm$ 1	55 $\pm$ 9	92 $\pm$ 2	88 $\pm$ 2	92 $\pm$ 1	87 $\pm$ 1	42 $\pm$ 10	62 $\pm$ 7
acetyl	2	0	89 $\pm$ 1	54 $\pm$ 9	92 $\pm$ 2	88 $\pm$ 2	91 $\pm$ 1	88 $\pm$ 1	56 $\pm$ 10	70 $\pm$ 6
butenoyl	4	1	91 $\pm$ 1	59 $\pm$ 8	93 $\pm$ 1	87 $\pm$ 2	92 $\pm$ 1	87 $\pm$ 1	60 $\pm$ 4	78 $\pm$ 3
hexanoyl	6	0	87 $\pm$ 2	42 $\pm$ 9	93 $\pm$ 1	87 $\pm$ 1	92 $\pm$ 1	88 $\pm$ 1	53 $\pm$ 7	65 $\pm$ 7
octanoyl	8	0	85 $\pm$ 3	35 $\pm$ 8	94 $\pm$ 1	85 $\pm$ 3	91 $\pm$ 1	89 $\pm$ 1	38 $\pm$ 5	57 $\pm$ 3
decanoyl	10	0	79 $\pm$ 3	23 $\pm$ 4	92 $\pm$ 1	77 $\pm$ 7	54 $\pm$ 9	88 $\pm$ 2	15 $\pm$ 2	24 $\pm$ 2
lauroyl	12	0	60 $\pm$ 9	20 $\pm$ 1	91 $\pm$ 2	67 $\pm$ 7	86 $\pm$ 3	88 $\pm$ 1	19 $\pm$ 3	31 $\pm$ 5
myristoyl	14	0	50 $\pm$ 6	20 $\pm$ 1	91 $\pm$ 2	57 $\pm$ 9	83 $\pm$ 3	87 $\pm$ 1	18 $\pm$ 2	25 $\pm$ 2
palmitoyl	16	0	24 $\pm$ 6	16 $\pm$ 2	89 $\pm$ 2	57 $\pm$ 10	65 $\pm$ 10	89 $\pm$ 1	20 $\pm$ 2	25 $\pm$ 3
palmitoleoyl	16	1	70 $\pm$ 6	24 $\pm$ 2	92 $\pm$ 2	73 $\pm$ 7	85 $\pm$ 3	89 $\pm$ 1	20 $\pm$ 2	29 $\pm$ 3
heptadecanoyl	17	0	30 $\pm$ 6	16 $\pm$ 1	89 $\pm$ 3	66 $\pm$ 11	73 $\pm$ 9	88 $\pm$ 1	17 $\pm$ 1	27 $\pm$ 2
stearoyl	18	0								
oleoyl	18	1	40 $\pm$ 6	18 $\pm$ 1	91 $\pm$ 3	47 $\pm$ 13	61 $\pm$ 11	88 $\pm$ 1	18 $\pm$ 1	26 $\pm$ 2
linoleoyl	18	2								
arachidoyl	20	0	58 $\pm$ 4	16 $\pm$ 2	87 $\pm$ 3	57 $\pm$ 12	77 $\pm$ 5	83 $\pm$ 6	16 $\pm$ 1	27 $\pm$ 2
arachidonoyl	20	4	84 $\pm$ 2	33 $\pm$ 8	94 $\pm$ 1	72 $\pm$ 11	90 $\pm$ 2	87 $\pm$ 2	20 $\pm$ 2	53 $\pm$ 4
MRS 2179			20 $\pm$ 2	20 $\pm$ 2	49 $\pm$ 9	31 $\pm$ 2	90 $\pm$ 1	89 $\pm$ 1	20 $\pm$ 2	29 $\pm$ 4
AR-C 69931			18 $\pm$ 1	17 $\pm$ 1	58 $\pm$ 10	23 $\pm$ 5	72 $\pm$ 1	67 $\pm$ 1	30 $\pm$ 7	36 $\pm$ 9

Effect of CoA derivatives on whole blood platelet aggregation in response to ADP and collagen: mean $\pm$ SEM, n=6

TABLE 2

The effect of different concentrations of CoA derivatives on ADP induced platelet aggregation measured at fixed time points

CoA derivatives	No.C in acyl group	No. C=C in acyl group	0.3μM ADP (%agg±sem) n=6			0.3μM ADP (%agg±sem) n=6			3μM ADP (%agg±sem) n=6		
			1min			4min			1min		
Vehicle alone	0	0	58±12			27±3			92±1		
			10μM	30μM	100μM	10μM	30μM	100μM	10μM	30μM	100μM
Palmitoyl	16	0	37±13	29±7	17±1	23±2	20±1	16±1	89±2	80±6	44±9
heptadecanoyl	17	0	33±11	23±5	17±1	21±1	18±2	18±2	88±3	79±8	37±9
stearoyl	18	0	35±10	20±3	17±2	20±1	16±1	17±2	87±3	75±8	26±7
									30±11	19±4	19±6

TABLE 3

CoA derivatives	No. C in acyl group	No. C=C in acyl group	3 μ M ADP (%agg $\pm$ sem) n=7		4 μ g/ml Collagen/Aspirin (%agg $\pm$ sem) n=6	
			1min	4min	2min	4min
Vehicle alone	0	0	92 $\pm$ 1	83 $\pm$ 3	82 $\pm$ 3	86 $\pm$ 2
palmitoyl	16	0	32 $\pm$ 9	9 $\pm$ 1	15 $\pm$ 1	27 $\pm$ 3
stearoyl	18	0	17 $\pm$ 6	10 $\pm$ 1	19 $\pm$ 2	26 $\pm$ 3
oleoyl	18	1	57 $\pm$ 13	10 $\pm$ 1	19 $\pm$ 3	36 $\pm$ 6
linoleoyl	18	2	65 $\pm$ 8	19 $\pm$ 4	20 $\pm$ 3	32 $\pm$ 5

In a second aspect, the present invention provides the use of a natural or synthetic coenzyme A or a derivative, mimetic, analogue, agonist, or antagonist thereof in the preparation of a medicament for the treatment of clotting disorders.

Preferably, the clotting disorder is a thrombosis or a thrombotic event such as myocardial infarction, ischaemic stroke, fissure of atherosclerotic plaques, occlusion of coronary, pulmonary or cerebral arteries, embolism, or ischaemic vascular disease.

Preferably, the natural or synthetic coenzyme A comprises an acyl-coenzyme A, for example acetyl-, butenoyl-, hexanoyl-, octanoyl-, decanoyl-, lauroyl-, myristoyl-, palmitoyl-, palmitoleoyl-, heptadecanoyl-, steroyl-, and arachidoyl- coenzyme A. The acyl-coenzyme A is preferably natural acyl-coenzyme A or a derivative, mimetic, analogue, agonist, or antagonist thereof.

In a further aspect, the present invention provides a method of treatment of clotting disorders, the method comprising the administration of an effective dose of a platelet aggregation modulator to a patient in need of treatment, in which the platelet aggregation modulator comprises natural or synthetic coenzyme A or a derivative, mimetic, analogue, agonist, or antagonist thereof.

Embodiments of the invention will now be described, purely by way of non-limiting example, with reference to and as illustrated by Figures 1 to 5 of the accompanying drawings, of which

Figure 1 is a graph showing platelet aggregation in the presence of 1 μ M ADP and 10 or 100 μ M of palmitoyl-coenzyme A;

Figure 2 is a graph showing platelet aggregation in the presence of 10 μ M ADP and 10 or 100 μ M of palmitoyl-coenzyme A;

Figure 3 is a graph showing the dose response curve for platelet aggregation at a range of ADP concentrations over 30 seconds;

Figure 4 is a graph showing the dose response curve for platelet aggregation at a range of ADP concentrations over 2 minutes;

Figure 5 is a bar chart showing platelet aggregation in PRP measured by light transmission (at 6 minutes) in response to a range of concentrations of collagen in the absence and presence of AR-C69931MX (1 μ M), palmitoyl-coenzyme A (100 μ M), MRS2179 (100 μ M) and the agents in combination;

Figures 6 are bar charts which show platelet aggregation without (6a) and with (6b) aspirin induced by collagen and the effects of various antagonists; where the mean (SEM), n=6;

Figure 7 is a bar chart showing platelet aggregation induced by serotonin plus ADP and the effects of various antagonists, where the mean (SEM), n=6 and concentrations are in μM ;

Figure 8 is a graph showing calcium ion mobilisation and the effects of various antagonists where mean (SEM), n=6 and concentrations are in μM ;

Figure 9 is a bar chart showing platelet aggregation induced by ADP and the effects of various antagonists, where the mean (SEM), n=6 and concentrations are in μM ; and

Figure 10 is a graph showing calcium ion mobilisation and the effects of various antagonists where the mean (SEM), n=6 and concentrations are in μM .

Example 1 - Coenzyme A, Acetyl-CoA and Palmitoyl-CoA Inhibit ADP Induced Platelet Aggregation

Studies of platelet aggregation in hirudinized whole blood from human volunteers. The use of hirudin is uncommon but is preferred to sodium citrate (which is the usual choice of anticoagulant for studies of platelet function) because it maintains the concentrations of plasma divalent cations at physiological levels. Platelet aggregation was measured using a platelet counting technique.

Studies of platelet aggregation in platelet-rich plasma derived from hirudinized whole blood from human volunteers. Platelet aggregation was measured using a light-absorbance aggregometry.

Studies of platelet shape change in platelet-rich plasma derived from hirudinized whole blood from human volunteers. Shape change was measured using the Biola aggregometer and by flow cytometry.

Studies of changes in intracellular Ca^{2+} in platelets in platelet-rich plasma derived from hirudinized whole blood from human volunteers. Ca^{2+} fluxes were measured by flow cytometry.

Studies of changes in the levels of cAMP in platelets in platelet-rich plasma derived from hirudinized whole blood from human volunteers.

Studies of vasodilator-stimulated phosphoprotein (VASP) phosphorylation in platelets in platelet-rich plasma derived from hirudinized whole blood from human volunteers. A flow cytometric technique was used.

Results obtained for CoA and its derivatives were compared with those obtained using the P2Y₁ antagonist MRS2179 (a selective P2Y₁ antagonist) and the P2Y₁₂ antagonist AR-C69931MX (cangrelor, a selective P2Y₁₂ antagonist).

Three agents (CoA, acetyl-CoA and palmitoyl-CoA) were tested.

Results obtained were as follows:

The substances inhibited ADP-induced platelet aggregation in hirudinised whole blood in a competitive fashion. Palmitoyl-CoA was the most potent of the agents that were tested.

The substances inhibited ADP-induced platelet aggregation in platelet-rich plasma derived from hirudinised whole blood. Again, palmitoyl-CoA was the most potent of the agents that were tested.

The agents proved to be selective inhibitors of ADP-induced platelet function in hirudinised whole blood and in platelet-rich plasma in that their effects on aggregation induced by 5-hydroxytryptamine (5-HT), platelet activating factor (PAF) and thrombin receptor activating peptide (TRAP) were identical to those of MRS2179 and AR-C69931MX used in combination; they only reduced the ADP component of the aggregation response.

The agents had no effect on the platelet shape change induced by agents other than ADP including 5-HT, PAF and TRAP.

The agents had no effect on increases in intracellular Ca²⁺ brought about by agents other than ADP including 5-HT, PAF and TRAP.

An effect of the substances at platelet P2Y₁ receptors was demonstrated by the following observations:

- 1) Like MRS2179, the agents inhibited ADP-induced shape change that is produced solely via P2Y₁ receptors under the conditions of the experiments that were performed.
- 2) Like MRS2179, the agents inhibited ADP-induced increase in intracellular Ca²⁺ that is produced solely via P2Y₁ receptors under the conditions of the experiments that were performed.

An effect of the substances at platelet P2Y₁₂ receptors was demonstrated by the following observations:

- 1) The agents inhibited the ADP-induced reduction in cAMP in forskolin-stimulated platelets; this occurs solely via P2Y₁₂ receptors under the conditions of the experiments that were performed.
- 2) The agents reduced inhibition of VASP phosphorylation by ADP in PGE₁-stimulated platelets; this occurs solely via P2Y₁₂ receptors under the conditions of the experiments that were performed.
- 3) The Ca²⁺ response to the combination of ADP and TRAP occurs in two phases mediated by P2Y₁ followed by P2Y₁₂. The combination of MRS2179 and AR-C69931MX reduces the Ca²⁺ response to that seen with TRAP alone. The CoAs behaved like the combination of MRS2179 and AR-C69931MX in this experiment.
- 4) The agents behaved like a combined P2Y₁ and P2Y₁₂ antagonist in its effects on collagen-induced platelet aggregation.

The fact that the agents did not act as antagonists at platelet P2X₁ receptors was demonstrated by the following observation:

- 1) The agents had no effect on the platelet shape change induced by α,β -methylene-ATP in platelet-rich plasma prepared under special conditions that prevent this receptor from desensitisation.
- 2) The agents had no effect on the Ca²⁺ mobilisation induced by α,β -methylene-ATP in platelet-rich plasma prepared under special conditions that prevent this receptor from desensitisation .

The present inventors therefore conclude that CoA inhibits platelet function through antagonism at both P2Y₁ and P2Y₁₂ receptors on platelets.

Example 2 - Palmitoyl-coenzyme A Behaves Like a Combined P2Y₁ and P2Y₁₂ Antagonist

Studies were performed in hirudinized whole blood or platelet-rich plasma (PRP) from normal healthy volunteers. Measurements of platelet aggregation, platelet shape change, intracellular cAMP and intracellular Ca²⁺ were performed. The results were compared with those obtained using the P2Y₁ antagonist MRS2179 and the P2Y₁₂ antagonist AR-C69931MX (cangrelor).

Three agents (coenzyme A, acetyl-coenzyme A and palmitoyl-coenzyme A) inhibited ADP-induced platelet aggregation in a competitive fashion. Palmitoyl-coenzyme A was the most potent. Palmitoyl-coenzyme A proved to be a selective inhibitor of ADP-induced aggregation in that its effects on aggregation induced by 5-HT and by PAF were identical to those of MRS2179 and AR-C69931MX; it only reduced the ADP component of the aggregation response. An effect at platelet P2Y₁ receptors was demonstrated: like MRS2179, palmitoyl-coenzyme A inhibited ADP-induced shape change and also ADP-induced increases in intracellular Ca²⁺. An effect at platelet P2Y₁₂ receptors was also demonstrated: palmitoyl-coenzyme A inhibited the ADP-induced reduction in cAMP in forskolin-stimulated platelets that occurs via this receptor. The inventors also examined its effect on changes in intracellular Ca²⁺ in response to the combination of ADP and thrombin receptor activating peptide (TRAP) which occurs in two phases. The first appears to be mediated by P2Y₁ in that it is inhibited by MRS2179, and the second by P2Y₁₂ and inhibited by AR-C69931MX. The combination of MRS2179 and AR-C69931MX reduces the Ca²⁺ response to that seen with TRAP alone. Palmitoyl-coenzyme A behaved like the combination of MRS2179 and AR-C69931MX. It also behaved like a combined P2Y₁ and P2Y₁₂ antagonist in its effects on collagen-induced platelet aggregation. Palmitoyl-coenzyme A had no effect on the platelet shape change induced by α,β -methylene-ATP indicating that it does not act at P2X₁ receptors.

The present inventors conclude that palmitoyl-coenzyme A inhibits platelet function through antagonism at both P2Y₁ and P2Y₁₂ receptors on platelets.

This compound, or a suitable derivative, could have a therapeutic role in controlling ADP-induced platelet function in man.

Example 3 - Effect of Palmitoyl-coenzyme A on Platelet Aggregation

Effects on platelet aggregation, platelet shape change, changes in intracellular Ca^{2+} , and changes in intracellular cAMP and cAMP-dependent VASP phosphorylation were investigated. The studies were performed in whole blood or platelet-rich plasma (PRP) from normal healthy volunteers. The $[\text{Ca}^{2+}]_i$ measurements were performed in HEPES buffer containing 1mM calcium.

Figure 1 and 2 show the inhibitory effects of palmitoyl-coenzyme A (10 and 100 μM) on the platelet aggregation that occurred in whole blood in response to 1 and 10 μM ADP. The aggregation was measured using residual platelet counting. In each case aggregation was inhibited better using 100 μM than 10 μM palmitoyl-coenzyme A, and there was better inhibition of aggregation induced by 1 than by 10 μM ADP. Pre-incubation with the antagonist was not required for the inhibitory effects to be realised.

Figures 3 and 4 show the dose response curves for the inhibition of ADP-induced aggregation. The inventors measured aggregation at both 30s (Figure 3) and at 2 min (Figure 4). These were compared with data generated using MRS2179 used in place of palmitoyl-coenzyme A, and with AR-C69931. The pattern of inhibition for all three agents was very similar. In terms of potency palmitoyl-coenzyme A and MRS2179 appear to be similar.

MRS2179 is a selective antagonist at P2Y_1 receptors on platelets which is one of the two receptors that is required for aggregation induced by ADP. AR-C69931 is a selective antagonist at P2Y_{12} receptors; this is the other receptor that is required. Clearly palmitoyl-coenzyme A inhibited ADP-induced platelet aggregation in a similar way to each of these receptor antagonists.

The inventors also looked at the effects of palmitoyl-coenzyme A, MRS2179 and AR-C69931 on PAF-induced platelet aggregation in whole blood. As with most platelet agonists there is some involvement of ADP but this is only seen at 2 min, not 30s. All three antagonists produced virtually the same effect at 2 min

and there was no inhibition at 30s. The inventors obtained similar results when they used 5-HT to induce the aggregation response (results not provided).

Figure 5 shows the effects of palmitoyl-coenzyme A, MRS2179 and AR-C69931 on collagen-induced platelet aggregation. Here the experiments were performed in PRP and the measurements were via light transmission. There was some inhibition by all three antagonists. AR-C69931 was more effective than MRS2179 indicating the importance of P2Y₁₂ receptors in the collagen response. Interestingly, the inhibitory effect of palmitoyl-coenzyme A was more like that of AR-C69931 than MRS2179. In the same experiments the inventors also looked at the effects of combining different pairs of antagonists. It should be noted that the combination of MRS2179 and AR-C69931 was more effective than either of these antagonists used alone. Also MRS2179 and palmitoyl-coenzyme A in combination provided better inhibition than either agent used alone. Also AR-C69931 and palmitoyl-coenzyme A in combination provided better inhibition than either agent used alone. These data suggest that palmitoyl-coenzyme A may be acting at both types of ADP receptor rather than just one of them.

The inventors used the Biola laser aggregometer to obtain information on the effects of the antagonists on platelet shape change in PRP. The shape change induced by ADP was prevented by palmitoyl-coenzyme A and by MRS2179, but not by AR-C569921. There was no effect of any of the agents on the shape changes induced by PAF and by 5-HT and by TRAP (results not provided). Palmitoyl-coenzyme A had no effect at P2X₁ receptors since there was no inhibition of the shape change induced by α,β -methylene-ATP which is a selective agonist at this receptor. These data provide good evidence that palmitoyl-coenzyme A is acting at P2Y₁ receptors.

The inventors looked at the effects of palmitoyl-coenzyme A on the changes that occurred in $[Ca^{2+}]_i$ in platelets induced by ADP, TRAP and the combination of these. The results were compared with those obtained using MRS2179 and/or AR-C6993. Palmitoyl-coenzyme A appears to be working as an inhibitor of both P2Y₁ and P2Y₁₂. Incidentally, here the inventors used the palmitoyl-coenzyme A at a concentration of 10 μ M rather than 100 μ M as it was more potent in the HEPES buffer that was used than it was in plasma.

In further experiments using PAF alone and 5-HT alone as platelet agonists none of the antagonists produced any inhibition of increases in $[Ca^{2+}]$, that occurred (results not provided).

The inventors carried out some further experiments to look for effects of palmitoyl-coenzyme A at P2Y₁₂ receptors. ADP acting at P2Y₁₂ brings about a reduction in cAMP in platelets in forskolin-stimulated platelets and P2Y₁₂ antagonists such as AR-C69931MX prevent this. The inventors found that palmitoyl-coenzyme A inhibited the ADP-induced reduction cAMP in forskolin-stimulated platelets, but it was not quite as good as AR-C69931. MRS2179 had no effect. Further experiments on VASP phosphorylation (which is dependent on cAMP) provided almost identical data.

Overall these results are compatible with good antagonism by palmitoyl-coenzyme A at P2Y₁ and with the additional antagonism at P2Y₁₂.

Example 4

Additional studies were performed to look further at the effect of a range of different acyl-coenzyme A's on platelet function. The inventors wanted further investigate the importance of both P2Y₁ and P2Y₁₂ receptors in platelet aggregation induced by different platelet agonists and combinations of agonists, and to assess the impact of different acyl groups in coenzyme A on the results obtained.

In these experiments platelet aggregation was measured in human whole blood (collected into hirudin as anticoagulant) or in platelet-rich plasma (PRP) derived from this. Aggregation was measured using a platelet counting technique (whole blood and PRP) and by conventional light absorbance aggregometry (PRP). The agonists used were adenosine diphosphate (ADP), collagen, thrombin receptor activating peptide (TRAP), serotonin, combinations of ADP and TRAP, and combinations of serotonin and TRAP. The coenzyme A's that examined were coenzyme A, acetyl coenzyme A (2:0), butenoyl coenzyme A (4:1), hexanoyl coenzyme A (6:0), octanoyl coenzyme A (8:0), decanoyl coenzyme A (10:0), lauroyl coenzyme A (12:0), myristoyl coenzyme A

(14:0), palmitoyl coenzyme A (16:0), palmitoleoyl coenzyme A (16:1), heptadecanoyl coenzyme A (17:0), stearoyl coenzyme A (18:0), oleoyl coenzyme A (18:1), linoleoyl coenzyme A (18:2), arachidoyl coenzyme A (20:0) and arachidonoyl coenzyme A (20:4). The results were compared with those obtained in the presence of MRS2179 (a selective P2Y₁ antagonist), AR-C69931 (a selective P2Y₁₂ antagonist) and combinations of these. Examples of the results obtained are provided in Figures 6-10 and Tables 1-3.

Studies of Ca²⁺ mobilisation were also performed using platelet labelled with the Ca²⁺ indicator Fluo-3. Here platelets were stimulated with the combination of ADP and TRAP and the effects of the various antagonists compared.

The inventors found that when ADP contributes to platelet stimulation in combination with another agonist (e.g. during collagen-induced platelet aggregation, during TRAP-induced platelet aggregation or when platelets respond to the combination of ADP and TRAP or serotonin and TRAP) both P2Y₁ and P2Y₁₂ receptors contribute to the overall extent of the aggregation response. Thus antagonism at both receptors provides more complete inhibition of aggregation than antagonism at one receptor alone. See Figure 6a for collagen-induced aggregation and the effects of palmitoyl coenzyme A (used at 100µM), the maximal effect of MRS2179 (used alone at 100µM), and the maximal effect of AR-C69931 (used alone at 1µM). It can be seen that combinations of MRS2179 and AR-C69931, of MRS2179 and palmitoyl coenzyme A, and of AR-C69931 and palmitoyl coenzyme A all increased the degree of inhibition of platelet aggregation achieved. Equivalent results were obtained in the presence of aspirin, used to obviate additional contributions to aggregation via TXA₂ synthesis (Figure 6b). Similar results were also obtained when the combination of ADP (0.3µM) and serotonin (10µM) were used (Figure 7). Results obtained for Ca²⁺ mobilisation induced by the combination of ADP (0.3µM) and TRAP (20µM) are shown in Figure 8. Here the effects of palmitoyl coenzyme A (10µM) were equivalent to those of the combination of MRS2179 (100µM) and AR-C69931 (1µM).

The inventors also found that increasing the length of the acyl group up to about n = 18 carbons (stearoyl coenzyme A) increased the degree of inhibition of aggregation induced by ADP, collagen, TRAP, ADP and TRAP, or ADP and

serotonin (See e.g. Tables 1 and 2). However, the presence of double bonds in the acyl groups reduces the degree of inhibition of aggregation compared with the parent saturated derivative, e.g. stearoyl (18:0) compared with oleoyl (18:1) compared with linoleoyl -coenzyme A (18:2) or arachidoyl (20:0) compared with arachidonoyl -coenzyme A (20:4). See Table 1 and 3 and Figure 9. Similar results were obtained for Ca^{2+} mobilisation induced by ADP (0.3 μM) and TRAP (20 μM) (Figure 10).

In conclusion these additional experiments have further emphasised the importance of both P2Y_1 and P2Y_{12} receptors in platelet function, have confirmed antagonism by coenzyme A's via both receptor subtypes, and have provided additional information on the impact of different acyl groups on coenzyme A on the capacity of these molecules to inhibit platelet function via interaction with P2Y_1 and P2Y_{12} receptors.

CLAIMS

1. A platelet aggregation modulator comprising coenzyme A a substituted coenzyme A or a derivative, mimetic, analogue, agonist, or antagonist thereof.
2. A platelet aggregation modulator according to Claim 1, in which the coenzyme A a substituted coenzyme A or its derivative, mimetic, analogue, agonist, or antagonist is natural or synthetic.
3. A platelet aggregation modulator according to Claim 1 or Claim 2, in which the coenzyme A a substituted coenzyme A or its derivative, mimetic, analogue, agonist, or antagonist is synthetically produced and is modified to attenuate any unwanted contraindications.
4. A platelet aggregation modulator according to any one of claims 1 to 3, in which the coenzyme A derivative comprises an acyl-coenzyme A.
5. A platelet aggregation modulator according to claim 4, in which the acyl moiety is C₁-C₂₀ in length.
6. A platelet aggregation modulator according to claim 4, in which the acyl moiety is C₁-C₁₈ in length.
7. A platelet aggregation modulator according to any one of claims 4-6, in which the acyl-coenzyme A is acetyl acetyl-, butenoyl-, hexanoyl-, octanoyl-, decanoyl-, lauroyl-, myristoyl-, palmitoyl-, palmitoleoyl-, heptadecanoyl-, steroyl-, and arachidoyl- coenzyme A.
8. A platelet aggregation modulator according to any one of claims 1 to 7, in which the modulation is inhibition.

9. A platelet aggregation modulator according to claim 7, in which the inhibition is to prevent thrombosis and/or thrombotic events.
10. A platelet aggregation modulator according to claim 8 or claim 9, in which the inhibition is to prevent myocardial infarction, ischaemic stroke, fissure of atherosclerotic plaques, occlusion of coronary, pulmonary or cerebral arteries, embolism, or ischaemic vascular disease.
11. A platelet aggregation modulator according to any one of the preceding claims, in which the platelet aggregation modulator binds at least one of the P2Y₁ and the P2Y₁₂ receptors.
12. A platelet aggregation modulator according to any one of claims 1 to 10, in which the platelet aggregation modulator binds at both the P2Y₁ and the P2Y₁₂ receptors on the platelet.
13. The use of a natural or synthetic coenzyme A or a derivative, mimetic, analogue, agonist, or antagonist thereof in the preparation of a medicament for the treatment of clotting disorders.
14. Use according to claim 13, in which the clotting disorder is a thrombosis or a thrombotic event.
15. Use according to claim 13 or claim 14, in which the clotting disorder or thrombotic event is myocardial infarction, ischaemic stroke, fissure of atherosclerotic plaques, occlusion of coronary, pulmonary or cerebral arteries, embolism, or ischaemic vascular disease.
16. Use according to any one of claims 13 to 15, in which the natural or synthetic coenzyme A comprises an acyl-coenzyme A.

17. Use according to claim 16, in which the acyl-coenzyme A is acetyl-, butenoyl-, hexanoyl-, octanoyl-, decanoyl-, lauroyl-, myristoyl-, palmitoyl-, palmitoleoyl-, heptadecanoyl-, steroyl-, and arachidoyl- coenzyme A.
18. Use according to any one of claims 13 to 17 in which the medicament further comprises one or more additional platelet aggregation modulators.
19. A method of treatment of clotting disorders, the method comprising the administration of an effective dose of a platelet aggregation modulator to a patient in need of treatment, in which the platelet aggregation modulator comprises natural or synthetic coenzyme A or a derivative, mimetic, analogue, agonist, or antagonist thereof.
20. The method according to claim 19, in which the method further comprises administration of one or more additional platelet aggregation modulators before, after or in combination with the platelet aggregation modulator comprising natural or synthetic coenzyme A or a derivative, mimetic, analogue, agonist, or antagonist thereof.

FIG. 1

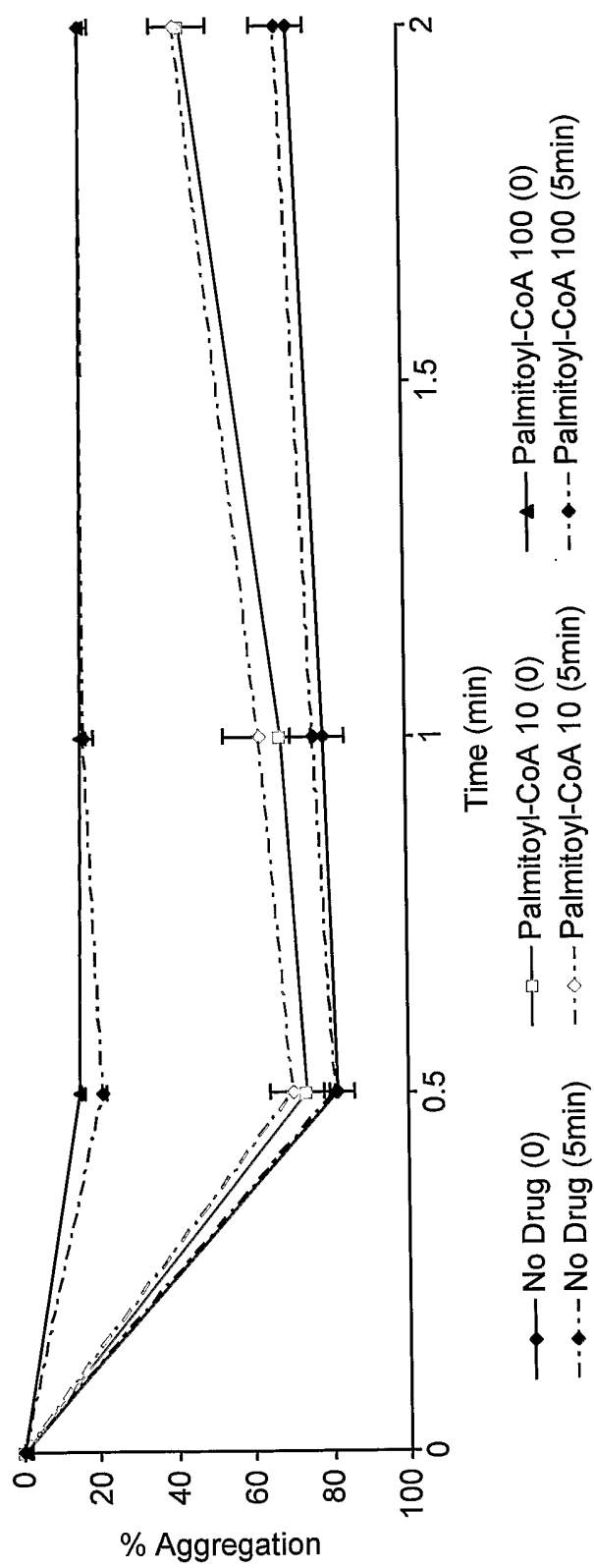
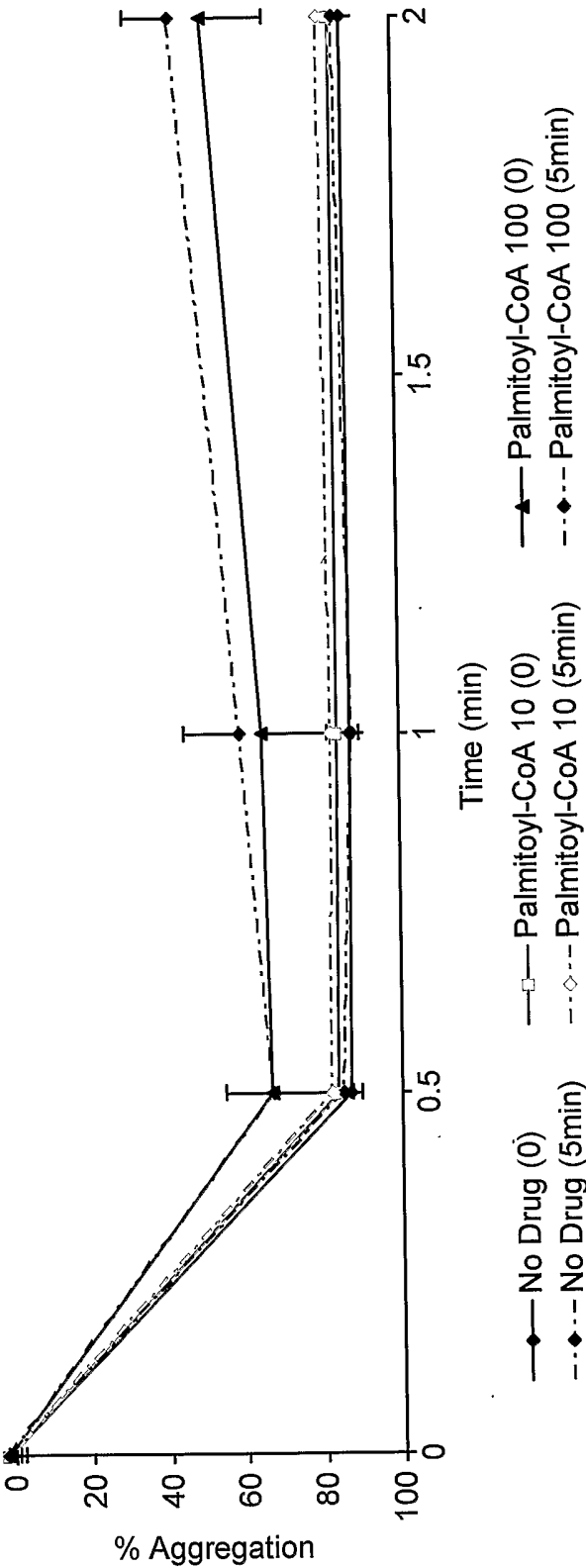


FIG. 2



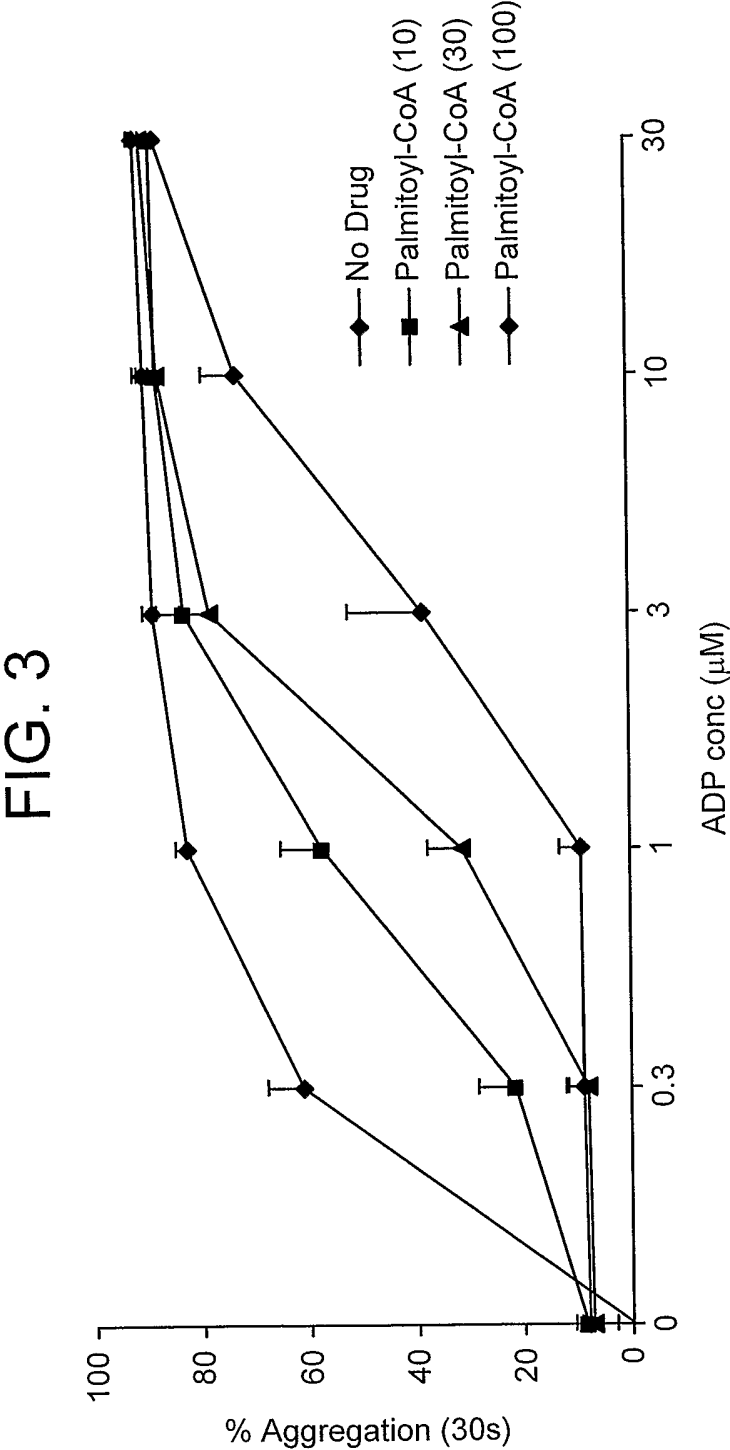
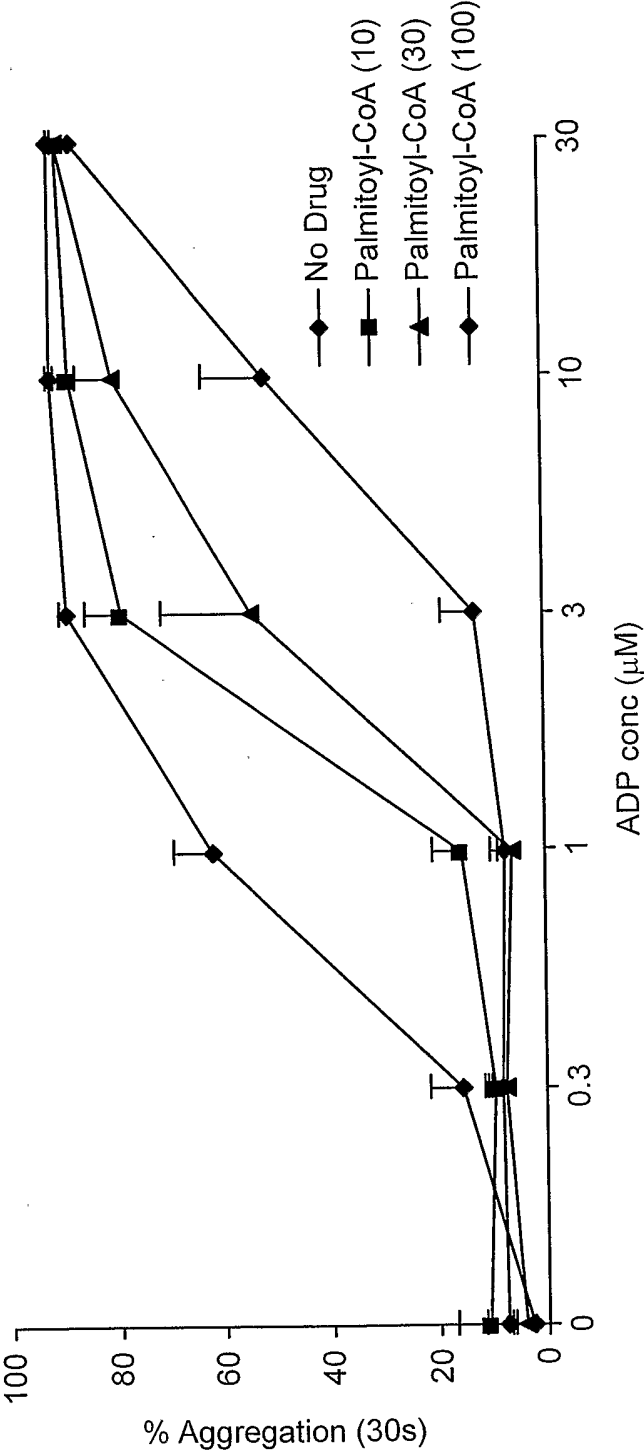
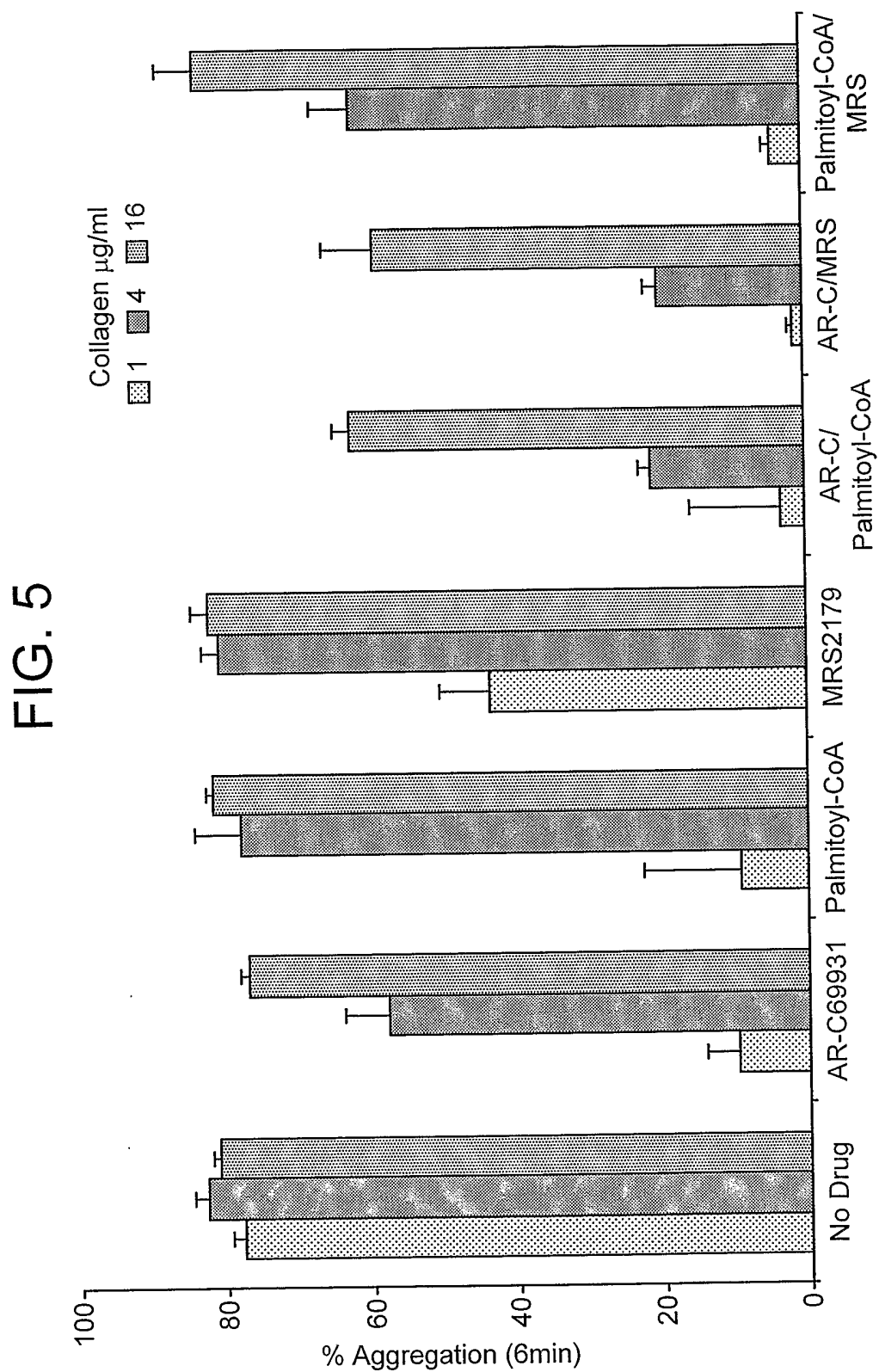


FIG. 4



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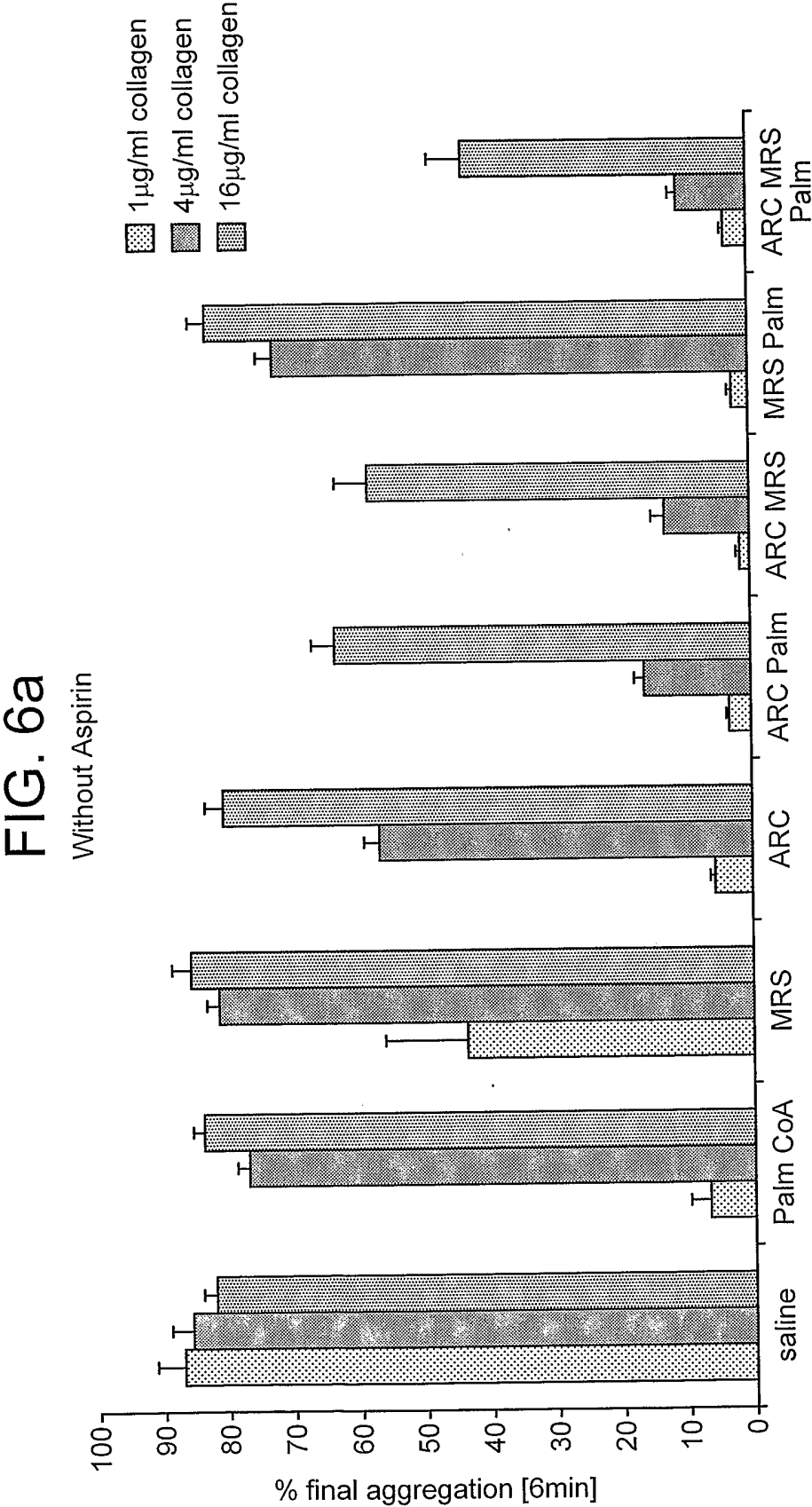


FIG. 6b
With Aspirin

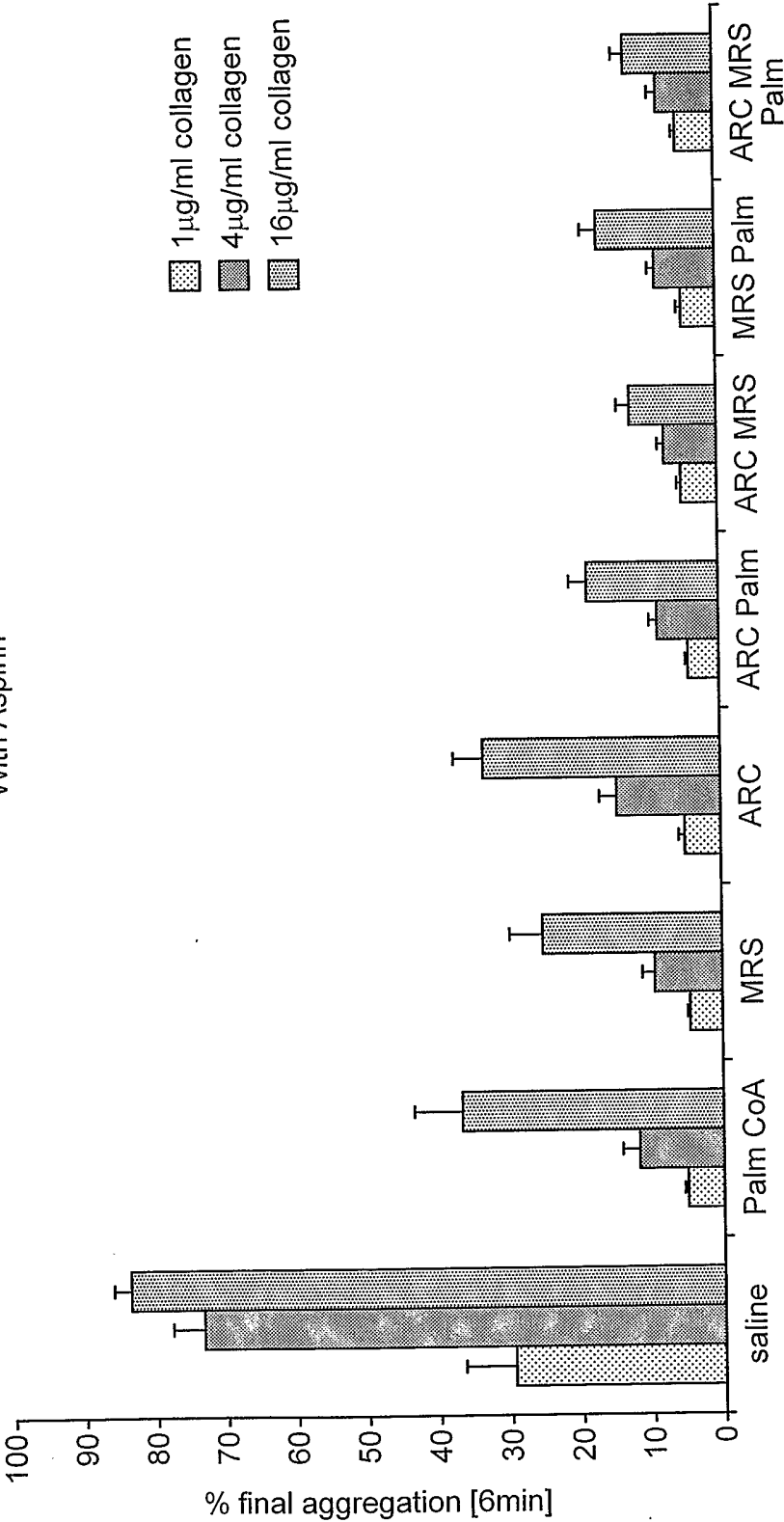
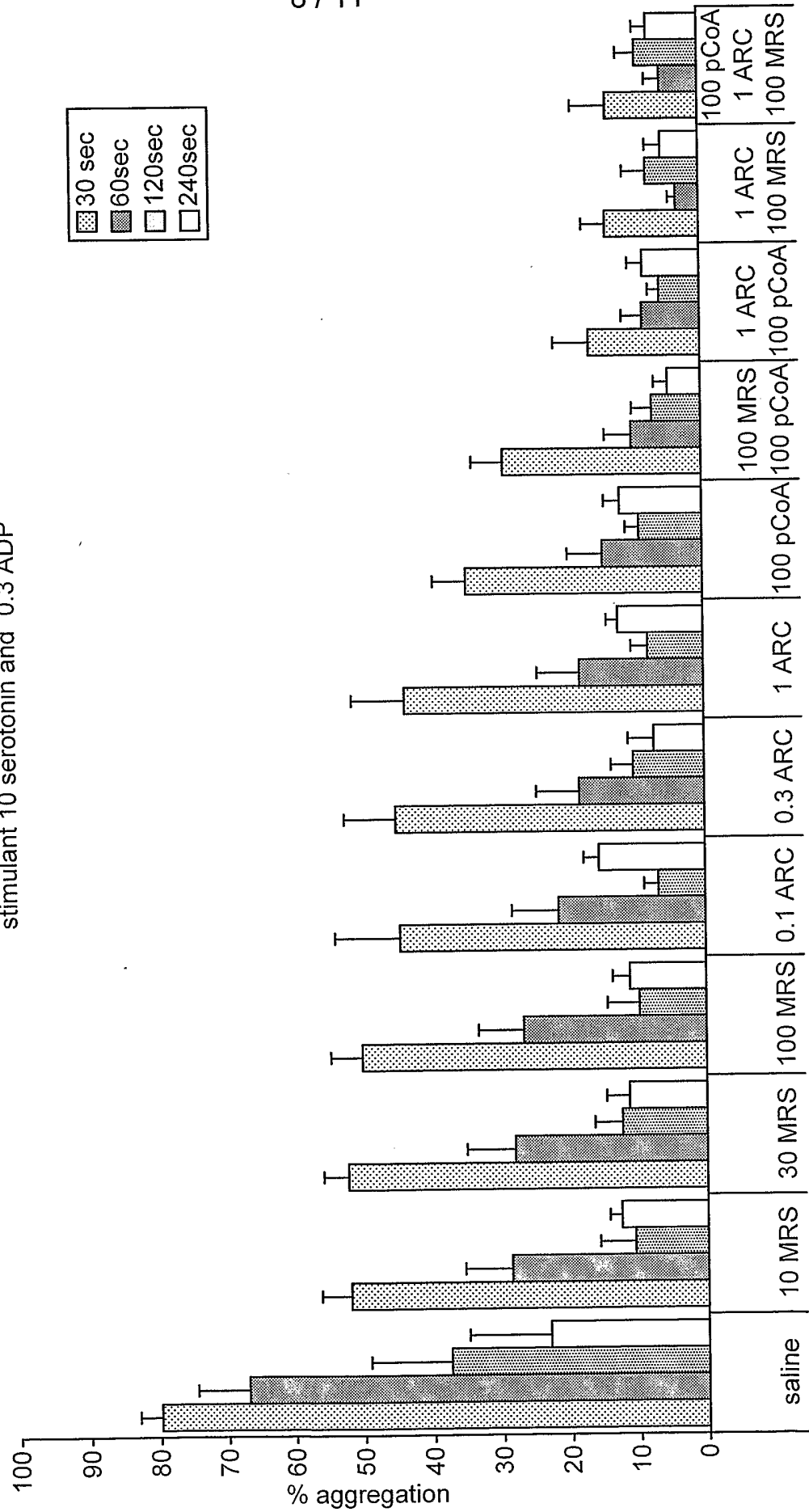


FIG. 7
stimulant 10 serotonin and 0.3 ADP



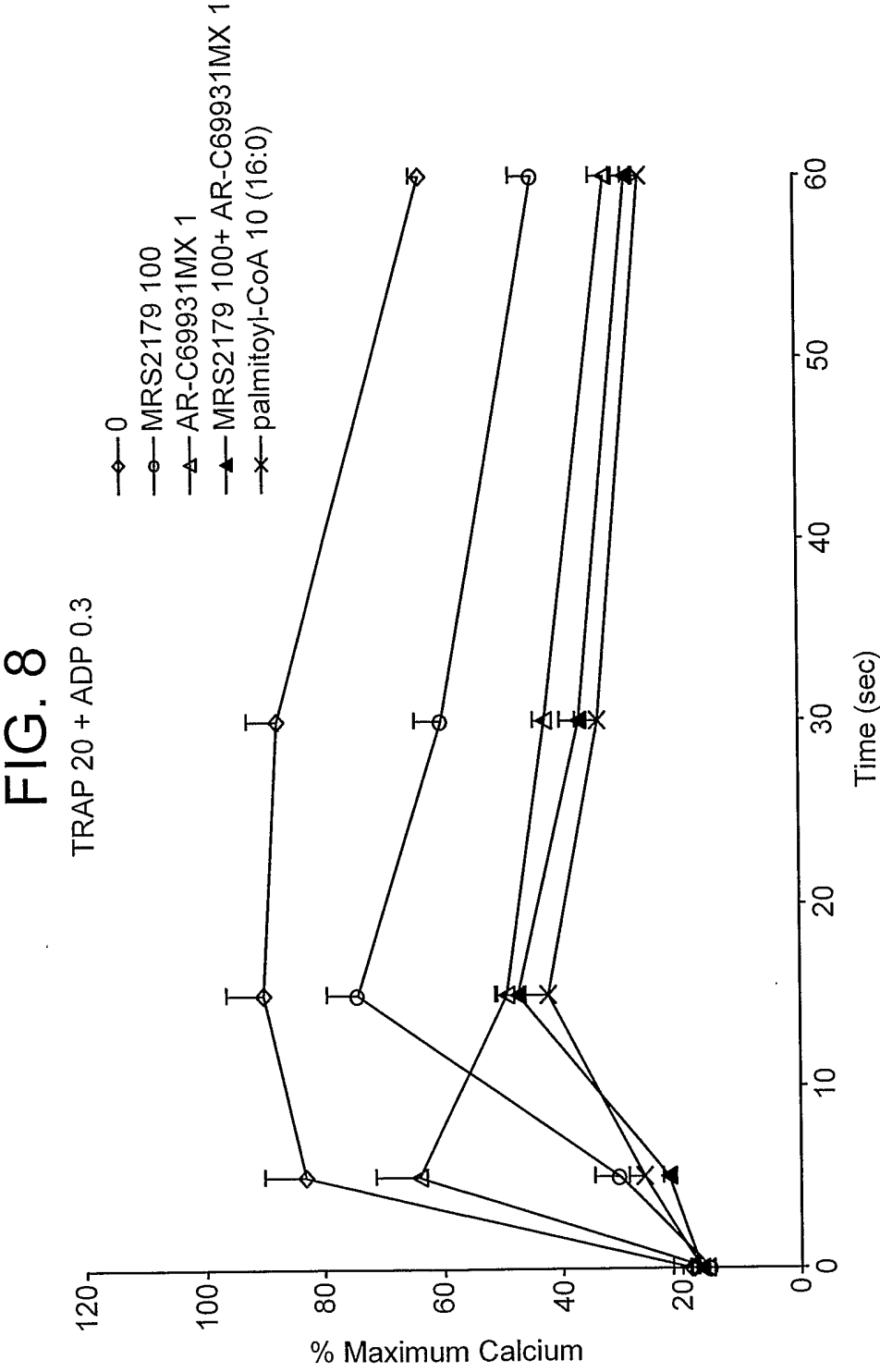
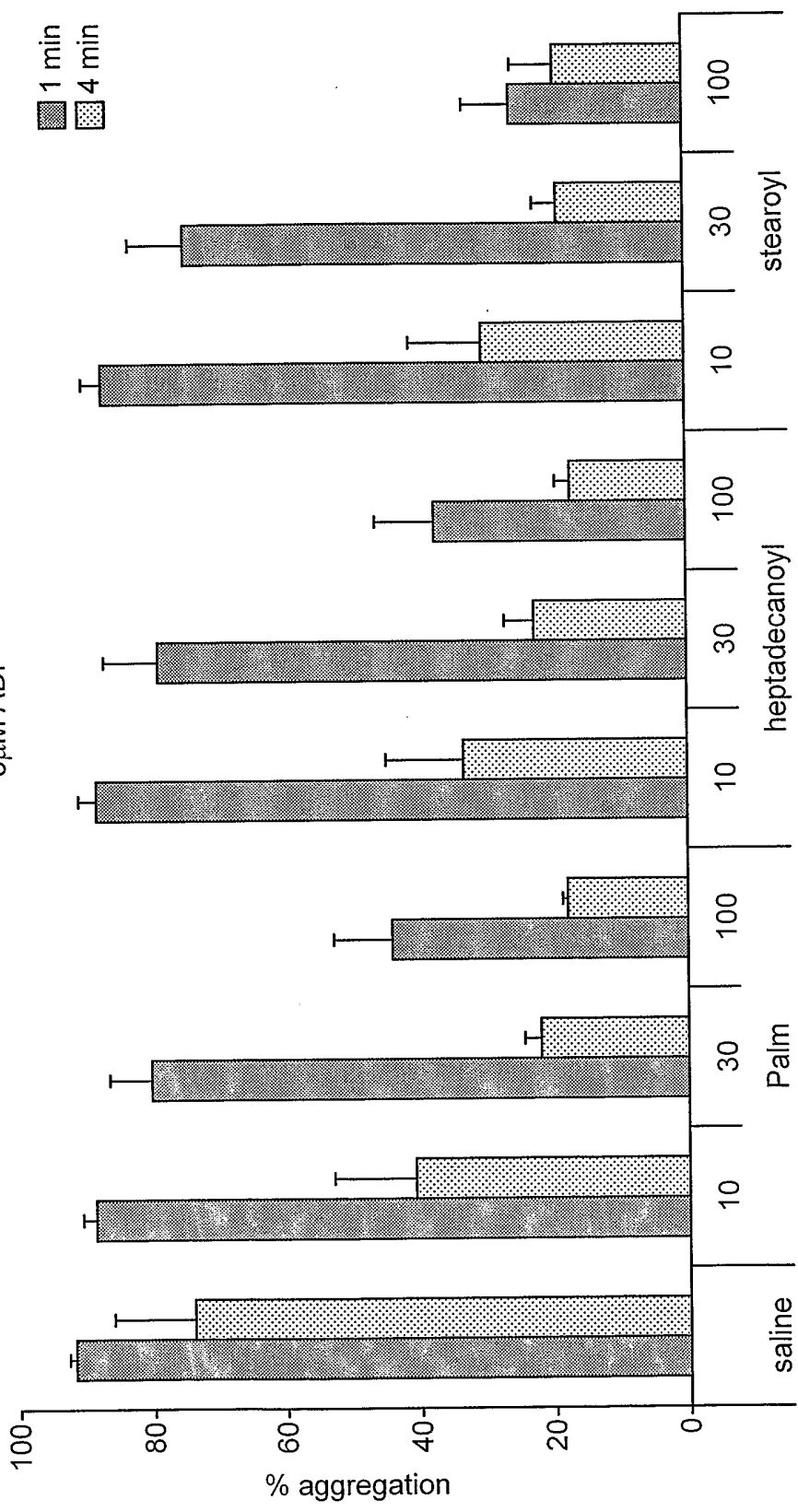


FIG. 9
Platelet aggregation in whole blood
3 μ M ADP



INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2005/004480

A. CLASSIFICATION OF SUBJECT MATTER
A61K31/22 A61P7/02

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 A61P

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, EMBASE, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	the whole document	1-20
X	US 6 509 035 B1 (REN GENFU ET AL) 21 January 2003 (2003-01-21) column 1, line 38 - line 48 claims 1-19	1-3,8-12
X	LIN C Y ET AL: "INHIBITION OF PLATELET AGGREGATION BY ACYL COENZYME A THIO ESTERS" BIOCHIMICA ET BIOPHYSICA ACTA, vol. 428, no. 1, 1976, pages 45-55, XP009061398 ISSN: 0006-3002	1-12
Y	the whole document	1-20
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☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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Date of the actual completion of the international search

10 February 2006

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INTERNATIONAL SEARCH REPORT

International application No
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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Information on patent family members

International application No
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