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(54) Title: NOVEL ANTI-PROLIFERATIVE EFFECTS OF SODIUM BUTYRATE (57) Abstract The present invention provides a method of inhibiting the proliferation of smooth muscle cells comprising the step of contacting said cells with a pharmacologically effective amount of sodium butyrate. Also provided is a method of inhibiting the formation of cerebrovascular disease in a human comprising the step of administering to said human a pharmacologically effective amount of sodium butyrate.		

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-1-

NOVEL ANTI-PROLIFERATIVE EFFECTS OF SODIUM BUTYRATE**BACKGROUND OF THE INVENTION****Field of the Invention**

5 The present invention relates generally to the fields of neurology and protein chemistry. More specifically, the present invention relates to a novel anti-proliferative effects of sodium butyrate.

Description of the Related Art

10 Atherosclerotic plaque development involves several synchronized events such as adhesion, migration, proliferation and transformation of cells and these events are mediated by growth factors and cytokines. Recently, attention has been focused on platelet-derived growth factor (PDGF) in the etiology
15 of atherosclerosis because of its mitogenic, chemotactic, and vasoconstrictor effects on vascular smooth muscle cells (VSMC). In view of the fact that vascular smooth muscle cell proliferation contributes to hypertension, atherosclerosis and restenosis after angioplasty, development of new pharmaceutical
20 agents to inhibit vascular smooth muscle cell proliferation is highly desirable.

 Sodium butyrate, a natural bacterial fermentation product produced in mammalian colon has been shown to have a potent anti-proliferative effect on several cancer cells. In
25 addition to its antineoplastic activity, sodium butyrate induces changes in cellular morphology, alters the expression of cellular genes, modulates hormone action and hormone receptors as well as growth factor receptors. Sodium butyrate has also been shown to inhibit the high fat diet-induced mammary tumorigenesis.
30 Additionally, sodium butyrate has been used in preliminary clinical trails to treat certain acute leukemias and stable butyrate derivatives are developed with a view to use these compounds to treat mammary carcinoma.

 The prior art is deficient in the lack of effective
35 means of inhibiting the proliferation of vascular smooth muscle

-2-

cells and retarding the atherosclerotic process involved in cerebrovascular disease. The present invention fulfills this longstanding need and desire in the art.

SUMMARY OF THE INVENTION

5 In one embodiment of the present invention, there is provided a method of inhibiting the proliferation of smooth muscle cells comprising the step of contacting said cells with a pharmacologically effective amount of sodium butyrate.

10 In another embodiment of the present invention, there is provided a pharmaceutical composition, comprising pharmacologically effective amount of sodium butyrate and a pharmaceutically acceptable carrier.

15 In yet another embodiment of the present invention, there is provided a method of inhibiting the formation of atherosclerotic plaques in a human comprising the step of administering to said human a pharmacologically effective amount of sodium butyrate.

20 In still yet another embodiment of the present invention, there is provided a method of inhibiting the formation of cerebrovascular disease in a human comprising the step of administering to said human a pharmacologically effective amount of sodium butyrate.

25 Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

30 So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, 35 that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

-3-

Figure 1 shows the influence of sodium butyrate on platelet derived growth factor (PDGF)-BB-induced protein tyrosine phosphorylation. Quiescent smooth muscle cells in serum-free DMEM are exposed for 5 minutes with no addition (Lane 1), 5 mM sodium butyrate (Lane 2), preincubation in 5 mM sodium butyrate for 30 minutes and then with 5 mM sodium butyrate plus 50 ng/ml PDGF -BB (Lane 3), 50 ng/ml PDGF-BB (Lane 4) or 5 mM sodium butyrate plus 50 ng/ml PDGF -BB (Lane 5) at 37°C. After incubation, cells were lysed. Equal amounts of proteins were used for western blotting and immunoprecipitation with anti-P-tyr antibody. Whole-cell proteins and immunoprecipitates are analyzed by electrophoresis on 7.5% SDS-polyacrylamide gels, transferred to PVDF membrane and immunoblotted with appropriate antibody. Immunoreactivity is detected by ECL. Figure 1A shows the Anti-P-tyr immunoblot of whole-cell proteins. Figure 1B shows the Anti- β -PDGFR immunoblot of anti-P-Tyr immunoprecipitates.

Figure 2 shows the effect of sodium butyrate on PDGF -BB-activated β -PDGFR autophosphorylation and its association with tyrosine phosphorylated proteins. Density-arrested cells were treated with similar additives as described in the description to Figure 1 for 5 minutes at 37°C. Cells lysates were prepared and processed for immunoprecipitation and immunoblotting. Similar amounts of cell lysates are used for immunoprecipitation with anti- β -PDGFR antibody. Figure 2A shows the anti-P-tyr immunoblot of anti- β -PDGFR immunoprecipitates. Figure 2B shows the anti- β -PDGFR immunoblot of anti- β -PDGFR immunoprecipitates.

Figure 3 shows the effect of sodium butyrate on PDGF -BB-stimulated tyrosine phosphorylation of GTPase activating protein (GAP) and its association with β -PDGFR. Quiescent smooth muscle cells were incubated in serum-free DMEM with similar additions as described in the description to Figure 1. Equal amounts of cell lysates were immunoprecipitated with anti-GAP and processed for immunoblotting. Figure 3A shows the anti-P-tyr immunoblot of anti-GAP immunoprecipitates. Figure 3B shows the anti-GAP immunoblot of anti-GAP immunoprecipitates.

-4-

Figure 4 shows the influence of sodium butyrate on PDGF-BB-provoked tyrosine phosphorylation of phospholipase C γ (PLC γ) and co-precipitation of β -PDGFR with PLC γ . Quiescent smooth muscle cells were treated as described above for 5 minutes at 37°C. Equal amounts of cell lysates are immunoprecipitated with anti-PLC γ and processed for immunoblotting. Figure 4A shows the Anti-P-tyr immunoblot of anti-PLC γ immunoprecipitates. Figure 4B shows the Anti-PLC γ immunoblot of anti-PLC γ immunoprecipitates.

Figure 5 shows the PDGF-BB-Induced β -PDGFR degradation. Serum-starved smooth muscle cells were incubated in serum-free DMEM containing no additions, 50 ng/ml PDGF -BB, 5 mM sodium butyrate or 50 ng/ml PDGF -BB plus 5 mM sodium butyrate for indicated periods of time. Cell lysates were prepared and equal amounts of cell lysates are subjected to polyacrylamide gel electrophoresis. After electrophoresis β -PDGFR degradation is detected by probing with anti β -PDGFR antibody.

Figure 6 shows the sodium butyrate effect on PDGF-BB-induced and uninduced mitogen activated protein kinase (mitogen activated protein-kinase) activity. Density arrested smooth muscle cells were exposed to treatments as described above for 5 minutes at 37°C. Cell lysates were prepared and equal amounts of cell lysates were used for immunoprecipitation of mitogen activated protein-Kinase. Mitogen activated protein Kinase activities in the immuno-complexes were measured by incubating with MBP in the presence of [γ -³²P] ATP. Proteins were separated by SDS-PAGE on 12% polyacrylamide gels and autoradiographed. The phosphorylated MBP bands were excised and incorporated radioactivities are measured. Figure 6A shows the auto-radiogram of phosphorylated MBP. Figure 6B shows the quantitation of MBP phosphorylation by Cerenkov counting.

Figure 7 shows the influence of sodium butyrate on serum and PDGF -BB-induced transcription of *c-fos*, *c-jun* and *c-myc*. Effect of sodium butyrate on serum and PDGF-BB-induced transcription of *c-fos* (A), *c-jun* (B) and *c-myc* (C) are assessed at the time of their maximal transcription (*c-fos*, 30 minutes; *c-jun* and *c-myc*, 60 minutes). Serum-deprived smooth muscle cells

-5-

in serum-free DMEM were incubated with no addition (Lane 3), 10% fetal calf serum (Lane 1), 10% fetal calf serum plus 5 mM sodium butyrate (Lane 2), 5 mM sodium butyrate (Lane 4), preincubation in 5 mM sodium butyrate for 30 minutes and subsequent incubation in 5 mM sodium butyrate plus 50 ng/ml PDGF -BB (Lane 5), 50 ng/ml PDGF -BB (Lane 6) or 50 ng/ml PDGF -BB plus 5 mM sodium butyrate (Lane 7) for 30 minutes or 60 minutes. After required time of incubation, total RNA are isolated and the levels of *c-fos*, *c-jun* and *c-myc* transcripts are measured. The RNA blots were probed with G3PDH probe to normalize the quantity of RNA loaded on to each lane.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to a method of inhibiting the proliferation of smooth muscle cells comprising the step of contacting said cells with a pharmacologically effective amount of sodium butyrate. In this method of the present invention, the amount of sodium butyrate desirable to produce an anti-proliferative effect in smooth muscle cells is from about 0.5 mM to about 5 mM.

The present invention also provides a pharmaceutical composition, comprising pharmacologically effective amount of sodium butyrate and a pharmaceutically acceptable carrier.

The present invention is also directed to a method of inhibiting the formation of atherosclerotic plaques in a human comprising the step of administering to said human a pharmacologically effective amount of the composition of claim 3.

The present invention is also directed to a method of inhibiting the formation of cerebrovascular disease in a human comprising the step of administering to said human a pharmacologically effective amount of the composition of claim 3. To produce this effect, the sodium butyrate composition is administered in a dose of from about 100 mg/kg to about 1000 mg/kg.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

-6-

EXAMPLE 1**Materials**

Recombinant platelet derived growth factor (PDGF)-AA, (PDGF)-AB and (PDGF)-BB were from Gibco BRL Life Technologies. Sodium butyrate was from Fluka. Anti-phosphotyrosine, anti- β PDGF-receptor, anti-GTPase activated protein and anti-PLC- γ were purchased from Upstate Biotechnology Incorporated. Anti-mitogen activated protein-Kinase, anti-RafI and protein A/G plus-agarose conjugate were from Santa Cruz Biotechnology. [Methyl- ^{3} H]-thymidine, Myo -[2- ^{3} H] inositol and [γ - ^{32} P]-ATP were from Amersham Corporation.

EXAMPLE 2**Cell culture**

A7r5 rat aortic smooth muscle cells were obtained from American Type Culture Collection (Rockville, MD). These cells were grown in Dulbecco's Modified Eagle's medium (DMEM) fortified with 10% fetal calf serum, 50 units/ml penicillin and 50 μ g/ml streptomycin. Cells were seeded into 25 cm² flasks for the [^{3} H]-thymidine and myo [^{3} H]-inositol incorporation studies. For other studies cells were plated in 150 mm culture dishes. A7r5 cells were grown to 90% confluency at 37°C in a humidified atmosphere of 5% CO₂. Culture media were changed every other day. Experiments were performed using 90% confluent cells. Quiescent cells were obtained by incubating 90% confluent cells either in 0.1% serum containing medium for 48 hours or in serum-free medium for 24 hours. For DNA synthesis measurements 10 ng/ml PDGF -AA, -AB or -BB were used. Concentration of PDGF -BB used in inositol phosphate production studies was 30 ng/ml. In protein phosphorylation, protein association, *in vitro* kinase assays and gene transcription experiments, 50 ng/ml PDGF-BB was used. A concentration of sodium butyrate was 5 mM unless otherwise mentioned.

EXAMPLE 3**Measurements of DNA synthesis**

Quiescent cells were stimulated with PDGF in a serum-free medium containing 1 μ Ci/ml [^{3} H]-thymidine in the presence or absence of sodium butyrate. After 24 hours of incubation, the

-7-

cells were washed three times with phosphate buffered saline (PBS) and fixed in 2 ml of 5% ice-cold trichloroacetic acid (TCA) for 1 hour at 4°C. Fixed cells were washed three times with 5% ice-cold TCA and dissolved in 0.2 N NaOH/0.1% SDS. Amount of radioactivity incorporated was measured.

EXAMPLE 4

Phosphoinositide assay

Quiescent cells, prelabeled with myo-[³H] inositol (4 μCi/ml) for 48 hours were incubated in PBS containing 20 mM lithium chloride for 10 minutes. After 10 minutes, phosphoinositide assay was initiated by the addition of test compound and terminated after 1 minute by adding 2 ml of 5% TCA. Trichloroacetic acid supernatants, collected by centrifugation were extracted three times with ether and neutralized before separating inositol phosphates on anion exchange resin (AG 1-x48; 200-400 mesh; Formate form). Inositol monophosphate (IP), inositol biphosphate (IP₂) and inositol triphosphates (IP₃) were separated according to the protocol described by Berridge et. al., Biochem. J. 212, 473-482 (1983). Radioactivity in each of these inositol phosphates was determined by liquid scintillation counting.

EXAMPLE 5

Immunoblots

Cells were grown in 150 mm culture plates and washed with ice-cold PBS three times and lysed in 1 ml of RIPA buffer (20 mM Tris, pH. 7.4, 137 mM NaCl, 10% glycerol, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 2 mM EDTA, 1 mM PMSF, 20 μM leupeptin and 1 mM Sodium orthovanadate) by gently rocking for 20 minutes at 4°C. Lysates were then sheared with syringe and needle and clarified by centrifugation. The clear lysates were normalized for total cellular protein and equal amount of protein from each lysate was fractionated on SDS-polyacrylamide gel and transferred to immobilon PVDF membrane and blocked for 1 hour in blocking buffer containing 5% non-fat dry milk and 0.1% Tween 20 in Tris-buffered saline (TBS). Blots were washed three times with Tris-buffered saline containing 0.1% Tween 20 (TBST) and probed with appropriate primary antibody for 1 hour in TBS.

-8-

Blots were washed three times with TBST and then incubated with appropriate second antibody conjugated to horseradish peroxidase. Immunoreactive proteins were identified using ECL chemiluminescence detection kit from Amersham corporation.

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EXAMPLE 6**Immunoprecipitations**

Cell lysates were prepared as described above using RIPA buffer. Equal amount of proteins were incubated with β PDGF-receptor, ras GTPase activated protein, PLC- γ or P-Tyr antibody and protein A/G plus agarose-conjugates for about 12 hours at 4°C. Immunoprecipitated complexes were collected by centrifugation, washed once with RIPA buffer, twice with 0.5 M LiCl in 0.1 M Tris, pH.7.4 and once with 10 mM Tris, pH.7.4. Immunoprecipitated proteins were eluted with SDS-PAGE buffer and separated on 7.5% gels. Separated proteins were transferred to immobilon PVDF membrane and used for immunoblotting with suitable antibodies.

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15**EXAMPLE 7****Mitogen-activated protein kinase assay**

Cell lysates were prepared by using nonionic detergent buffer containing 1% Triton X-100, 20 mM Tris, pH-8.0, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM PMSF, 20 μ M leupeptin and 1 mM sodium orthovanadate as described above. Similar amounts of proteins were incubated with polyclonal mitogen activated protein-Kinase antibody for 2 hours at 4°C and protein A/G plus agarose-conjugate is added for the last 30 minutes. Immunocomplexes were collected, washed twice with nonionic detergent buffer followed by one wash in TBS supplemented with 1 mM sodium orthovanadate and 5 mM benzamidine. Mitogen activated protein-kinase activity was measured by incubating the immunoprecipitation with 40 μ l of kinase reaction mix (30 mM Tris, pH.8.0) 10 mM $MgCl_2$, 20 μ M ATP, 1 mM DTT, 5 mM benzamidine, 10 μ Ci of [γ - ^{32}P] ATP and 6 μ g of myelin basic protein and incubating for 30 minutes at 37°C. The reaction was terminated by adding 20 μ l of 4x SDS-PAGE buffer. Reaction products were analyzed by SDS-PAGE on a 12% gel followed by autoradiography.

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-9-

Phosphorylated MBP was excised from the gel and the radioactivity was measured.

EXAMPLE 8

RNA isolation and Northern analysis

5 Quiescent smooth muscle cells grown in 150 mm culture dishes were exposed to different treatments for times indicated in the relevant result section. Total RNA was isolated using TRI reagent method (Molecular Research Center, Cincinnati, OH). For Northern analysis, 15 µg of total RNA was denatured in sample
10 loading buffer containing 1 µg/ml ethidium bromide and electrophoresed through 1% agarose formaldehyde gel. RNA was transferred to S&S MaxS nytran membrane by downward alkaline transfer method using S&S Turbo Blotter transfer system. Transferred RNA was fixed to nytran membrane by UV irradiation.
15 Random primed c-fos, c-jun and c-myc probes were used for hybridization according to the protocol of Gilbert and Church, Proc. Natl. Acad. Sci. 81:1991-1995 (1984). Blots were exposed to Hyperfilm-MP (Amersham Corp.) with intensifying screen at -70°C for 1-3 days.

EXAMPLE 9

Measurements of DNA synthesis

Incorporation of [³H]-thymidine into DNA was used as a measure of cell proliferation. Growth arrested smooth muscle cells were exposed to PDGF-AA, -AB or -BB with or without 5 mM
25 sodium butyrate for 24 hours in the presence of [³H]-thymidine. After 24 hours, the amount of [³H]-thymidine incorporated was measured. Table I shows that although PDGF is a potent mitogen for smooth muscle cells, not all three isoforms are equally potent. PDGF-AA was the least potent and PDGF -BB was the most
30 potent of the three isoforms of PDGF. Accordingly, PDGF-AA, PDGF-AB and PDGF-BB stimulated DNA synthesis by 3-, 5- and 9-fold respectively compared to uninduced smooth muscle cells. However, in the presence of 5 mM sodium butyrate, PDGF-induced DNA synthesis is inhibited more than 80% irrespective of the
35 isoform used. At 5 mM concentration, sodium butyrate has no significant effect on DNA synthesis compared to uninduced smooth muscle cells.

-10-

TABLE I

Inhibition of PDGF-AA, PDGF-AB and PDGF-BB stimulated rat aortic smooth muscle cell proliferation by sodium butyrate

	Addition	dpm x 10 ⁻³ / flask
5	None	559 ± 154
	Sodium butyrate	650 ± 154
	PDGF-AA	1759 ± 314
	PDGF-AA + Sodium butyrate	677 ± 78
	PDGF-AA	2882 ± 229
10	PDGF-AB + Sodium butyrate	445 ± 46
	PDGF-BB	5168 ± 604
	PDGF-BB + Sodium butyrate	645 ± 23

Following growth arrest in serum-free DMEM medium for 24 hours, cells were induced to proliferate in serum-free DMEM medium containing [³H]-thymidine with PDGF-AA, PDGF-AB and PDGF-BB in the presence or absence of 5 mM sodium butyrate. Each value is mean ± S.D. of a minimum of four measurements and representative of three experiments.

Since PDGF BB is the most effective isoform of PDGF, it was used to demonstrate the effective concentration of sodium butyrate on PDGF-BB-induced proliferation (Table II). The effect of sodium butyrate on PDGF-BB-induced DNA synthesis was measured between the concentration of 1 μM to 10 mM. Between 1 μM and 0.5 mM, sodium butyrate had no effect on PDGF-BB-induced DNA synthesis (data not shown). Between 0.5 mM and 1 mM sodium butyrate there was about 20% inhibition of PDGF-BB-induced DNA synthesis. Higher than 1 mM concentration of sodium butyrate exhibit significant inhibition of PDGF-BB-induced DNA synthesis. Between 5 mM and 8 mM, there was complete inhibition of PDGF -BB-induced DNA synthesis. However, at these concentrations the smooth muscle cells appeared phenotypically normal although there was a decrease in PDGF-induced cellular protein content (data not shown). Since 5 mM concentration of sodium butyrate almost

-11-

completely inhibited PDGF-BB-induced DNA synthesis without any visual adverse effect on smooth muscle cells, this concentration of sodium butyrate was selected to illustrate the mechanism of inhibition of PDGF -BB-induced proliferation.

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TABLE II

Effect of sodium butyrate on PDGF-BB-induced proliferation of rat aortic smooth muscle cells.

<u>Sodium butyrate</u>		<u>dpm x 10⁻³ / flask</u>	
		<u>-PDGF-BB</u>	<u>+PDGF-BB</u>
10	0	1031 ± 258	3904 ± 184
	1 mM	1268 ± 177	3071 ± 370
	3 mM	1218 ± 63	1236 ± 151
	5 mM	1173 ± 99	1054 ± 29
	8 mM	882 ± 145	777 ± 71

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Rat aortic smooth muscle cells are grown to 90% confluency and then rendered quiescent by incubating in serum-free DMEM medium for 24 hours. Quiescent cells are stimulated in serum-free medium containing [³H]-thymidine with PDGF-BB in the presence or absence of different concentrations of sodium butyrate. Data presented are mean ± S.D. and representative of two independent experiments.

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EXAMPLE 10PDGF-BB induced down-regulation of β -PDGF-receptor (β -PDGF-R)

In view of understanding the mechanism of inhibition of PDGF -BB-induced proliferation by sodium butyrate afforded by the present invention, the influence of sodium butyrate on PDGF-BB stimulated β -PDGF-R modification was next shown because the PDGF -BB signal is transduced through the receptor into the cell. Cell lysates obtained from smooth muscle exposed to no addition, PDGF -BB, sodium butyrate and PDGF-BB plus sodium butyrate for different lengths of time were analyzed by SDS-PAGE followed by immunoblotting with anti- β -PDGF-R to study the receptor down-regulation (Figure 1). As shown in Figure 1A, β -PDGF-R down-

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-12-

regulation occurred after 2 hours of incubation of smooth muscle cells with PDGF-BB. This time-dependent, PDGF-BB-activated, down-regulation of β -PDGF-R was unaffected by sodium butyrate (Figure 1C). Cells exposed to PDGF-BB plus sodium butyrate exhibit a similar pattern of β -PDGF-R down-regulation as PDGF-BB treated cells. On the other hand, β -PDGF-R undergo no degradation when smooth muscle cells were exposed to sodium butyrate alone (Figure 1B) indicating sodium butyrate did not interfere with ligand-stimulated receptor down-regulation

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EXAMPLE 11

PDGF-BB-induced protein tyrosine phosphorylation

Tyrosine phosphorylation of proteins has been implicated in mitogenic signal pathway. Therefore, the PDGF-BB stimulated protein phosphorylation and its response to sodium butyrate treatment was probed. Total cellular lysate as well as anti-P-tyrosine immunoprecipitates were prepared from smooth muscle cells and subject to immunoblotting with anti-P-tyrosine and anti-PDGFR, respectively (Figures 2A and 2B). Cells exposed to PDGF-BB or PDGF-BB plus sodium butyrate exhibit increased protein tyrosine phosphorylation. In addition there were no obvious differences in the protein tyrosine phosphorylation profile between the two treatments (Figure 2A). Similarly, pre-treatment of cells for 30 minutes with sodium butyrate and subsequent exposure to PDGF-BB plus sodium butyrate had no effect on PDGF-BB-induced protein tyrosine phosphorylation profile (Figure 2A). In the absence of PDGF-BB there was no increased phosphorylation of protein.

PDGF-BB-induced, β -PDGF-R autophosphorylation was also unaffected by sodium butyrate irrespective of whether the cells were exposed to sodium butyrate before PDGF-BB or together with PDGF-BB (Figure 2B). In the absence of PDGF-BB as well as in the presence of sodium butyrate alone there is no β -PDGF-R reaction with anti-P-tyrosine antibody, indicating that sodium butyrate was not interfering with PDGF-BB-induced tyrosine phosphorylation of β -PDGF-R as well as cellular proteins.

-13-

EXAMPLE 12**Association of signaling molecules with tyrosine phosphorylated β -PDGF-R**

PDGF-treatment of cells has been shown to result in PDGF-R association and tyrosine phosphorylation of signaling molecules such as PLC γ , Raf 1, ras GTPase activated protein and PI-3 kinase. To ascertain whether inhibition of PDGF-BB-induced proliferation of smooth muscle cells by sodium butyrate involves disruption of this process, β -PDGF-R immunoprecipitates were examined for the presence of PLC γ and ras GTPase activated protein. β -PDGF-R immunoprecipitation were prepared from smooth muscle cells and analyzed by immunoblotting using anti-P-tyrosine. As shown in Figure 3A, treatment of cells with no additions or sodium butyrate alone neither induced β -PDGF-R phosphorylation nor protein association. On the other hand, PDGF-BB induced not only β -PDGF-R phosphorylation but also promoted association and phosphorylation of PLC γ , GTPase activated protein and a 74 Kd protein with β -PDGF-R (Figure 3A). Presence of β -PDGF-R, (Figure 3B), PLC γ and GTPase activated protein (data not shown) were confirmed by re-probing the blot with corresponding antibodies. The identity of 74 Kd phosphorylated protein was not confirmed, but presumably is Raf-1 kinase. Incubation of smooth muscle cells with PDGF-BB plus sodium butyrate or preincubation with sodium butyrate alone for 30 minutes and subsequent incubation with PDGF-BB plus sodium butyrate did not alter PDGF-BB-stimulated β -PDGF-R phosphorylation and its association and phosphorylation of PLC γ , GTPase activated protein and 74 Kd protein. Thus, inhibition of PDGF-BB-induced proliferation of smooth muscle cells by sodium butyrate did not involve a signal transduction pathway associated with PLC γ and ras GTPase activated protein. This was further confirmed by the analysis of ras GTPase activated protein and PLC γ immunoprecipitates.

-14-

EXAMPLE 13**Tyrosine phosphorylation of ras GTPase activated protein and association with β -PDGF-R**

Immunoprecipitates of ras GTPase activated protein were prepared from cell lysates and analyzed by immunoblotting with anti-P-tyrosine or anti-ras GTPase activated protein (Figures 4A and 4B). Anti-P-tyrosine immunoblot identified β -PDGF-R, PLC γ , ras GTPase activated protein and a protein of 74 Kd in the samples that were exposed to PDGF-BB with or without sodium butyrate as well as in samples that are pretreated with sodium butyrate and then incubated in PDGF-BB plus sodium butyrate. Thus, sodium butyrate had no effect on β -PDGF-R induced protein association and phosphorylation of ras GTPase activated protein (Figure 4A). Sodium butyrate by itself had no effect either on protein phosphorylation or on protein association. Therefore, no proteins were identified in cells that were exposed to sodium butyrate, as in, no addition treatments. When the blot was re-probed with anti-ras GTPase activated protein, ras GTPase activated protein was visible in all the treatments (Figure 4B).

EXAMPLE 14**Phosphorylation of PLC γ and its association with β -PDGF-R**

The effect of sodium butyrate on PDGF-BB-induced phosphorylation of PLC γ and its association with activated β -PDGF-R was shown by immunoprecipitating smooth muscle cell lysate with anti-PLC γ and probing with anti-P-tyrosine or anti-PLC γ antibody (Figure 5A and B). As expected, smooth muscle cells exhibited no phosphorylated proteins after exposure to no addition or sodium butyrate. Exposure of smooth muscle cells to PDGF-BB, activates association and phosphorylation of β -PDGF-R, PLC γ and a protein of 74Kd (Figure 5A). The identity of β -PDGF-R (data not shown) and PLC γ (Figure 5B) was confirmed by probing the blot with corresponding antibodies. Exposure of smooth muscle cells to PDGF-BB plus sodium butyrate or pretreatment of smooth muscle cells with sodium butyrate for 30 minutes and subsequent incubation in PDGF-BB plus sodium butyrate, has no effect on PDGF-BB-induced association and phosphorylation of β -PDGF-R and PLC γ . Thus, the mechanism of inhibition of PDGF-BB-

-15-

induced proliferation by sodium butyrate was not through the signaling pathway mediated by PLC γ . Additional support was obtained by determining PDGF-BB stimulated, PLC γ -mediated IP₃ production. Exposure of smooth muscle cells to PDGF-BB results in an approximate 30% increase in IP₃ formation which is increased to 60% by incubating smooth muscle cells in the presence of PDGF-BB plus sodium butyrate (Table III). Thus, the signaling pathway that associates with PLC γ was not the target for the sodium butyrate inhibition of PDGF-BB-induced proliferation.

TABLE III

Effects of Sodium Butyrate on PDGF-BB-induced inositol phosphate production.

Addition	<u>dpm x 10⁻³ / flask</u>		
	<u>IP</u>	<u>IP₂</u>	<u>IP₃</u>
None	105 $\pm$ 10	11 $\pm$ 1.4	3.5 $\pm$ 0.7
Sodium butyrate	99 $\pm$ 14	10 $\pm$ 0.2	3.3 $\pm$ 0.2
PDGF-BB	110 $\pm$ 14	12 $\pm$ 0.9	4.7 $\pm$ 0.5
PDGF-BB +			
Sodium butyrate	117 $\pm$ 9	12 $\pm$ 0.5	5.7 $\pm$ 0.4

In TABLE III, rat aortic smooth muscle cells are pre-labeled with [³H] myoinositol for 48 hours and then exposed to PDGF-BB, sodium butyrate or sodium butyrate plus PDGF-BB for 1 minute. Inositol phosphates were separated and measured. Values are the mean $\pm$ S.D. of at least four measurements and representative of two experiments.

EXAMPLE 16PDGF-BB-induced mitogen activated protein kinases activity

Mitogen activated protein-kinases are serine and threonine kinases that are strongly activated by PDGF. The effect of sodium butyrate on PDGF-BB-induced mitogen activated protein kinase activity was measured by immunocomplex kinase assay. Mitogen activated protein-kinase was immunoprecipitated

-16-

from the smooth muscle cells cell lysates using an anti-mitogen activated protein-kinase antibody and the immunocomplex was subjected to *in vitro* kinase reaction using myelin basic protein as a substrate (Figure 6). Surprisingly, exposure of smooth muscle cells to sodium butyrate also stimulates mitogen activated protein-kinase activity by about 2.7-fold over no addition control. Addition of PDGF-BB to smooth muscle cells stimulates mitogen activated protein-kinase activity by about 11.4-fold. This PDGF-BB stimulated mitogen activated protein-kinase activity was unaffected when smooth muscle cells were exposed to PDGF-BB plus sodium butyrate. But, preincubation of smooth muscle cells with sodium butyrate for 30 minutes and subsequent addition of PDGF-BB plus sodium butyrate resulted in a significant decrease in PDGF-BB-induced mitogen activated protein-kinase activity. There was only a 4.6-fold increase in activity over the "no addition" control suggesting that mitogen activated protein-kinase activities were differentially modified by sodium butyrate.

EXAMPLE 17

Serum and PDGF-BB-induced transcription of *c-fos*, *c-jun* and *c-myc*

Exposure of cells to serum or growth factors causes increased rapid transcription of nuclear protooncogenes. The effect of sodium butyrate on serum and PDGF-BB-induced transcription of *c-fos*, *c-jun* and *c-myc* was demonstrated. Figure 7 shows that treatment of serum-starved smooth muscle cells with serum caused increased transcription of all three nuclear protooncogenes compared to the no addition control. The serum induced effect was more dramatic on *c-fos* and *c-jun* than on *c-myc*. Addition of sodium butyrate along with serum diminished the serum induced transcription of *c-fos*, *c-jun* and *c-myc*. Similar to serum, PDGF-BB also induced the transcription of all three protooncogenes. Sodium butyrate diminished the PDGF-BB stimulated transcription of *c-fos*, *c-jun* and *c-myc*. irrespective of whether the cells were pretreated with sodium butyrate and then exposed to sodium butyrate plus PDGF-BB or treated simultaneously with PDGF-BB plus SB. However, inhibition of *c-myc* transcription is more severe than inhibition of *c-fos* and *c-*

-17-

jun transcription.

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and
5 publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and
10 obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred
15 embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

WHAT IS CLAIMED IS:

-18-

Claims

1. A method of inhibiting the proliferation of smooth muscle cells comprising the step of contacting said cells with a pharmacologically effective amount of sodium butyrate.

5 2. The method of claim 1, wherein said amount of sodium butyrate is from about 0.5 mM to about 5 mM.

3. A pharmaceutical composition, comprising pharmacologically effective amount of sodium butyrate and a pharmaceutically acceptable carrier.

10 4. A method of inhibiting the formation of atherosclerotic plaques in a human comprising the step of administering to said human a pharmacologically effective amount of the composition of claim 3.

15 5. The method of claim 4, wherein said composition is administered in a dose of from about 100 mg/kg to about 1000 mg/kg.

6. The method of claim 5, wherein said composition is administered intravenously.

20 7. The method of claim 5, wherein said composition is administered as a dietary supplement.

8. The method of claim 7, wherein said dietary supplement comprises sodium butyrate administered as approximately 5% to about 30% of total daily fat intake.

25 9. A method of inhibiting the formation of cerebrovascular disease in a human comprising the step of administering to said human a pharmacologically effective amount of the composition of claim 3.

-19-

10. The method of claim 9, wherein said composition is administered in a dose of from about 100 mg/kg to about 1000 mg/kg.

11. The method of claim 9, wherein said composition
5 is administered intravenously.

12. The method of claim 9, wherein said composition is administered as a dietary supplement.

13. The method of claim 12, wherein said dietary
supplement comprises sodium butyrate administered as
10 approximately 5% to about 30% of total daily fat intake.

14. A dietary supplement consisting essentially of sodium butyrate.

15. The dietary supplement of claim 14, wherein said
15 contains from about 1 mg sodium butyrate to about 20 mg sodium butyrate.

AMENDED CLAIMS

[received by the International Bureau on 6 June 1996 (06.06.96) ;
original claim 1 amended; remaining claims unchanged (1 page)]

1. A method of inhibiting the proliferation of smooth muscle cells in a patient in need thereof comprising administering to said patient a pharmacologically effective
5 amount of sodium butyrate.

2. The method of claim 1, wherein said amount of sodium butyrate is from about 0.5 mM to about 5 mM.

3. A pharmaceutical composition, comprising pharmacologically effective amount of sodium butyrate and a
10 pharmaceutically acceptable carrier.

4. A method of inhibiting the formation of atherosclerotic plaques in a human comprising the step of administering to said human a pharmacologically effective amount of the composition of claim 3.

15 5. The method of claim 4, wherein said composition is administered in a dose of from about 100 mg/kg to about 1000 mg/kg.

6. The method of claim 5, wherein said composition is administered intravenously.

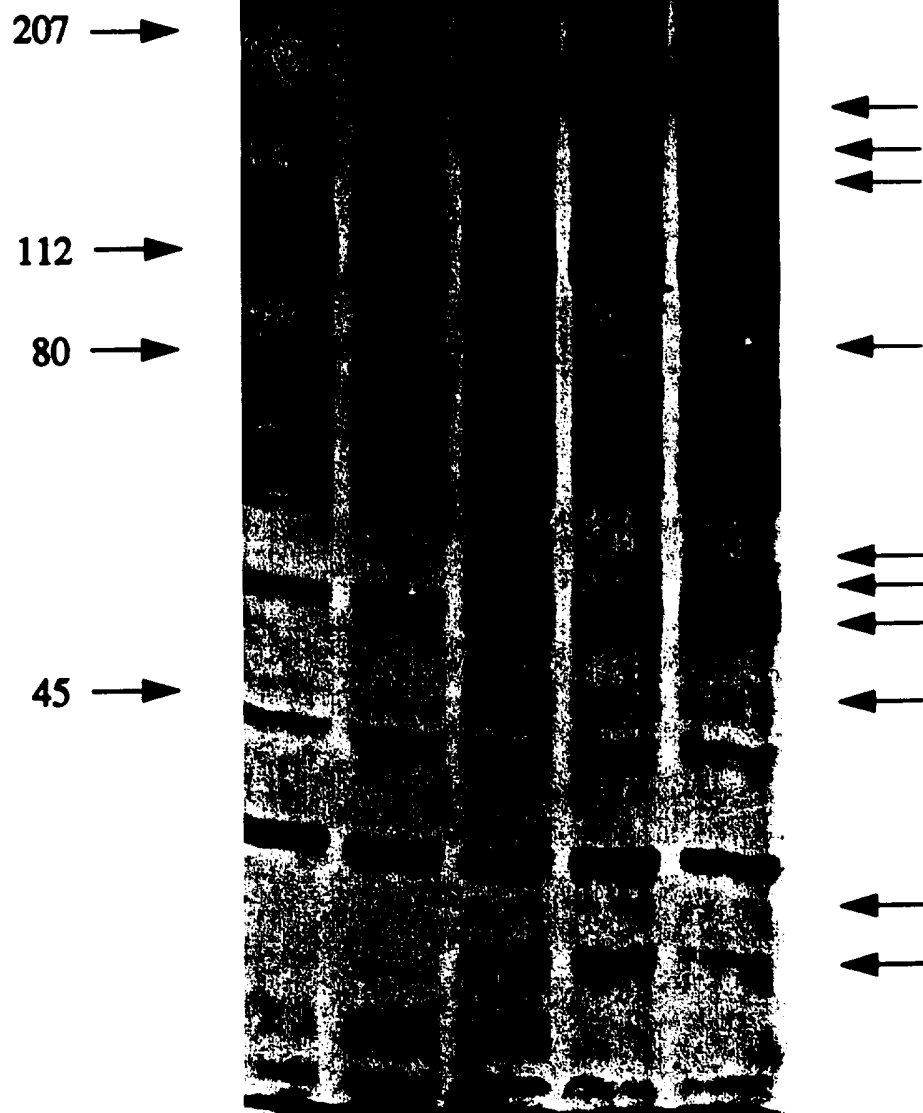
20 7. The method of claim 5, wherein said composition is administered as a dietary supplement.

8. The method of claim 7, wherein said dietary supplement comprises sodium butyrate administered as approximately 5% to about 30% of total daily fat intake.

25 9. A method of inhibiting the formation of cerebrovascular disease in a human comprising the step of administering to said human a pharmacologically effective amount of the composition of claim 3.

1/11

1 2 3 4 5



Blt. Ab:

Anti - P - Tyr

FIG. 1A

2/11

IP Ab: Anti - P - Tyr

1 2 3 4 5

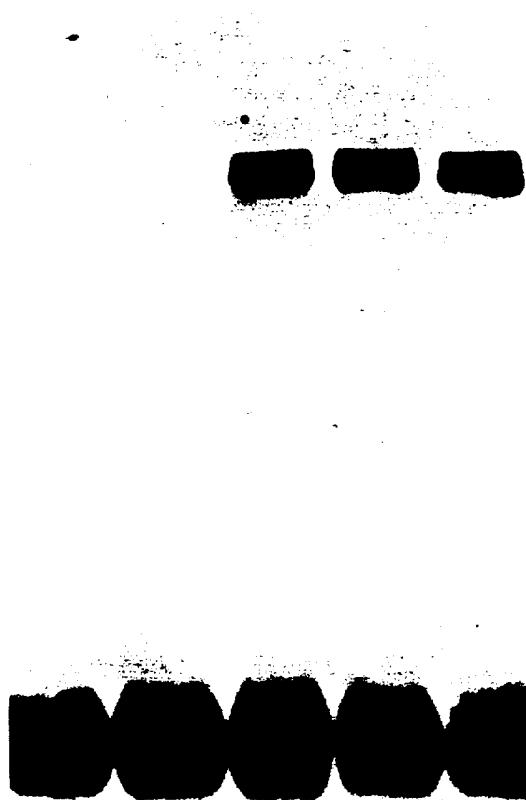
207 →

112 →

80 →

45 →

← β-PDGFR

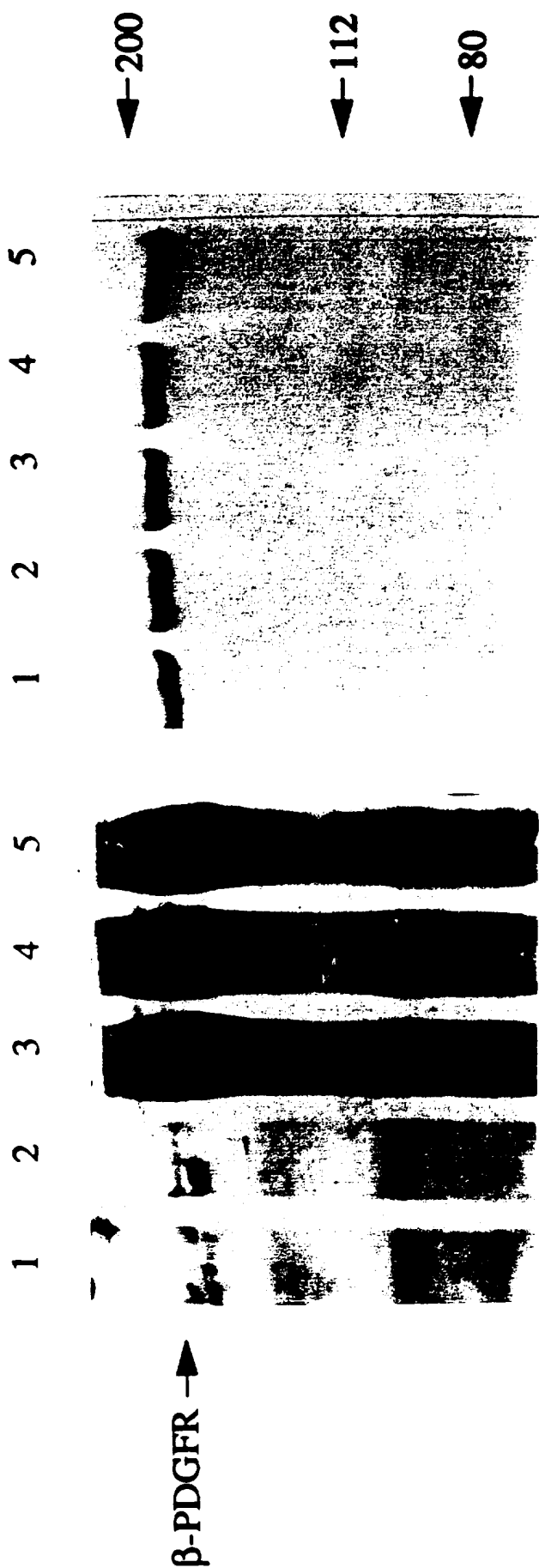


Anti - β -PDGFR

FIG. 1B

3/11

IP Ab: Anti - β - PDGFR



Anti - β - PDGFR

Anti - P - Tyr

Blt. Ab:

FIG. 2B

FIG. 2A

IP Ab: Anti - GAP

1

2

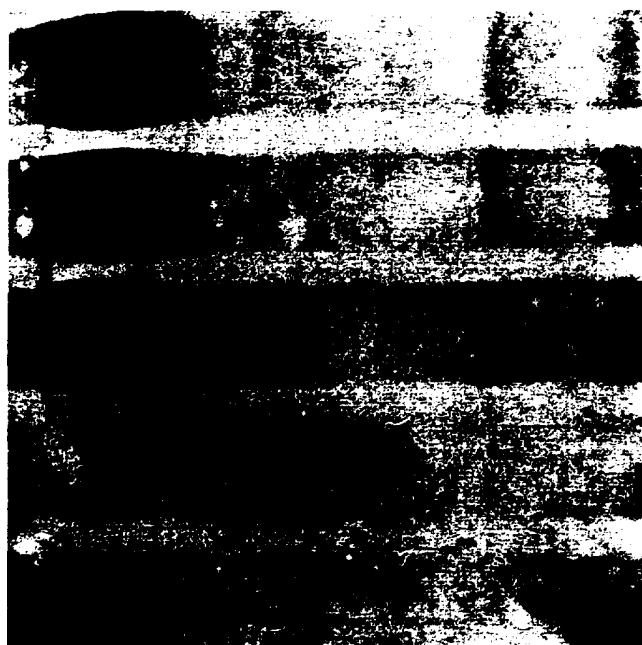
3

4

5

β-PDGFR →

GAP →



Blot. Ab:

Anti - P - Tyr

FIG. 3A

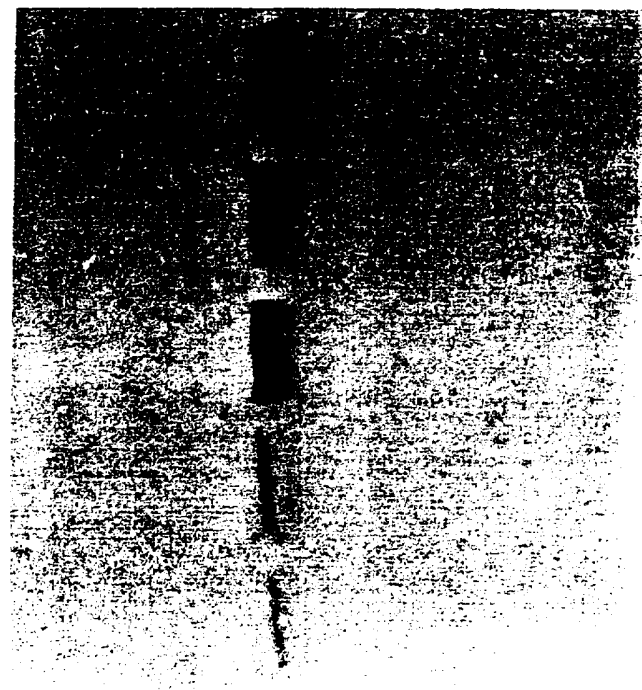
5

4

3

2

1



Anti - GAP

FIG. 3B

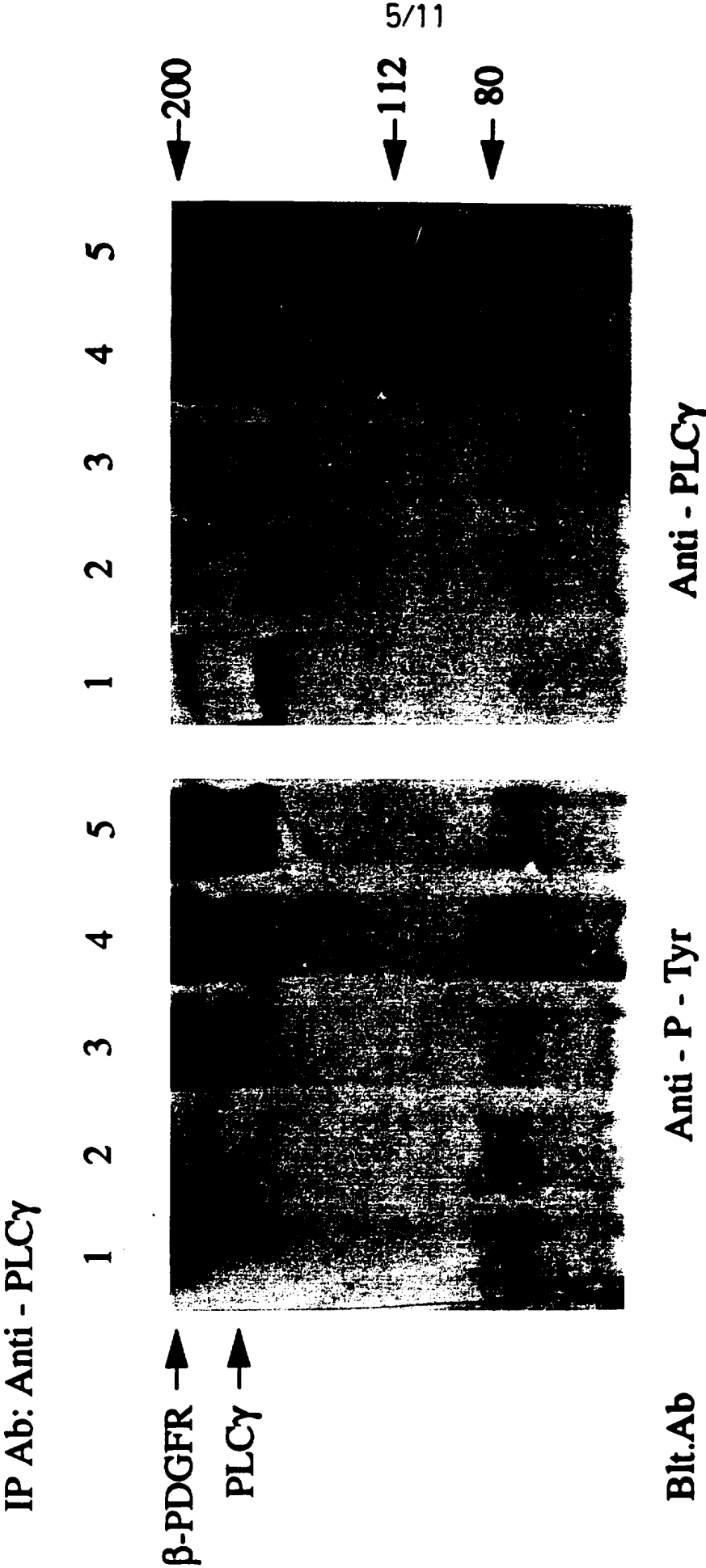
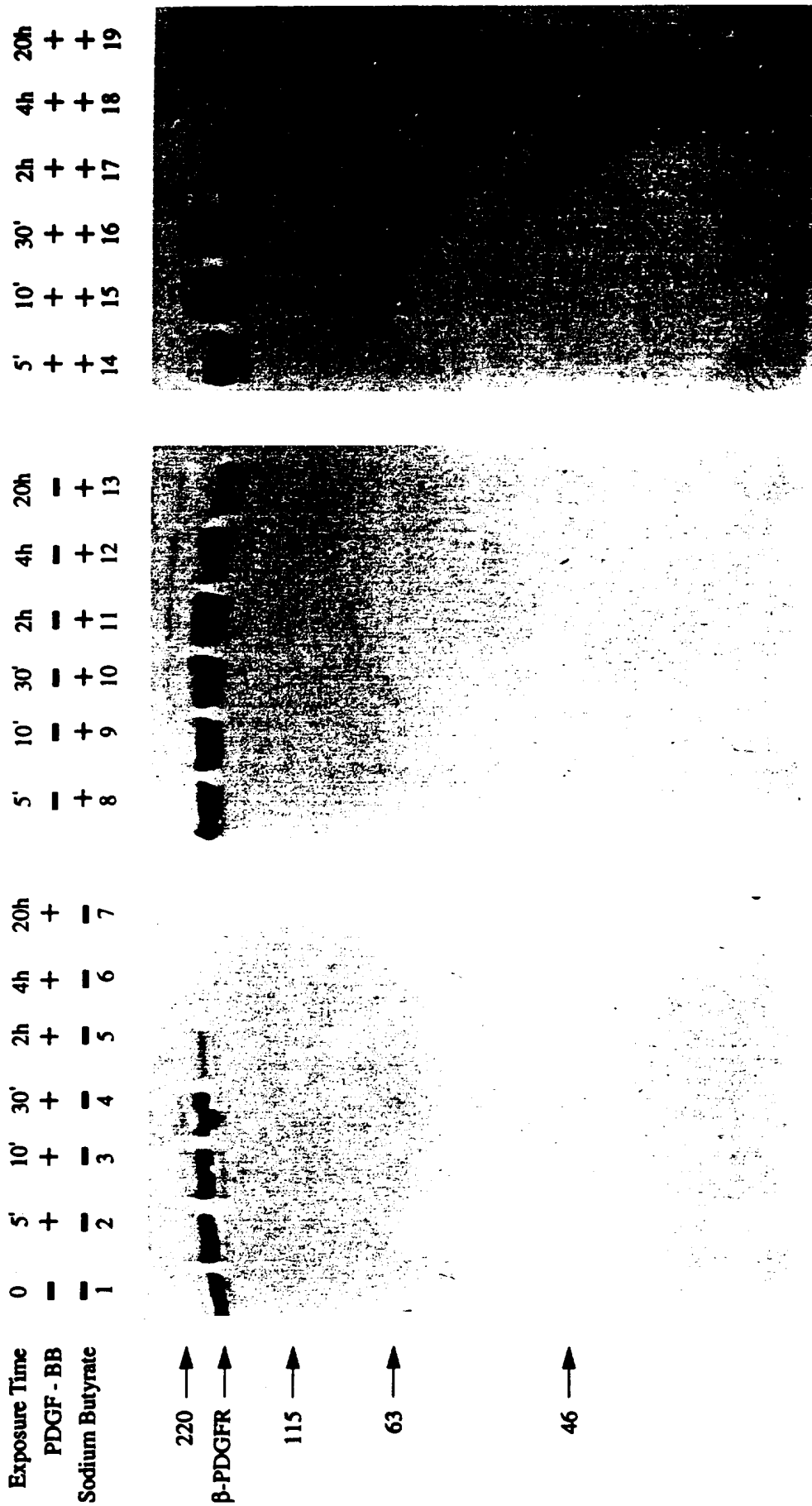


FIG. 4B

FIG. 4A

6/11



Anti - β - PDGFR

FIG. 5

Blot. Ab:

7/11

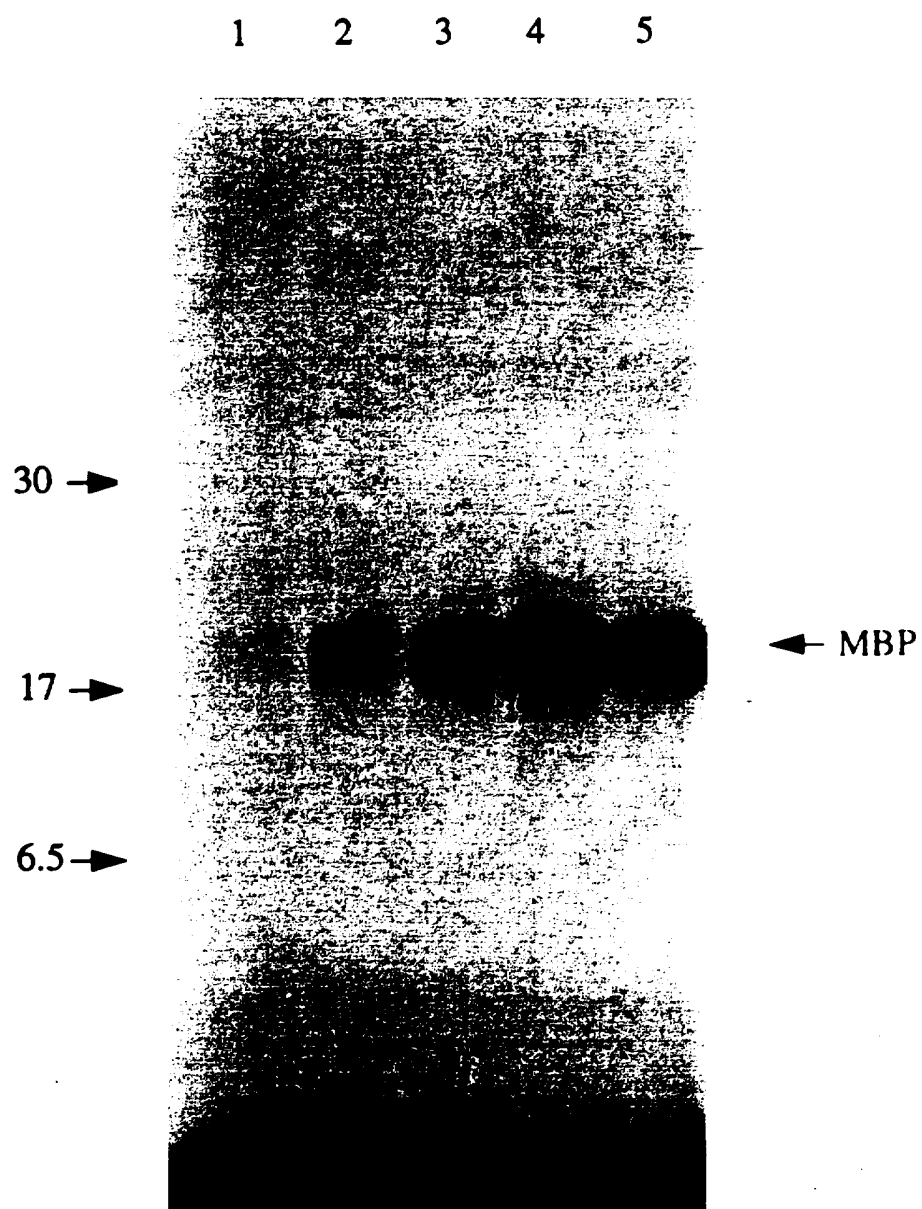


FIG. 6A

8/11

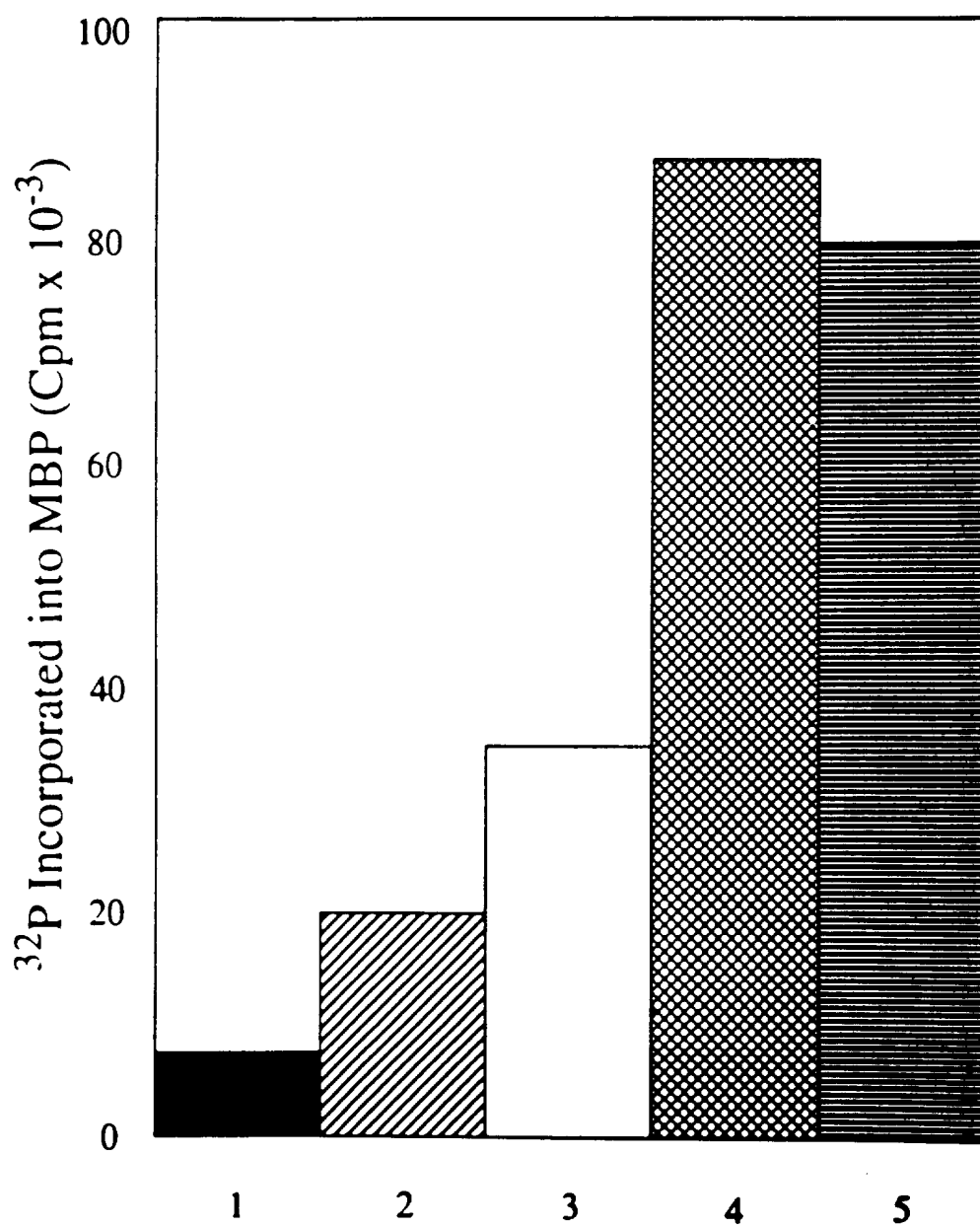


FIG. 6B

9/11

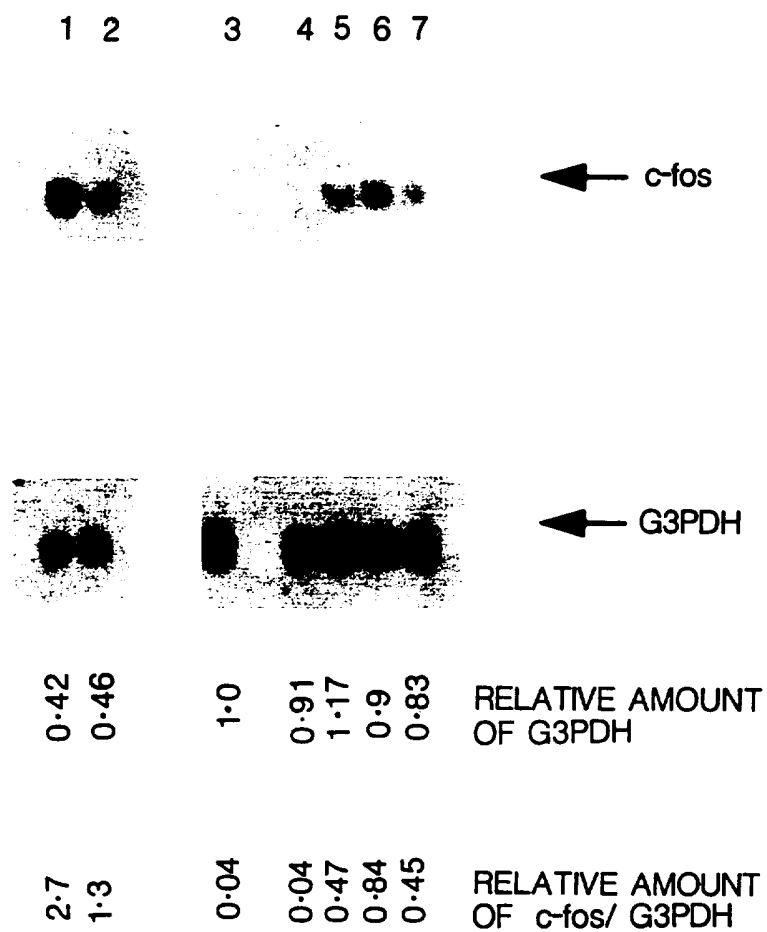


FIG. 7A

10/11

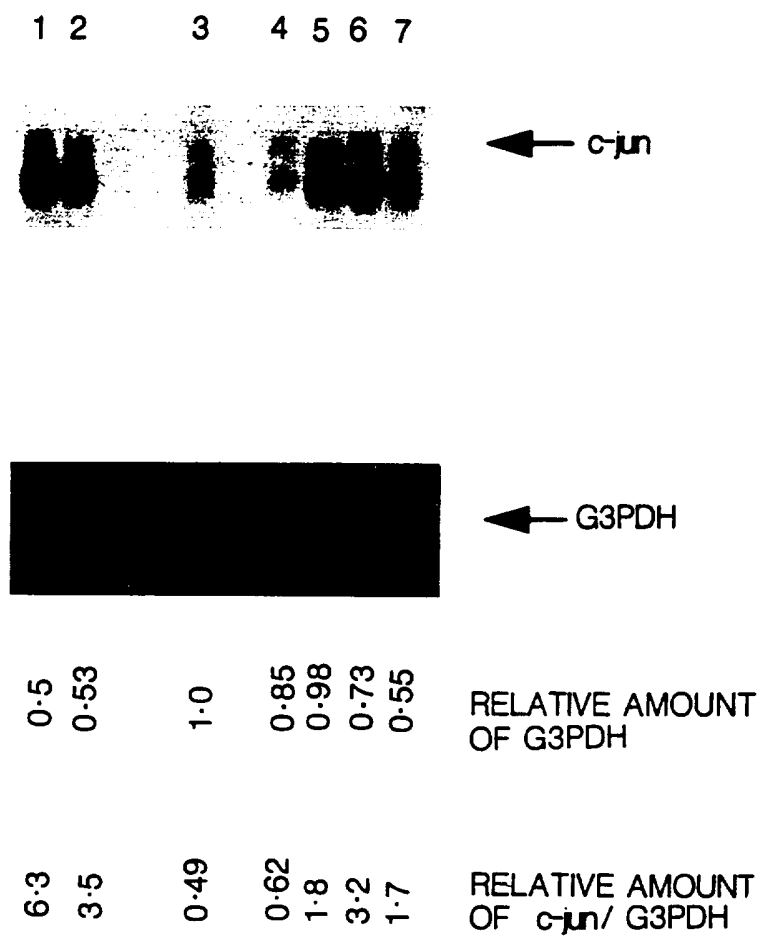


FIG. 7B

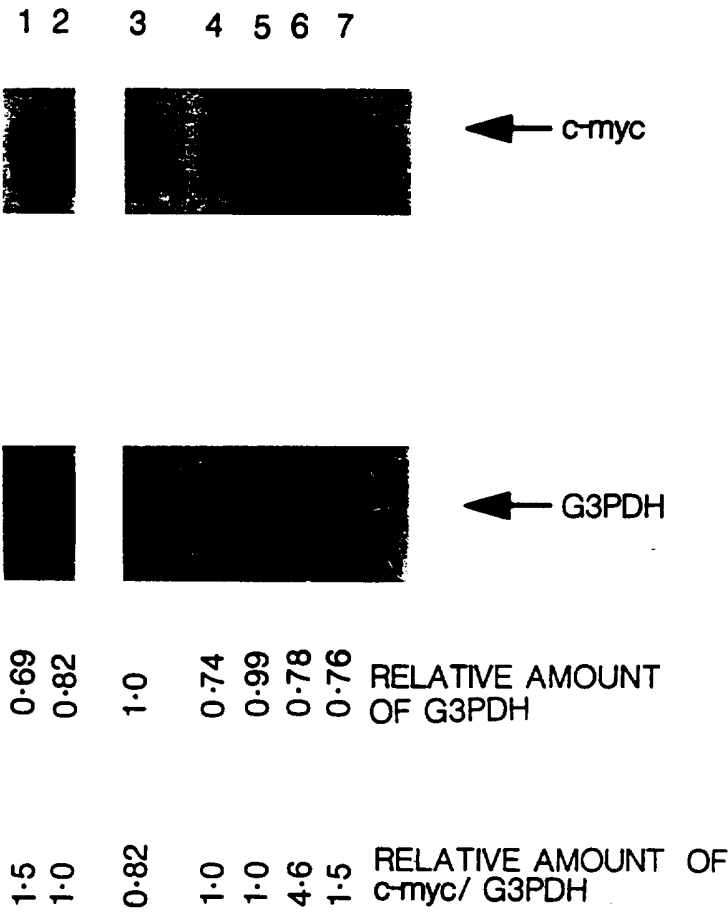


FIG. 7C

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/16949

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/19

US CL :514/557

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)

U.S. : 514/557

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 practicable, search terms used)

APS, STN (CA, BIOSIS, MEDLINE)

search terms: butyrate, butyric acid, butanoate, butanoic acid, smooth muscle cell proliferation or growth, atherosclerosis, arteriosclerosis, cerebrovascular disease, stroke

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A --- X	Chemical Abstracts, Volume 94, No. 23, issued 08 June 1981, Dexter et al, "Induction by butyrate of differentiated properties in cloned murine rhabdomyosarcoma cells" see page 109, column 1, abstract no. 186197p, Differentiation, 18(2), 115-122.	1, 2, 4-13 ----- 3, 14, 15
A --- X	Biological Abstracts, Volume 80, No. 12, issued 1985, Knudsen, "The calcium-dependent myoblast adhesion that precedes cell fusion is mediated by glycoproteins.", see page 174, column 2, abstract no. 102425, J. Cell Biol., 101(3), 891-897.	1, 2, 4-13 ----- 3, 14, 15



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* 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
A document defining the general state of the art which is not considered to be of particular relevance	*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
E earlier document published on or after the international filing date	*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
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)	*Z* document member of the same patent family
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	

Date of the actual completion of the international search

28 MARCH 1996

Date of mailing of the international search report

16 APR 1996

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

WILLIAM JARVIS

Telephone No. (703) 308-1235

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/16949

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

- I. Claims 1-3, drawn to a method and product
- II. Claims 4-8, drawn to a method
- III. Claims 9-13, drawn to a method
- IV. Claims 14 and 15, drawn to a product

Although the groups are linked by "sodium butyrate", this is not a special technical feature as defined by PCT Rule 13.2 since it does not define a contribution over the prior art. Furthermore, the three methods lack unity of invention since they involve significantly different uses of sodium butyrate. The two products lack unity of invention since a pharmaceutical composition and a dietary supplement are designed for different purposes. Clearly, lack of unity is clearly present in the claims.