(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 February 2010 (25.02.2010)

PCT

(10) International Publication Number
WO 2010/020647 A2

(51) International Patent Classification:
G01N 33/68 (2006.01) *A61P 19/08* (2006.01)
A61K 31/00 (2006.01) *A61P 19/10* (2006.01)
A61P 19/00 (2006.01)

(21) International Application Number:
PCT/EP2009/060691

(22) International Filing Date:
18 August 2009 (18.08.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
08290783.3 18 August 2008 (18.08.2008) EP

(71) Applicant *(for all designated States except US)*: **CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE (CNRS)** [FR/FR]; 3, rue Michel Ange, F-75016 Paris (FR).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **BLANGY, Anne** [FR/FR]; Enclos Laffoux no.4 11 avenue de Castelnau, F-34090 Montpellier (FR).

(74) Agent: **FAIVRE PETIT, Frédérique**; Cabinet Regimbeau, 20, rue de Chazelles, F-75847 Paris Cedex 17 (FR).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2010/020647 A2

(54) **Title**: NEW METHOD FOR IDENTIFYING COMPOUNDS USEFUL FOR TREATING AND/OR PREVENTING DISEASE-ASSOCIATED BONE LOSS

(57) **Abstract**: The present invention concerns a method for identifying a compound which inhibits the activation of RAC GTPase by DOCK5 protein comprising the steps of (i) coexpressing the DOCK5 and the RAC proteins in a cell, wherein said D0CK5 protein induces the conversion of inactive RAC, which inactive RAC is bound to GDP, to active RAC, which active RAC is bound to GTP, (ii) contacting or not said cell with said compound, (iii) determining the conversion of inactive RAC to active RAC in the presence or absence of said compound, and (iv) selecting the compound inhibiting the conversion of inactive RAC to active RAC. Said compound is useful for treating disease-associated bone loss.

NEW METHOD FOR IDENTIFYING COMPOUNDS USEFUL FOR TREATING AND/OR PREVENTING DISEASE - ASSOCIATED BONE LOSS

Field of the invention

The invention relates to the field of diseases associated with bone loss, and more specifically to a new method for identifying compounds useful for treating and/or preventing diseases associated with bone loss.

Background of the Invention

Bone is a dynamic tissue that is continually remodeled throughout life depending on factors such as nutrition and the load the bone must carry. Normal bone formation depends on the delicate balance between new bone addition and old bone resorption. Bone formation is based on the deposition of bone matrix by osteoblasts and bone resorption and more specifically mineralized tissue, chiefly calcium carbonate and calcium phosphate resorption in vertebrates is achieved by osteoclasts. Typically, in a normal adult, about 5-10% of bone is replaced by these processes annually.

These osteoclasts are multinucleated cells of up to 400μm related to macrophage and other cells that develop from monocyte cells, which are actively motile cells that migrate along the surface of bone. Like macrophage, osteoclasts are derived from haematopoietic progenitor cells. The bone resorption is initiated when an osteoclast attaches to the surface of mineralized bone, forms a tight "sealing zone" and secretes necessary acids and proteases that initiate the resorption of mineralized tissue from the bone. After a period of several hours to days, the osteoclast detaches from the bone, leaving a pit on the bone surface. Under normal conditions, the pit is a target for osteoblasts, which deposit a material that ultimately becomes new bone.

Bone loss can result when the bone resorptive process is dominant over the bone formative process. Diseases associated with bone loss are usually accompanied by increased osteoclast activation. Such diseases include any bone loss resulting notably from an estrogen deficiency after the menopause but not only and comprise osteoporosis, osteopenia due to bone metastases, periarticular erosions in rheumatoid arthritis, primary

hyperparathyroidism, hypercalcemia of malignancy, Paget's disease of bone, periodontal disease, immobilization induced osteopenia, and glucocorticoid treatment.

As an example, there are currently 20 million people with detectable fractures of the vertebrae due to osteoporosis in the United States. In addition, there are 250,000 hip fractures per year attributed to osteoporosis. This clinical situation is associated with a 12% mortality rate within the first two years, while 30% of the patients require nursing home care after the fracture.

Since diseases of bone loss are associated with increased activity of osteoclast, it is important to understand the mechanisms by which osteoclasts are activated in these disease states, and to devise rational and therapeutic means to inhibit or reduce this activation.

Thus, the aim of the present invention is to elaborate new screening methods which can be useful for treating and/or preventing bone loss diseases, and to use such compounds to prepare a drug for treating and/or preventing bone loss diseases.

Description of the invention

The inventors have presently identified the DOCK5 protein is implicated in sealing zone formation and consequently in bone resorption. Thus, DOCK5 corresponds to a new therapeutic target for treating and/or preventing bone loss diseases. Finally, the inventors have used yeast exchange assay (YEA) for identifying inhibitors of DOCK5, which inhibitors can be useful for treating and/or preventing bone loss diseases.

Thus, in a first object, the present invention is directed to a method for identifying a compound which inhibits the activation of RAC GTPase, more specifically RAC1/2 GTPase, by DOCK5 protein comprising the steps of:

- coexpressing the DOCK5 and the RAC proteins in a cell, wherein said DOCK5 protein induces the conversion of inactive RAC, which inactive RAC is bound to GDP, to active RAC, which active RAC is bound to GTP,

- contacting or not said cell with said compound,

- determining the conversion of inactive RAC to active RAC, more specifically the conversion of inactive RAC1/2 to active RAC1/2, in the presence or absence of said compound, and

- selecting the compound inhibiting the conversion of inactive RAC to active RAC, more specifically the conversion of inactive RAC1/2 to active RAC1/2.

The selected compound is useful for treating disease associated with bone loss. In fact, the inventors have established that the conversion of inactive RAC to active RAC by DOCK5 is associated with the sealing zone formation.

According to the present invention "RAC1/2" means "RAC1 and/or RAC2". In fact, the inhibition of the activation of RAC1 GTPase and/or of RAC2 GTPase give rise to the same kind of results, while both RAC1 and RAC2 are involved in (and thus necessary for) the osteoclast differenciation and resorption functions.

Advantageously, the present invention is directed to a method for identifying a compound which inhibits the activation of RAC1/2 GTPase and which is useful for treating disease associated with bone loss by DOCK5 protein comprising the steps of:

- coexpressing the DOCK5 and the RAC proteins in a cell, wherein said DOCK5 protein induces the conversion of inactive RAC, which inactive RAC is bound to GDP, to active RAC, which active RAC is bound to GTP.

- contacting or not said cell with said compound,

- determining the conversion of inactive RAC to active RAC in the presence or absence of said compound,

- selecting the compound inhibiting the conversion of inactive RAC to active RAC since this conversion is associated with the sealing zone formation, and

- testing the inhibition of bone resorption, corresponding to the testing of mineralised matrix resorption by osteoclasts, by the selected compounds.

As an example of disease associated with bone loss, one can cites menopause, osteoporosis, osteopenia due to bone metastases, periarticular erosions in rheumatoid arthritis, primary hyperparathyroidism, hypercalcemia of malignancy, Paget's disease of bone, periodontal disease, immobilization induced osteopenia, or in glucocorticoid treatment. Preferably, said disease associated with bone loss is osteoporosis.

Results from the cellular and bone resorption assay systems used herein are widely accepted in the art as predictive of in vivo effects. As the bone resorption assay uses material that includes bone marrow isolated cells, it is an ex vivo assay. Thus, the showing that the inhibition of RAC activation by DOCK5 inhibits bone resorption in these assays is evidence of the clinical utility of inhibitors of this specific activation for treating osteoporosis. Various scientific publications, such as Carano et al. (1990); Blair & Schlesinger (1992); Schlesinger & Blair (1992); Vaananen et al., 1990; all support the fact that such assays are accepted as being predictive of in vivo activity.

Methods for determining the conversion of inactive RAC to active RAC are well known from the skilled person. As an example of such methods, one can cites the methods disclosed in the examples and in COTE & VUORI (*J. Cell. Sci.,* vol.115, p: 4901-4913, 2002).

In a preferred embodiment, the method of the invention further comprises the step of testing the inhibition of bone resorption by the selected compound.

In another preferred embodiment, the method of the invention includes a further step of comparing the conversion of inactive RAC to active RAC in presence of the tested compound and in the absence of said compound.Said inhibition of bone resorption can be simply tested by method well known from the skilled person, such as the one disclosed in the examples, wherein mineralised matrix resorption by osteoclasts is tested by culturing said osteoclasts on calcium phosphate substrates and mineralised matrix resorption is determined by VON KOSSA staining.

As used herein, the term "compound" refers to a natural or synthetic compound, such as chemical or peptidic compound.

Preferably, the compounds are chosen in the group consisting in:

- 4-[5-(4-bromophenyl)-3-(4-nitrophenyl)-4,5-dihydro-1H-pyrazol-1-yl]-4-oxobutanoic acid;
- 2,2,2-trichloro-N-(1,1-dioxido-2,3-dihydro-3-thienyl)-N-(4-methylphenyl)acetamide;
- 3-(3-chlorophenyl)-7-methyl-4-methylene-3,4-dihydro-2(1H)-quinazolinone;
- 3-[4-(3-bromobenzylidene)-3-methyl-5-oxo-4,5-dihydro-1H-pyrazol-1-yl]benzoic acid;
- N-2,1,3-benzothiadiazol-4-yl-5-bromo-2-furamide;
- 1-acetyl-4-(2-chloro-4-nitrophenyl)-2-methylpiperazine;
- 3-(3-methoxybenzylidene)-5-(4-methylphenyl)-2(3H)-furanone;
- 3-[5-(3,4-dichlorophenyl)-2-furyl]acrylic acid;
- (2-chloro-4-{[5-(2-chlorophenyl)-6-(ethoxycarbonyl)-7-methyl-3-oxo-5H-[1,3]thiazolo[3,2-a]pyrimidin-2(3H)-ylidene]methyl}-6-methoxyphenoxy)acetic acid;
- 4-{[4-(diphenylmethyl)-1-piperazinyl]sulfonyl}-2,1,3-benzothiadiazole;
- 4-[4-phenyl-5-(2-thienyl)-1H-imidazol-2-yl]-1,2-benzenediol;
- N-(3,4-dimethoxyphenyl)-4-[methyl(phenylsulfonyl)amino]benzamide;
- 1-[(2-hydroxyphenyl)carbonothioyl]-3-phenyl-5-(trifluoromethyl)-4,5-dihydro-1H-pyrazol-5-ol;
- 2-methoxyethyl 4-[(4-tert-butylbenzoyl)amino]benzoate;
- N-(2,3-dichlorophenyl)-3-(5-methyl-2-furyl)acrylamide;

- N-(4-fluorophenyl)-3-[3-(trifluoromethyl)phenyl]acrylamide;

- 3-(2-furylmethyl)-2-(2-hydroxyphenyl)-2,3-dihydro-4(1H)-quinazolinone;

- N-(4-ethoxyphenyl)-2-{[5-(4-methoxyphenyl)-1,3,4-oxadiazol-2-yl]thio}acetamide;

- 5-(4-nitrobenzylidene)-2-thioxo-3-[3-(trifluoromethyl)phenyl]-1,3-thiazolidin-4-one;

- (3,5-dichlorophenyl)[(phenylsulfonyl)carbonyl]amine;

- N-(2-bromophenyl)-3-(5-methyl-2-furyl)acrylamide;

- 2-(2-chlorophenoxy)-N-[2-chloro-5-(trifluoromethyl)phenyl]acetamide;

- N-[4-(4-acetyl-1-piperazinyl)phenyl]propanamide;

- 8-[(dimethylamino)methyl]-9-hydroxy-2-methyl-4H-pyrido[1,2-a]pyrimidin-4-one;

- 4-tert-butyl-N-[1-{[(2-methoxyphenyl)amino]carbonyl}-2-(2-thienyl)vinyl]benzamide;

- 2-chloro-N-(3-chloro-4-methoxyphenyl)benzamide;

- 2,6-di-tert-butyl-4-(2,3-dihydro-1H-perimidin-2-yl)phenol;

- 3-benzyl-2-(2,6-dichlorophenyl)-2,3-dihydro-4(1H)-quinazolinone;

- 1-(3,4-dichlorobenzyl)-1H-indole-3-carbaldehyde;

- N-[5-(1-adamantyl)-1,3,4-thiadiazol-2-yl]-N'-phenylurea;

- N-(3,4-dichlorophenyl)-N'-{5-[(4-methylphenoxy)methyl]-1,3,4-thiadiazol-2-yl}urea;

- N-(2,3-dihydro-1,4-benzodioxin-6-yl)-2-(1-naphthyloxy)acetamide;

- N-[4-(4-acetyl-1-piperazinyl)phenyl]-4-ethoxy-3-nitrobenzamide;

- N-(2-chlorophenyl)-3-(4-fluorophenyl)acrylamide;

- 1-[(dimethyl-lambda~4~-sulfanylidene)amino]-2-methoxy-4-nitrobenzene;
- 5-benzylidene-1-(2-chlorophenyl)-2,4,6(1H,3H,5H)-pyrimidinetrione;
- 4-ethyl-5,6-dimethyl-2-phenylpyrimidine;
- 2-(3-chlorobenzylidene)-1H-indene-1,3(2H)-dione;
- 5-{5-[(3-methyl-5-oxo-1-phenyl-1,5-dihydro-4H-pyrazol-4-ylidene)methyl]-2-furyl}-1H-isoindole-1,3(2H)-dione;
- N-(2,5-dimethylphenyl)-3-(4-methoxyphenyl)acrylamide;
- 2-({2-[(4-nitrophenyl)amino]ethyl}amino)ethanol;
- N-(3-methoxyphenyl)-4-propoxybenzamide;
- 2-(4-hydroxyphenyl)-3-phenyl-2,3-dihydro-4(1H)-quinazolinone;
- 4-methyl-1-(2-nitrobenzoyl)piperidine;
- 2-hydroxy-N'-[(2-methylphenyl)sulfonyl]benzohydrazide;
- 4-(1,3-benzothiazol-2-yl)butanoic acid;
- 4-(3-methylbenzylidene)-1-phenyl-3,5-pyrazolidinedione;
- 4-(2,4-dichlorophenoxy)-N-(2-ethoxyphenyl)butanamide;
- N-(2-methoxyphenyl)-N'-(phenylsulfonyl)benzenecarboximidamide;
- N-[2-(2-chloro-5-iodophenyl)-1,3-benzoxazol-5-yl]-2-methylpropanamide;
- 5-(4-butoxyphenyl)-3-cyclohexyl-1,2,4-oxadiazole;
- N-(3,4-dichlorophenyl)-N'-4H-1,2,4-triazol-4-yl urea;
- 6-chloro-4-phenyl-3-[3-(3,4,5-trimethoxyphenyl)acryloyl]-2(1H)-quinolinone;
- 6-bromo-4-phenyl-3-[3-(3,4,5-trimethoxyphenyl)acryloyl]-2(1H)-quinolinone; and

- N-(1H-1,2,3-benzotriazol-1-ylmethyl)-4-nitro-1,2,5-oxadiazol-3-amine.

More preferably, the compounds are chosen in the group consisting in:

- 4-[5-(4-bromophenyl)-3-(4-nitrophenyl)-4,5-dihydro-1H-pyrazol-1-yl]-4-oxobutanoic acid
- 2,2,2-trichloro-N-(1,1-dioxido-2,3-dihydro-3-thienyl)-N-(4-methylphenyl)acetamide
- 3-(3-chlorophenyl)-7-methyl-4-methylene-3,4-dihydro-2(1H)-quinazolinone
- 3-[4-(3-bromobenzylidene)-3-methyl-5-oxo-4,5-dihydro-1H-pyrazol-1-yl]benzoic acid
- N-2,1,3-benzothiadiazol-4-yl-5-bromo-2-furamide
- 1-acetyl-4-(2-chloro-4-nitrophenyl)-2-methylpiperazine
- 3-(3-methoxybenzylidene)-5-(4-methylphenyl)-2(3H)-furanone
- 3-[5-(3,4-dichlorophenyl)-2-furyl]acrylic acid
- (2-chloro-4-{[5-(2-chlorophenyl)-6-(ethoxycarbonyl)-7-methyl-3-oxo-5H-[1,3]thiazolo[3,2-a]pyrimidin-2(3H)-ylidene]methyl}-6-methoxyphenoxy)acetic acid
- 4-{[4-(diphenylmethyl)-1-piperazinyl]sulfonyl}-2,1,3-benzothiadiazole
- 4-[4-phenyl-5-(2-thienyl)-1H-imidazol-2-yl]-1,2-benzenediol
- N-(3,4-dimethoxyphenyl)-4-[methyl(phenylsulfonyl)amino]benzamide
- 1-[(2-hydroxyphenyl)carbonothioyl]-3-phenyl-5-(trifluoromethyl)-4,5-dihydro-1H-pyrazol-5-ol
- 2-methoxyethyl 4-[(4-tert-butylbenzoyl)amino]benzoate
- N-(2,3-dichlorophenyl)-3-(5-methyl-2-furyl)acrylamide

- N-(4-fluorophenyl)-3-[3-(trifluoromethyl)phenyl]acrylamide
- 3-(2-furylmethyl)-2-(2-hydroxyphenyl)-2,3-dihydro-4(1H)-quinazolinone
- 2,6-di-tert-butyl-4-(2,3-dihydro-1H-perimidin-2-yl)phenol
- 3-benzyl-2-(2,6-dichlorophenyl)-2,3-dihydro-4(1H)-quinazolinone
- 1-(3,4-dichlorobenzyl)-1H-indole-3-carbaldehyde
- N-(4-ethoxyphenyl)-2-{[5-(4-methoxyphenyl)-1,3,4-oxadiazol-2-yl]thio}acetamide
- 5-(4-nitrobenzylidene)-2-thioxo-3-[3-(trifluoromethyl)phenyl]-1,3-thiazolidin-4-one
- (3,5-dichlorophenyl)[(phenylsulfonyl)carbonyl]amine
- N-(2-bromophenyl)-3-(5-methyl-2-furyl)acrylamide
- 2-(2-chlorophenoxy)-N-[2-chloro-5-(trifluoromethyl)phenyl]acetamide
- N-[4-(4-acetyl-1-piperazinyl)phenyl]propanamide
- 8-[(dimethylamino)methyl]-9-hydroxy-2-methyl-4H-pyrido[1,2-a]pyrimidin-4-one
- 4-tert-butyl-N-[1-{[(2-methoxyphenyl)amino]carbonyl}-2-(2-thienyl)vinyl]benzamide
- 2-chloro-N-(3-chloro-4-methoxyphenyl)benzamide
- N-[5-(1-adamantyl)-1,3,4-thiadiazol-2-yl]-N'-phenylurea
- N-(3,4-dichlorophenyl)-N'-{5-[(4-methylphenoxy)methyl]-1,3,4-thiadiazol-2-yl}urea
- N-(2,3-dihydro-1,4-benzodioxin-6-yl)-2-(1-naphthyloxy)acetamide
- N-[4-(4-acetyl-1-piperazinyl)phenyl]-4-ethoxy-3-nitrobenzamide
- N-(2-chlorophenyl)-3-(4-fluorophenyl)acrylamide

- 1-[(dimethyl-lambda~4~-sulfanylidene)amino]-2-methoxy-4-nitrobenzene

- 5-benzylidene-1-(2-chlorophenyl)-2,4,6(1H,3H,5H)-pyrimidinetrione; and

- 4-ethyl-5,6-dimethyl-2-phenylpyrimidine.

As used herein, the expression "DOCK5 protein" refers to a polypeptide comprising at least the DHR2 domain of the protein DOCK5 corresponding to the amino acid 1132 to 1661 of the DOCK5 protein from *Mus musculus* SEQ ID NO:1 and derivatives thereof.

Therefore, the present invention is directed to a method for identifying a compound which inhibits the activation of RAC GTPase, more specifically RAC1/2 GTPase, by DOCK5 protein comprising the steps of:

- coexpressing a polypeptide comprising at least the DHR2 domain of the protein DOCK5 and the RAC proteins in a cell, wherein said polypeptide induces the conversion of inactive RAC, which inactive RAC is bound to GDP, to active RAC, which active RAC is bound to GTP,

- contacting or not said cell with said compound,

- determining the conversion of inactive RAC to active RAC, more specifically the conversion of inactive RAC1/2 to active RAC1/2, in the presence or absence of said compound, and

- selecting the compound inhibiting the conversion of inactive RAC to active RAC, more specifically the conversion of inactive RAC1/2 to active RAC1/2.

The full length Dock5 protein has an aminoterminal SH3 domain, between aminoacids K11 and E68, followed by the DHR1 domain, between aminoacids G440 and E682, and the DHR2 domain between aminoacids M1132 and Y1661 (Figure 3E).

Preferably, said DOCK5 protein corresponds to SEQ ID NO:1.

Again preferably, said DOCK5 protein corresponds to SEQ ID NO:4 corresponding to *Homo sapiens* DOCK5 protein.

As used herein, the expression "RAC protein" refers to SEQ ID NO:2 and derivatives thereof.

According to a preferred embodiment, said cell is an eukaryotic cell, preferably a yeast cell.

Advantageously, said method comprises the expression of any protein, capable to interact with the active RAC protein and not with inactive RAC protein. One skilled in the art knows such protein known as a GTPase effector. According to a preferred embodiment, the protein capable to interact with the active RAC protein is chosen in the group comprising PAK1 protein.

As used herein, the expression "PAK1 protein" refers to the SEQ ID NO:3 and derivatives thereof.

As used herein, the term "derivatives"' refer to a polypeptide having a percentage of identity of at least 80% with amino acid 1132 to 1661 of SEQ ID NO: 1, SEQ ID NO: 2, EQ ID NO:3, SEQ ID NO:4 or SEQ ID NO:9, or orthologs thereof, preferably of at least 90%, as an example of at least 95%, and more preferably of at least 99%.

As used herein, "percentage of identity" between two amino acids sequences or two nucleic sequences, means the percentage of identical amino-acids or nucleotides, between the two sequences to be compared, obtained with the best alignment of said sequences, this percentage being purely statistical and the differences between these two sequences being randomly spread over the amino acids sequences. As used herein, "best alignment" or "optimal alignment", means the alignment for which the determined percentage of identity (see below) is the highest. Sequences comparison between two sequences are usually realized by comparing these sequences that have been previously align according to the best alignment; this comparison is realized on segments of comparison in order to identify and compared the local regions of similarity. The best sequences alignment to perform comparison can be realized, beside by a manual way, by using the global homology algorithm developed by SMITH and WATERMAN (*Ad. App. Math.*, vol.2, p:482, 1981), by using the local homology algorithm developed by NEDDLEMAN and WUNSCH (*J. Mol. Biol.*, vol.48, p:443, 1970), by using the method of similarities developed by PEARSON and LIPMAN (*Proc. Natl. Acd. Sci. USA*, vol.85, p:2444, 1988), by using computer softwares using such algorithms (GAP, BESTFIT, BLAST P, BLAST N, FASTA, TFASTA in the Wisconsin Genetics software Package, Genetics Computer Group, 575 Science Dr., Madison, WI USA), by using the MUSCLE multiple alignment

algorithms (Edgar, Robert C., *Nucleic Acids Research*, vol. 32, p:1792, 2004). To get the best local alignment, one can preferably used BLAST software, with the BLOSUM 62 matrix, or the PAM 30 matrix. The identity percentage between two sequences of amino acids two nucleic sequence is determined by comparing these two sequences optimally aligned, the amino acids sequences being able to comprise additions or deletions in respect to the reference sequence in order to get the optimal alignment between these two sequences. The percentage of identity is calculated by determining the number of identical position between these two sequences, and dividing this number by the total number of compared positions, and by multiplying the result obtained by 100 to get the percentage of identity between these two sequences.

Advantageously, said cell further comprises a reporter gene under the control of a promoter sequence, and said RAC and PAK1 proteins are each fused either with a transactivation domain or with a DNA binding domain specific of said promoter sequence, wherein the interaction of RAC with PAK1 results in the induction of expression of the reporter gene.

The method corresponds to the Yeast Exchange Assay (YEA) as disclosed in DE TOLEDO *et al.* (*FEBS*, vol.480, p:287-292, 200) and International Patent application PCT WO 2005/064007 using the DOCK5 and the RAC protein.

Thus, the disclosure of YEA in Patent application PCT WO 2005/064007 (page 6, "description de l'invention" paragraph, to page 23) are incorporated herein by reference.

The term "reporter gene" is well known from the skilled person and can correspond to an auxotrophic marker or to a gene coding for a protein which can be simply detected such as GFP, luciferase or β-Gal.

In this embodiment, the determination of the conversion of inactive RAC to active RAC is done by determining the expression of the reporter gene. The inhibition of the expression of the reporter gene corresponding to an inhibition of the conversion of inactive RAC to active RAC.

In another embodiment, the present invention provides a method for the selection of compounds, which permit to decrease the level of expression of a DOCK5 gene (SEQ ID N°10) in diseases associated with bone loss comprising the step of:

a) contacting a test compound with an host cell expressing a reporter nucleic acid comprising a nucleic acid sequence coding for a reporter placed under the control of

a promoter, which promoter comprises all or part of the promoter sequence of DOCK5 gene or a derivative thereof, and

b) measuring the level of expression of the reporter.

As used herein, the term "derivatives"' refer to a nucleic sequence having a percentage of identity of at least 80% with the sequence of DOCK5 promoter, preferably of at least 90%, as an example of at least 95%, and more preferably of at least 99%. The percentage of identity is as defined above.

By "compound" or "test compound", one should understand compounds of different nature, structure and origin, particularly biological compounds, nuclear factors, cofactors, and the like, chemical, synthetic compounds and the like, which are tested for their capacity of enhancing the level of expression of said gene implicated in antimicrobial defence.

The concentration of said test compound can be adjusted by the skilled person according to the characteristics of said compound (its toxicity, ability to penetrate cells, etc.), the number of cells, the length of the incubation period, etc. Generally, the cells are exposed to concentrations of test compounds ranging from 1 nM to 1 mM. Of course it is possible to test other concentrations without deviating from the invention, and also to test simultaneously different test compound concentrations.

Different adjuvants and/or vectors and/or products facilitating the penetration of the test compounds into the host cell such as liposomes, cationic lipids or polymers can also be used, when necessary.

By "decreasing the level of expression of a DOCK5 gene", one should understand that the expression level of DOCK5 gene is diminished or inhibited compared to a control level.

It should be noticed that said expression level of the DOCK5 gene is correlated to the expression level of the reporter gene in the method of the invention. In fact, one of skilled in the art can deduce that a test compound can decrease the expression level of the DOCK5 gene from the capacity of said compound to obtain an diminished expression level of the reporter gene in the method of the invention.

In the present invention, the control level can be determined, by example, by measuring the expression level of the reporter gene in the absence of the test compound.

Thus, in a preferred embodiment, the method according to the invention further comprises a step c) of comparing the level of expression of the reporter gene as measured in step b) with the level of expression of the reporter gene in the absence of said test compound.

In another embodiment, the present invention provides a method for identifying a compound which inhibits the activation of RAC1/2 GTPaseby inhibiting the binding of ELMO1 protein (SEQ ID N°9) to the SH3 domain of DOCK5 comprising the steps of:

a) contacting a test compound with the ELMO1 protein or a derivative thereof;

b) determining the possible binding of said test compound to the ELMO1 protein or the derivative thereof; and optionally

c) selecting the compound inhibiting the conversion of inactive RAC1/2 to active RAC1/2.

As used herein, the expression "ELMO1 protein" refers to SEQ ID N°9 and derivatives thereof.

The binding between said ELMO1 protein and the tested compound can be measured by methods well known from one skilled in the art.

If the binding between said ELMO1 protein and said test compound is observed, it can thus be conclude that the compound is an inhibitor of the binding of ELMO1 and the SH3 domain of DOCK5, and that this compound is useful to inhibit the conversion of inactive RAC1/2 to active RAC1/2.

Optionally, said method can include a further step after step b) of contacting a polypeptide comprising at least the SH3 domain of DOCK5 or the derivative thereof with said test compound and ELMO1 protein, and comparing the binding between said ELMO1 protein and said polypeptide in the presence or in the absence of said compound.

Alternatively, the present invention provides a method for identifying a compound which inhibits the activation of RAC1/2 GTPase by inhibiting the binding of ELMO1 to the SH3 domain of DOCK5 comprising the steps of:

a) contacting a test compound with the ELMO1 protein or the derivative thereof and a polypeptide comprising at least the SH3 domain of DOCK5 or the derivative thereof;

b) measuring the binding between said ELMO1 protein and said polypeptide in the presence or in the absence of said compound; and optionally

c) selecting the compound inhibiting the conversion of inactive RAC1/2 to active RAC1/2.

The binding between said ELMO1 protein and said polypeptide can be measured by methods well known from one skilled in the art. If the binding between said ELMO1 protein and said polypeptide in the presence of the tested compound is lower than the one measured in absence of said compound, it can thus be conclude that the compound is an inhibitor of the binding of ELMO1 to the SH3 domain of DOCK5, and that this compound is useful to inhibit the conversion of inactive RAC1/2 to active RAC1/2.

Optionally, the compounds as described above are coupled with a bisphosphonate radical. The bisphosphonate radical permits a fast incorporation of the compound after its administration.

Another object of the present invention is a compound as described above for treating and/or preventing bone loss diseases in a subject in need thereof.

Therefore, the present invention relates to the use of at least one compound as described above in preparing a drug for treating and/or preventing bone loss disease in a subject in need thereof.

Another object of the present invention is a pharmaceutical composition comprising at least one compound as described above and, optionally, a pharmaceutically acceptable support for treating and/or preventing bone loss diseases in a subject in need thereof.

Therefore, the present invention relates to the use of a pharmaceutical composition comprising at least one compound as described above in preparing a drug for treating and/or preventing bone loss diseases in a subject in need thereof.

As examples of pharmaceutically acceptable supports, the composition can include emulsions, microemulsions, oil in water emulsions, anhydrous lipids and water in oil emulsions or other types of emulsions.

The inventive composition can further include one or more additives such as diluents, excipients, stabilizers and preservatives. Such additives are well known to those skilled in the art and are described notably in "*Ullmann's Encyclopedia of Industrial Chemistry, 6th Ed.*" (various editors, 1989-1998, Marcel Dekker) and in "*Pharmaceutical Dosage Forms and Drug Delivery Systems*" (ANSEL *et al.*, 1994, WILLIAMS & WILKINS).

As used in the present application, the term "subject" refers to a mammal such as a rodent, cat, dog, primate or human, preferably said subject is a human.

Another object of the invention relates to a therapeutic method for treating a subject and/or preventing bone loss diseases, comprising the administration of a therapeutically effective quantity of a pharmaceutical composition as described above.

A "therapeutically effective quantity" means a quantity that inhibits or reduces the osteoclats activation. Those skilled in the art will be able to determine said therapeutically effective quantity based on their general knowledge and on the methods described in the examples.

The compounds can be administered by any mode of administration such as, for example, by intramuscular, intravenous or oral route, etc.

The inventive compounds preferably will be administered at a concentration chosen by those skilled in the art according to the state of advancement of the disease and the targeting mode used, the age and the weight of the subject. Preferably, the compound will be administrated at a concentration of between 5 and 200μM, preferably at a concentration comprised between 10 and 100 μM.

In the following, the invention is described in more detail with reference to amino acid sequences, nucleic acid sequences and the examples. Yet, no limitation of the invention is intended by the details of the examples. Rather, the invention pertains to any embodiment which comprises details which are not explicitly mentioned in the examples herein, but which the skilled person finds without undue effort.

EXAMPLES

1) Dock 5 mRNA expression

The expression of Dock5 was established in different mouse tissue. For this, DNaseI-treated total RNA was extracted using the High pure RNA isolation kit (ROCHE DIAGNOSTICS). To generate cDNA, RNA was primed with 10-mer random primers and reverse transcription catalysed using SUPERSCRIPT II reverse transcriptase (INVITROGEN). Quantitative PCR was performed with a Light Cycler (ROCHE DIAGNOSTICS) or a Mx3000p PCR system (STRATAGENE) using the PLATINIUM *Taq* DNA polymerase (INVITROGEN) and SYBR GREEN I (BIOWITAKKER) as in described in COELHO *et al.* (*Proc. Natl. Acad. Sci. U.S.A.*, vol.102, p:11917-11922, 2005) with the primers Dock5-Up (TGGTGACACAGGGACAGTGG, SEQ ID NO:5) and Dock5-Do (CACCCCAACTAGCACGTGG, SEQ ID NO: 6) for Dock5, and Gapdh-Up (ACAGTCCATGCCATCACTGCC, SEQ ID NO: 7) and Gapdh-Do (GCCTGCTTCACCACCTTCTT, SEQ ID NO: 8) for Gapdh as a control.

The specificity was assessed by purification and sequencing of the PCR product. All real-time PCR measures to quantify cDNA were done in triplicate, and the 95% confidence limits of the ratios to Gapdh were determined by Student's *t*-test.The figure 1A and B show the expression of Dock5 in different mouse tissues In figure 1A, said expression has been normalised according to Dock5 osteoclasts' expression (i.e., Dock5 osteoclasts' expression corresponding to 100% level).

The analysed tissues of figure 1A are as follow: Muscle 1 (M1), Muscle 2 (M2), heart (H), mammary gland at 10.5 days of embryo's development (GM 10.5), mammary gland at 13.5 days of embryo's development (GM 13.5), mammary gland at 15.5 days of embryo's development (GM 15.5), mammary gland at 18.5 days of embryo's development (GM 18.5), mammary gland of juvenile mouse (GM j), mammary gland at lactation (GM l), brain (Br), kidney (Kd), uterus (Ut), liver (Lv), macrophage (Mac), Testis 1 (T1), Testis 2 (T2), spleen (Sp), colon (Co), bone marrow (Bm), placenta at 13.5 days of embryo's development (Pl 13.5), placenta at 15.5 days of embryo's development (GM 15.5), and osteoclasts (Os).

Furthermore, total RNA of bone marrow macrophages (ND), induced for osteoclastic differentitation (OC) or dendritic cell differentiation (DC) and from mensenchymal stem

cells (MSC J0) induced for osteoblastic differentiation (MSC J4) were extracted and level of Dock5 mRNA relative to Gapdh mRNA was determined by RT-PCR.

The results of figure 1 B show that Dock5 mRNA is not expressed in dendritic cells and osteoblasts.

The results show that Dock5 is predominantly expressed in osteoclats, but an important expression of Dock5 is also found in placenta (i.e., nearly 50%) and testis. The expression of Dock5 is reduced in bone marrow, colon, spleen and testis compared to osteoclasts (i.e., nearly 20%), whereas its expression in the other tested tissues is fewer (i.e., nearly 10%). Thus, the results established that the expression of Dock5 is very specific from the osteoclats.

2) obtaining of DOCK5 polyclonal antibody

A rabbit polyclonal antibody was raised to a mouse DOCK5 C-terminus peptide corresponding to amino acids 1658-1869 from mouse DOCK5 and purified by immunoaffinity. In fact, the amino acids sequences significantly differ between the differents members of the subgroup DOCK-A.

Osteoclastogenesis was induced by RANKL-stimulation in purified mouse bone marrow macrophages were purified and in RAW264.7 cell line as described in BRAZIER *et al.* (abovementioned, 2006), which cells were maintained in culture. At 0, 3 or 5 days of stimulation, the cells were subjected to SDS-PAGE and blotted on polyvinyl difluoride membrane (MILLIPORE IMMOBILON-P pore size 0.45 µm). After transfer, the membrane was incubated in TBS-T (Tris buffered saline containing 0.1% TWEEN) with 2% skim millk at room temperature for 30 min and then with rabbit antisera diluted 1:1000 in TBS-T overnight at 4°C. The bound antibodies were detected by peroxidase labelled anti-rabbit immunoglobulin chemoluminescence system (AMERSHAM) and LAS-1000 image analyser (FUJI FILM). As a control, the membrane was further incubated with GAPDH antibodies, the bound antibodies being detected as previously.

The Figure 2 A and B show the expression of DOCK5 and GAPDH proteins in purified mouse bone marrow macrophages at 0, 3 and 5 days from the RANKL-stimulated osteoclastogenesis.

The results established that a protein of 215 kDa was induced during RANKL-stimulated osteoclastogenesis of purified mouse bone marrow macrophages (figure 2) and of RAW264.7 cell line (data not shown). This size is compatible with the size of the DOCK5 protein deduced from its mRNA.

Furthermore, total proteins were extracted from mouse tissues and subjected to western blot with antibodies against Dock5 and against tubulin for normalization.

The analysed tissues of figure 2C are as follow Ey: Eye, Sp: Spleen, St: Stomac, Te: Testis, Pl: Placenta, Lu: Lung, Br: Brain, He: Heart, Li: Liver, Ki: Kidney; Mu: Muscle.

The results of figure 2A confirm that Dock 5 is predominantly expressed in osteoclasts, testis and placenta.

3) DOCK5 polyclonal antibody specificity

ShRNA target sequences were selected in mouse *Dock5* open reading frames, and the 65-mer sense and antisense strands of DNA oligonucleotides were designed according to the CLONTECH BIOINFORMATICS DATA server and are described in BRAZIER *et al.* (abovementioned, 2006). The oligonucleotide was then synthetised by INVITROGEN annealed and cloned in pSINREN-RETROQ vector containing a puromycin resistance selection marker according to the manufacturer's instructions (CLONTECH). The pSIREN-RETROQ-Luc vector (CLONTECH) targeting firefly luciferase was used as a control. Retrovirus packaging was done by co-transfection of pSIREN-RETROQ vectors, the Friend MLV-based Gag-Pol expression vector pC57GP (LASSAUX *et al.*, *J. Virol.*, vol.79, p:6560-6564, 2005), and the VSV-G envelope glycoprotein expression vector pCSIG (BATTINI *et al., Proc. Natl. Acad. Sci.*, vol.96, p:1385-1390, 1999) into 293T cells using Jet PI (QBIOGEN) according to manufacturer's instructions. Viral supernatants were harvested 3 days after transfection and filtered through a 0.45 µm pore size filter.

For infections, RAW264.7 cells were plated at 2.10^5 cells per 6-cm dish. The next day, the medium was replaced for 4h with 1.5 ml of viral supernatant and 0.5 ml of growth medium containing 8µg/ml polybrene. Cells were left to recover in growth medium for 24 h, and infected cells were selected by addition of puromycin (3 µg/ml) for another 24h. Infected RAW264.7 were scrapped and reseeded in growth medium at 5.10^4 cells/well of a

6-well plate for RANKL-stimulated osteoclastogenesis as described in BRAZIER *et al.* (abovementioned, 2006).

Then, the detection of the DOCK5 protein was realized with the rabbit polyclonal anti-DOCK5 as described previously.

The Figure 2 A shows the expression of DOCK5 and GAPDH proteins in RAW264.7 cell lines infected with retrovirus coding for either small hairpin RNA directed against firefly luciferase (shLuc) or dock5 (shDock5) at 0, 3 and 5 days from the RANKL-stimulated osteoclastogenesis.

As described previously, the results established that a protein of 215 kDa was induced during RANKL-stimulated osteoclastogenesis of RAW264.7 cell line infected with a retrovirus coding for a small hairpin RNA directed against firefly luciferase. For RAW264.7 cell line infected with a retrovirus coding for a small hairpin RNA directed against Dock5, no protein of 215 kDa was detected during RANKL-stimulated osteoclastogenesis. Finally, the results confirmed that the protein DOCK5, such as its corresponding RNA, is induced during osteoclastogenesis, and that the obtained rabbit polyclonal anti-DOCK5 antibody is specific of the DOCK5 protein.

4) Dock5 mediates Rac activation *in vivo*

We therefore examined whether the DOCK5 protein, and more specifically its DHR2 domain, could activate small GTPases of the Rho-family –i.e., RAC1/2 and cdc42-.

To this end a GFP protein fused to the DHR2 domain of DOCK5 (see Figure 3A) was generated.

In vivo GTP loading of Rac and cdc42 was analysed as previously described in COTE & VUORI (*J. Cell. Sci.,* vol.115, p: 4901-4913, 2002).

Briefly, 293-T cells were transfected in six-wells plates with a vector coding for the GFP fusion protein comprising the DHR2 domain of DOCK5 (DHR2) or with a vector coding for GFP (GFP). 48 hours after transfection, cells were lysed in MLB buffer (25mM HEPES, pH 7.5, 150 mM NaCl, 1% NP-40, 10 mM MgCl2, 1 mM EDTA and 10% glycerol). The clarified lysates were incubated for 30 minutes with the GST-PAK-PBD

fusion protein bound to Glutathione sepharose. The beads were washed extensively with MLB buffer and the bound GTP-loaded Rac and cdc-42 were detected by immunoblotting. Equal amount of input lysate were analysed by immunoblotting to verify the expression levels of Rac, cdc42, GFP-DHR2 and GFP proteins. GST-PAK-PBD was expressed and purified for these experiments as described previously in ABASSI & VUORI (*EMBO J.*, vol.21, p:4571-4582, 2002).

The figure 3B shows the expression levels of Rac, cdc42, GFP-DHR2 and GFP proteins in total cell lysates (total) and the protein detected after GTP-trapping.

The results show that the expression of the DHR2 domain in 293-T cells induces the activation of endogeneous Rac but has no effect on cdc42 (figure 3B). Finally, the results established that the DHR2 domain of DOCK5 is able to activate the Rac GTPase, whereas it has no effect on cdc42.

5) ELMO1 binds to the SH3 domain of DOCK5

293-T cells were cotransfected as described previously with a vector coding for the ELMO1 protein or deleted from the C-terminus (ΔT625) - (GUMIENNY *et al.*, *Cell*, vol.107, p:27-41, 2001) and a vector coding GFP fusion proteins comprising the Full length DOCK5 protein (FL), the DHR2 domain, the DOCK5 protein sequence deleted from (i) the amino acids 1 to 559 of its N-terminus extremity (ΔNter), including the SH3 domain and half of the DHR1 domain, or the DOCK5 protein sequence deleted from (ii) the amino acids 1 to 82 comprising the SH3 domain (ΔSH3) (see figure 3 E).

48 hours after transfection, cells were lysed in MLB buffer (25mM HEPES, pH 7.5, 150 mM NaCl, 1% NP-40, 10 mM MgCl2, 1 mM EDTA and 10% glycerol). The clarified lysates were immunoprecipitated with anti-GFP antibody and the bound ELMO1 protein was detected by immunoblotting. Equal amount of input lysate were analysed by immunoblotting to verify the expression levels of ELMO1 protein.

The figure 3C and 3F show the expression levels of ELMO1 protein in total cell lysates (total) and after immunopreciptation with anti-GFOP antibody (IP GFP), in cells cotransfeted with a vector coding for ELMO1 protein and full length DOCK5 (FL), the

DHR2 domain (DHR2), DOCK5 deleted from its SH3 domain (ΔSH3) or from its N-term domain (ΔNter).

The results show that deletion if Dock5 SH3 domain or coexpression of full lengh ELMO1 with full length Dock5 greatly increased its exchange activity on Rac thus establishing that the N-term domain of DOCK5, and more specifically its SH3 domain, is necessary for the binding of ELMO1 to DOCK5 (Figure 3C). Figure 3F shows that Dock5 N-terminal domain binds Elmo1 C-terminus.

6) The SH3 domain of DOCK5 inhibits Rac activation *in vivo*

In vivo GTP loading of Rac was determined as previously in the presence of different domains of the DOCK5 protein and, eventually, the simultaneous presence of the ELMO1 protein.

The figure 3D shows the expression levels of Rac in total cell lysates (total) and the RAC-GTP protein detected after GTP trapping in the cells transfected with a vector coding for the GFP protein (GFP), for the DHR2 domain of DOCK5 (DHR2), for the DOCK5 protein deleted from its SH3 domain (ΔSH3), for the DOCK protein (FL), eventually cotransfected with a vector coding for the ELMO1 protein (FL+Elmo1).

The results show as previously that the expression of the DHR2 domain is able to activate the Rac GTPase and that the SH3 domain inhibits this activation (Figure 3D). In fact, the deletion of the SH3 domain results in the activation of the Rac GTPase by the deleted DOCK5 protein. Finally, the binding of ELMO1 to the SH3 domain results in the activation of the Rac GTPase.

7) DOCK5 is a major activator of Rac in osteoclats.

RAW264.7 cell lines stimulated with RANKL were infected as described previously with a retrovirus coding for either small hairpin RNA directed against firefly luciferase (shLuc) or dock5 (shDock5).

Furthermore, the levels of active Rac in TCL from $Dock5^{+/+}$ and $Dock5^{-/-}$ osteoclasts were determined. $Dock5^{-/-}$ mice were obtained by gene trap (Laurin et al. 2008) to generate Dock5 deficient osteoclasts.

The *in vivo* GTP loading of Rac was determined as disclosed previously.

The figure 4 shows the average of three independent experiments with active Rac levels set to 1 in control shLuc and $Dock5^{+/+}$ osteoclasts. Error bars : SD.

The figure 4A show the expression levels of Rac in total cell lysates (total Rac) and the RAC-GTP protein detected after GTP trapping in the cells infected with a retrovirus coding for either small hairpin RNA directed against firefly luciferase (shLuc) or dock5 (shDock5).

The figure 4B shows that $Dock5^{-/-}$ osteoclasts have reduced active Rac levels compared to the control level of $Dock5^{+/+}$ osteoclasts.

The results established that the inhibition of DOCK5 expression results in a decrease of the levels of active RAC (i.e., 40%) in osteoclasts expressing Dock5 shRNAs and osteoclasts derived from Dock5 KO BMMs as compared to controls. Thus, DOCK5 is an essential exchange factor of RAC in osteoclasts.

8) DOCK5 is necessary for mineralised matrix resorption

RAW264.7 cell lines were infected as described previously with a retrovirus coding for either small hairpin RNA directed against firefly luciferase (shLuc) or dock5 (shDock5), and then osteoclastogenesis was stimulated with RANKL. The obtained cells were then cultured on calcium phosphate substrates to induce the formation of the actin ring. After 48 hours, cells were fixed and stained for actin using rhodamine-labeled Phalloidin to reveal the sealing zone (figure 5).

The figure 6 shows the polymerisation of actin in RAW264.7 cell lines stimulated with RANKL which have been infected with a retrovirus coding for either small hairpin RNA directed against firefly luciferase (shLuc) or dock5 (shDock5) and the mineralised matrix resorption in the presence of said osteoclasts.

The results show that in the osteoclasts, the DOCK5 protein is associated with the podosome and with the sealing zone (data not shown). The osteoclasts wherein DOCK5 expression was inhibited show a default of contraction and of sealing zone formation. The

measure of mineralised matrix resorption surface by VON KOSSA staining shows a strong decrease of the resorption by osteoclasts wherein DOCK5 expression was decreased.

9) Confirmation by osteoclasts from Dock5$^{-/-}$ mice

BMMs (bone marrow macrophages) isolated from Dock5$^{+/+}$ and Dock5$^{-/-}$ mice were differentiated into osteoclasts in the presence of 100 ng/ml RANKL and 10 ng/ml M-CSF. TCL (total cell extracts) were prepared at days 0, 3 and 4 and subjected to western blot with antibodies against Dock5 and ß-gal and against tubulin for normalization.

Osteoclasts derived from Dock5$^{-/-}$ BMMs express Dock5 truncated after aminoacid 1115, between DHR1 and DHR2 domains, and fused to a ß-geo cassette (Figure 7A).

Furthermore, the differentiated osteoclasts were fixed and stained with TRAP and Hoeschst at day 5 to determine the number of MNCs (multinucleated cells). Figure 7B (average and SD from four independent experiments **: significant difference, $p<0.01$, Mann & Whitney test) shows that the efficiency of TRAP positive MNCs formation was reduced in Dock5$^{-/-}$ BMMs as compared to Dock5$^{+/+}$ and osteoclasts were smaller.

Furthermore, in order to show that osteoclasts differentiated from Dock5$^{-/-}$ BMMs can't assemble a sealing zone, they were seeded on calcium-phosphate substrate to induce the formation of the actin ring. After 48 hours, cells were fixed and stained for actin using rhodamine-labeled Phalloidin (green) to reveal the sealing zone and with Hoeschst dye to stain nuclei (blue) (data not shown). It was observed that on calcium-phosphate substrates, sealing zone assembly and resorption was defective in Dock5$^{-/-}$ osteoclasts.

Finally, to demonstrate that Dock5$^{-/-}$ osteoclasts can't form resorption pit, derived from Dock5$^{-/-}$ BMMs were differentiated on bone sliced for 5 days, fixed and observed by scanning electron microscopy.

The results show that when seeded on bone slices, Dock5$^{-/-}$ osteoclasts did not form resorption pits.

Moreover, in order to show that Dock5$^{-/-}$ osteoclasts are defective for bone resorption, the levels of collagen degradation peptide (CTx) were determined in the medium of Dock5$^{+/+}$ and Dock5$^{-/-}$ osteoclasts after 5 days of differentiation and bone slices were stained. Figure 7C shows average and SD of three osteoclast-seeded wells from one experiment, representative of three independent experiments.

The measurement of collagen telopeptide (CTx) confirmed that the resorbing activity of Dock5$^{-/-}$ osteoclasts was defective (Figure 7C).

10) Suppression of Dock5 impairs RAC activation in osteoclasts.

The levels of osteoclastic markers in wild type and Dock5 deficient osteoclasts derived from BMM of Dock5$^{+/+}$ or Dock5$^{-/-}$ animals or from control and Dock5 shRNA expressing RAW264.7 cells. Total RNA was prepared from Dock5$^{+/+}$ and Dock5$^{-/-}$ BMMs grown for 5 days in the presence of M-CSF only (black bars) or in the presence of RANKL and M-CSF to obtain osteoclasts (white bars). The levels of indicated gene mRNAs relative to Gapdh mRNA were determined by RT-PCR.

The results of figure 8A show that the expression of osteoclast differentiation markers is normal in osteoclasts differentiated from Dock5$^{-/-}$ BMMs. This indicated osteoclast maturation was not affected and suggested Dock5 deficiency did not impair the capacity of osteoclasts to respond to M-CSF and RANKL in vitro.

Moreover, the ability of Dock5$^{-/-}$ preosteoclasts to respond to M-CSF and RANKL was not the result of a compensatory increase in Dock1 or Dock2 expression as their mRNA levels were identical as in Dock5$^{+/+}$ (Figure 8B).

Preosteoclasts prepared from Dock5$^{+/+}$ and Dock5$^{-/-}$ BMMs were stimulated with M-CSF or RANKL for the inducated amount of time. The levels of ERK, p38 and Akt phosphorylation in TCL were determined by western blot.

The results show that M-CSF-driven phosphorylation ERK and p38MAP kinase (Figure 8C) and RANKL-driven phosphorylation of Akt (Figure 8D) were unaffected in Dock5$^{-/-}$ preosteoclasts as compared to controls.

Finally, these results established that DOCK5 is a new therapeutic target for limiting bone loss in menopause, osteoporosis, osteopenia due to bone metastases, periarticular erosions in rheumatoid arthritis, primary hyperparathyroidism, hypercalcemia of malignancy, Paget's disease of bone, periodontal disease, immobilization induced osteopenia, or in glucocorticoid treatment. Because of the specific osteoclasts DOCK5

expression, the targeting of DOCK5 may limit side effects such as the ones observed with drugs for treating bone loss.

11) Identification of DOCK5 inhibitor

In order to identify DOCK5 inhibitors, which inhibitors can be useful for treating bone loss associated disease, we use the Yeast Exchange Assay (YEA) as disclosed in DE TOLEDO *et al.* (*FEBS*, vol.480, p:287-292, 200) and International Patent application PCT WO 2005/064007.

Briefly, we transform a yeast strain TAT7 (Mata, trp1, his3, leu2, ura3, ade2, LYS:: (LexAop)4-HIS3, URA3:: (LexAop)8-lacZ) provided by J. CAMONIS) with vectors expressing the DHR2 domain of DOCK5 fused to a myc-tag (SEQ ID NO: ...), the wild type Rac GTPase fused to LexA and its effector PAK fused to the transactivation domain of GAL4.

In the obtained transformed yeast, the expression of the DHR2 domain of DOCK5 induces the activation of Rac, which activated Rac interacts with its effector PAK resulting in the expression of reporter genes β-Gal and His3 (see Figure 6).

In order to modify yeast cell membrane permeability, a mutation in the Erg6 gene has been introduced as disclosed in BLANGY *et al.* (*Biol. Cell.*, vol.98(9), p:511-22, 2006). This mutation of the Erg6 gene increases the entry of the screened compounds in the yeast cells, and thus enables to limit the concentration of the screened compounds.

For screening DOCK5 inhibitors, which can be useful for treating bone loss diseases, the transformed yeast is contacted with several chemical or peptidic molecules, and the chemical or peptidic molecules inhibiting the expression of reporter genes β-Gal and His3 are selected for further testing in the bone loss model disclosed in 8 and then in bone loss diseases models.

The yeast strain TAT7 was used to identify DOCK5 inhibitors. The strain was seeded, in a 96-well culture plate in a selective medium devoid of histidine or in a non selective medium where histidine is added. 2560 compounds were screened to select the ones which inhibit the growth of the strains in a selective medium without having effect on the growth in a non selective medium. DMSO was used as a control.

The compounds were tested at a concentration of 200µM in presence of 1% DMSO. The growth of the yeasts was measured by optical density at 600 nm at t= 2 hours, 15 hours, 20 hours and 24 hours after seeding. The inhibiting compounds were defined as follows:

-At time n, the growth derivative Cr (medium) = (OD600Tn-OD600T2)/Tn-T2 in test medium (-HIS) and in toxic medium (+HIS).

- At each time and for each plate, the Cr (-HIS) and Cr (+HIS) medium control was calculated on the control.

- the ratio R(compound)= Cr (-HIS) and Cr(+HIS) and R was calculated for each plate

- the inhibition rate was determined by dividing by the control ratio I(compound)= R(compound)/R(control)*

-the selected compounds are those showing a ratio I(compound) < 0.9 at each time.

Results are shown in table 2.

55 compounds were thus selected as inhibiting the activation of RAC1/2 by Dock5.

12) Toxicity test on osteoclast precursors.

The selected compounds were then tested for their toxicity on osteoclast precursors. Since these cells do not express Dock5, a Dock5 inhibitor should not affect their growth. RAW264.7 cells used as osteoclasts inhibitors were allowed to grow for 72 hours with 10 to 100µM of compound. The growth of the cells was compared to control cells which were grown with 0.5% DMSO.

The results are presented in table 2. The optimal concentration was the determined for the compounds which were not toxic (the concentration which does not affect the growth of the cells).

13) Toxicity test on differentiated osteoclasts.

The compounds were tested for their toxicity on differentiated osteoclasts at the concentration determined above. RAW264.7 cells differentiated in osteoclasts were allowed to grow for 72 hours in presence of the tested compounds. The tartrate-resistant acid phosphatase (TRAP) was then revealed in osteoclasts by coloration (SUDA et al., 1997). This osteoclasts specific labeling permits to visualize the cell morphology. The cell morphology was then compared to control cells which were allowed to grow in presence of 0.5% DMSO. The compounds were then classified in 3 categories:

- compounds which induce the death of all the osteoclasts after 72 hours (-)
- compounds which induce morphological anomalies and/or death of part of the osteoclasts (+/-)
- Compounds which do not induce visible modifications of the osteoclasts.

The results are shown in table 2.

14) Resorption inhibition test

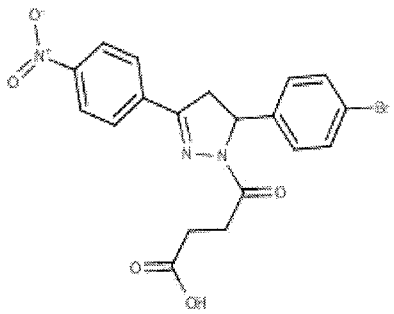
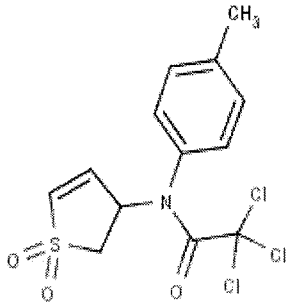
The identified compounds were used at the same concentration as defined above on osteoclats seeded on miner0alised matrix resorption surface of calcium phosphate (Osteologic Biocoat Clontech Reference 354609) during 72 hours. Then the mineralised matrix was coloured with silver nitrate in order to show the resorbed areas. The compounds were classified in 3 categories:

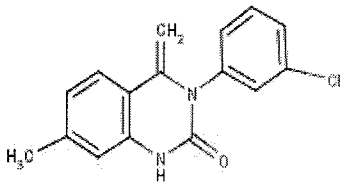
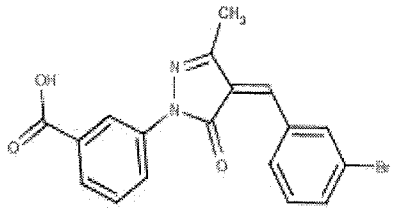
- Compounds that totally prevent resorption in 72 hours (-)
- Compounds that induce an attenuated resorption compared to the control (+/-)
- Compounds that do not visibly modify the osteoclats resorption activity compared to the control. (+)

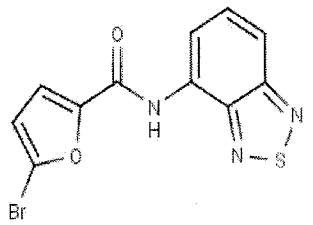
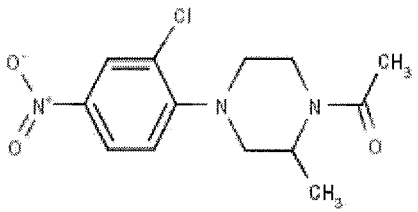
The compounds of the resorption categories (+/-) and (-) represent new inhibitors of the bone resorption. They were used at a concentration of 10 to 100 µM.

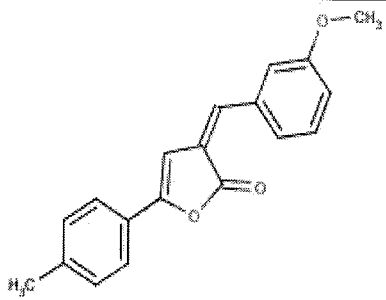
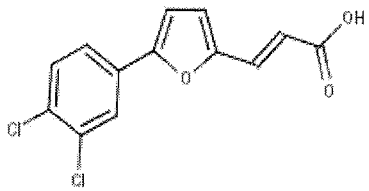
To confirm the results, the compounds were then tested *in vivo* in a mouse which presents osteoporose.

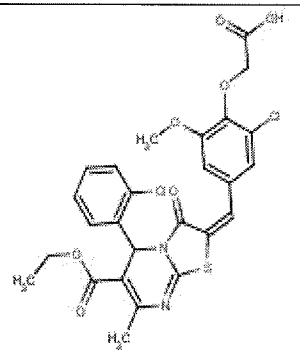
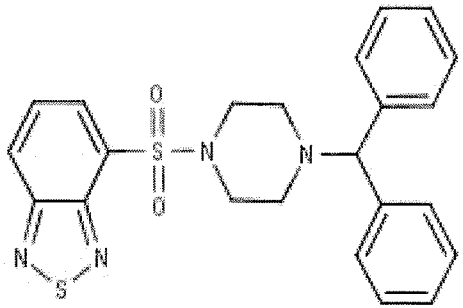
Table 1: compound identified by the screening method of the present invention

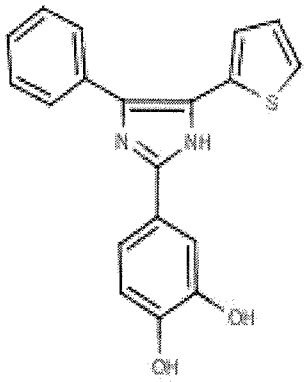
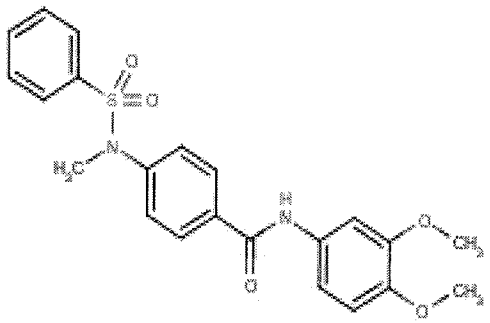
Structure	Mol Weight	Mol Formula	Mol Name	compound n°
	446,3	C19 H16 Br N3 O5	4-[5-(4-bromophenyl)-3-(4-nitrophenyl)-4,5-dihydro-1H-pyrazol-1-yl]-4-oxobutanoic acid	4
	368,7	C13 H12 Cl3 N O3 S	2,2,2-trichloro-N-(1,1-dioxido-2,3-dihydro-3-thienyl)-N-(4-methylphenyl)acetamide	5

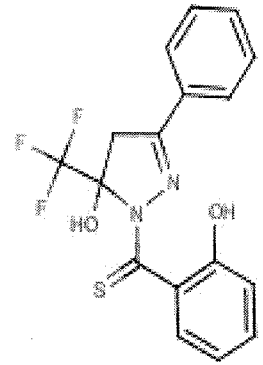
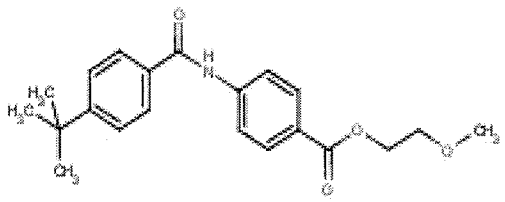
	284,7	C16 H13 Cl N2 O	3-(3-chlorophenyl)-7-methyl-4-methylene-3,4-dihydro-2(1H)-quinazolinone	11
	385,2	C18 H13 Br N2 O3	3-[4-(3-bromobenzylidene)-3-methyl-5-oxo-4,5-dihydro-1H-pyrazol-1-yl]benzoic acid	18

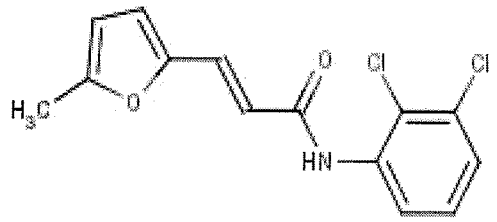
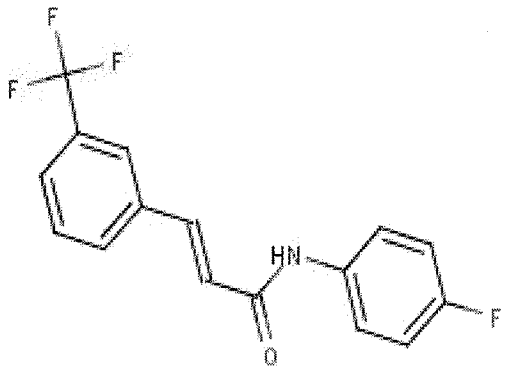
	324,2	C11 H6 Br N3 O2 S	N-2,1,3-benzothiadiazol-4-yl-5-bromo-2-furamide	20
	297,7	C13 H16 Cl N3 O3	1-acetyl-4-(2-chloro-4-nitrophenyl)-2-methylpiperazine	22

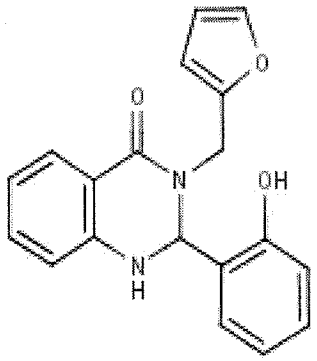
	292,3	C19 H16 O3	3-(3-methoxybenzylidene)-5-(4-methylphenyl)-2(3H)-furanone	23
	283,1	C13 H8 Cl2 O3	3-[5-(3,4-dichlorophenyl)-2-furyl]acrylic acid	24

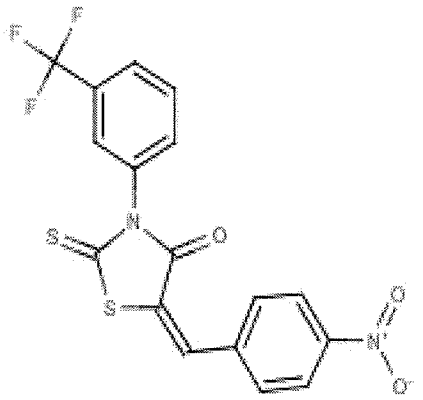
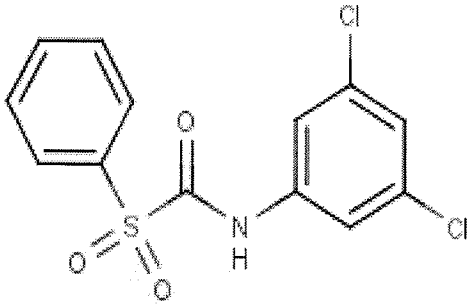
	577,4	C26 H22 Cl2 N2 O7 S	(2-chloro-4-{[5-(2-chlorophenyl)-6-(ethoxycarbonyl)-7-methyl-3-oxo-5H-[1,3]thiazolo[3,2-a]pyrimidin-2(3H)-ylidene]methyl}-6-methoxyphenoxy)acetic acid	25
	450,6	C23 H22 N4 O2 S2	4-{[4-(diphenylmethyl)-1-piperazinyl]sulfonyl}-2,1,3-benzothiadiazole	26

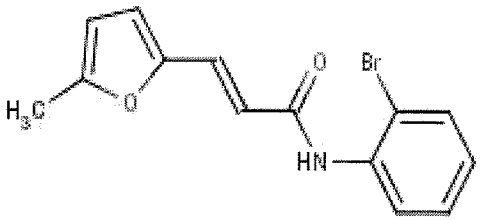
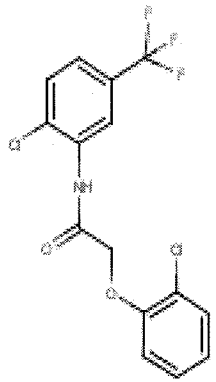
	334,4	C19 H14 N2 O2 S	4-[4-phenyl-5-(2-thienyl)-1H-imidazol-2-yl]-1,2-benzenediol	34
	426,5	C22 H22 N2 O5 S	N-(3,4-dimethoxyphenyl)-4-[methyl(phenylsulfonyl)amino]benzamide	37

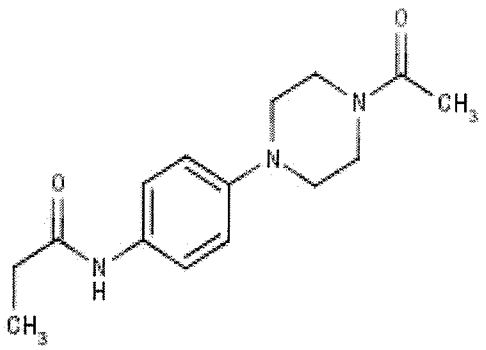
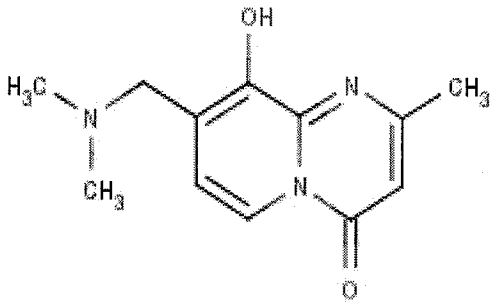
	366,4	C17 H13 F3 N2 O2 S	1-[(2-hydroxyphenyl)carbonothioyl]-3-phenyl-5-(trifluoromethyl)-4,5-dihydro-1H-pyrazol-5-ol	42
	355,4	C21 H25 N O4	2-methoxyethyl 4-[(4-tert-butylbenzoyl)amino]benzoate	44

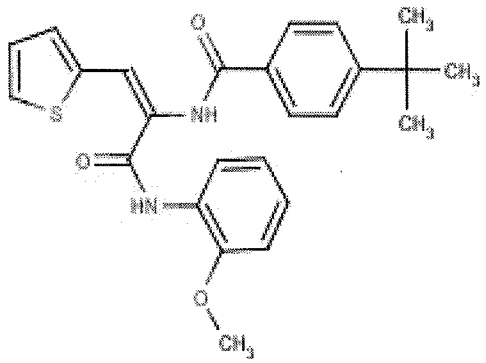
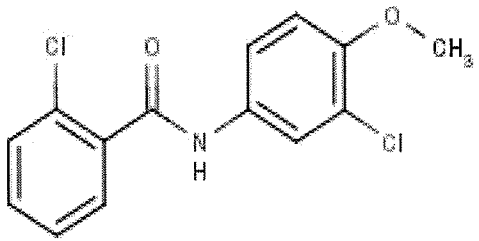
	296,2	C14 H11 Cl2 N O2	N-(2,3-dichlorophenyl)-3-(5-methyl-2-furyl)acrylamide	47
	309,3	C16 H11 F4 N O	N-(4-fluorophenyl)-3-[3-(trifluoromethyl)phenyl]acrylamide	54

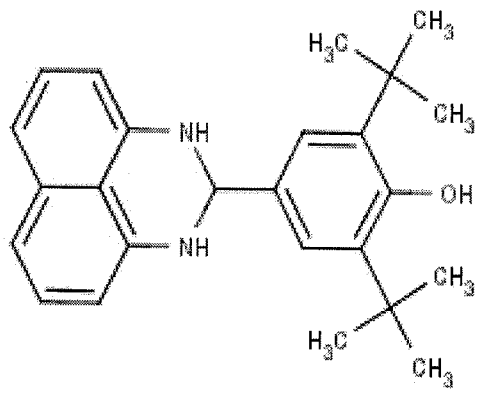
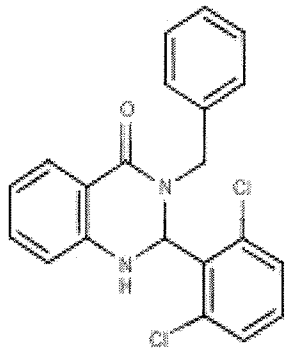
	320,3	C19 H16 N2 O3	3-(2-furylmethyl)-2-(2-hydroxyphenyl)-2,3-dihydro-4(1H)-quinazolinone	55
	385,4	C19 H19 N3 O4 S	N-(4-ethoxyphenyl)-2-{[5-(4-methoxyphenyl)-1,3,4-oxadiazol-2-yl]thio}acetamide	3

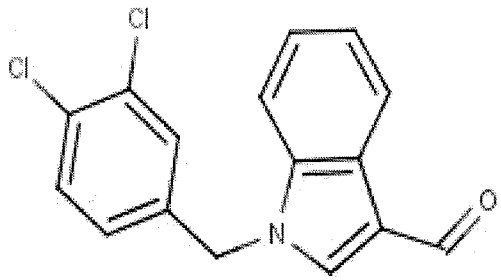
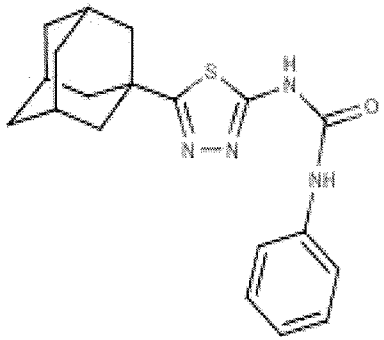
	410,4	C17 H9 F3 N2 O3 S2	5-(4-nitrobenzylidene)-2-thioxo-3-[3-(trifluoromethyl)phenyl]-1,3-thiazolidin-4-one	16
	330,2	C13 H9 Cl2 N O3 S	(3,5-dichlorophenyl)[(phenylsulfonyl)carbonyl]amine	21

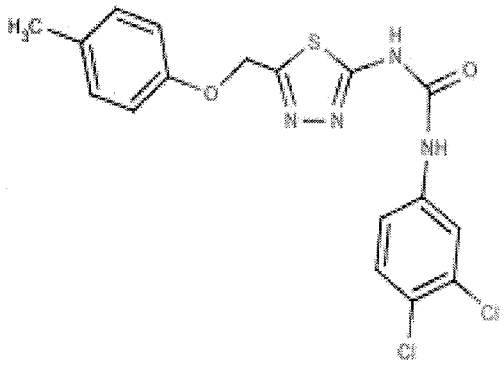
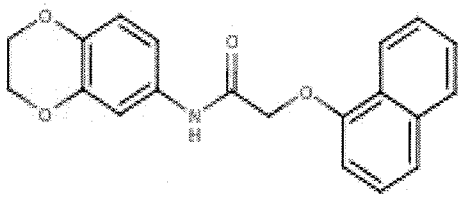
	306,2	C14 H12 Br N O2	N-(2-bromophenyl)-3-(5-methyl-2-furyl)acrylamide	6
	364,1	C15 H10 Cl2 F3 N O2	2-(2-chlorophenoxy)-N-[2-chloro-5-(trifluoromethyl)phenyl]acetamide	12

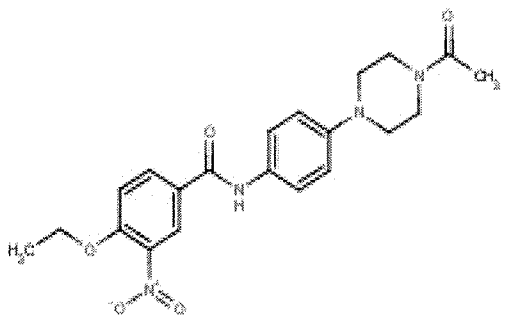
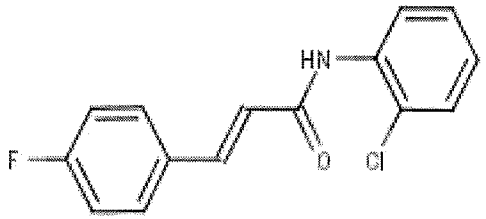
	275,3	C15 H21 N3 O2	N-[4-(4-acetyl-1-piperazinyl)phenyl]propanamide	13
	233,3	C12 H15 N3 O2	8-[(dimethylamino)methyl]-9-hydroxy-2-methyl-4H-pyrido[1,2-a]pyrimidin-4-one	14

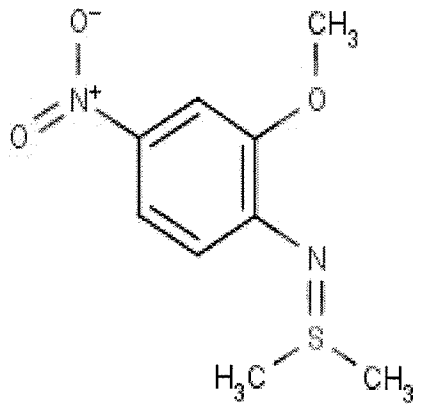
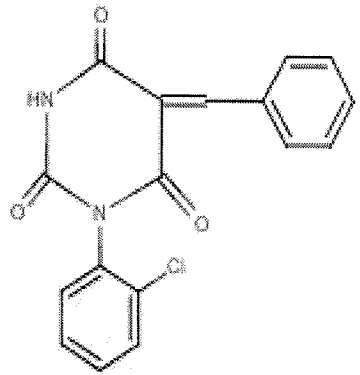
	434,6	C25 H26 N2 O3 S	4-tert-butyl-N-[1-{[(2-methoxyphenyl)amino]carbonyl}-2-(2-thienyl)vinyl]benzamide	46
	296,2	C14 H11 Cl2 N O2	2-chloro-N-(3-chloro-4-methoxyphenyl)benzamide	51

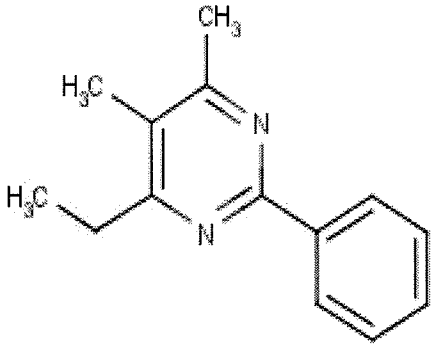
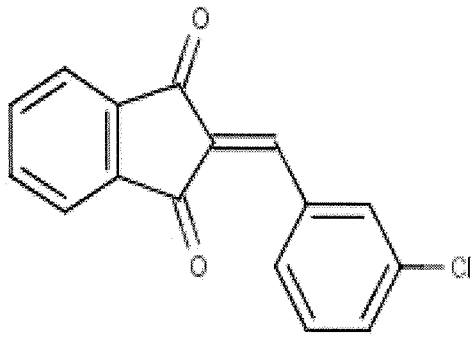
	374,5	C25 H30 N2 O	2,6-di-tert-butyl-4-(2,3-dihydro-1H-perimidin-2-yl)phenol	30
	383,3	C21 H16 Cl2 N2 O	3-benzyl-2-(2,6-dichlorophenyl)-2,3-dihydro-4(1H)-quinazolinone	33

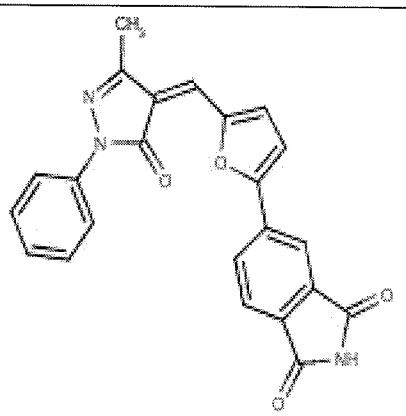
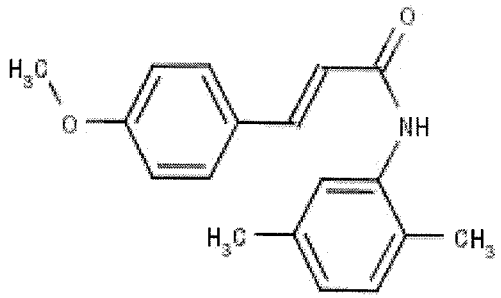
	304,2	C16 H11 Cl2 N O	1-(3,4-dichlorobenzyl)-1H-indole-3-carbaldehyde	35
	354,5	C19 H22 N4 O S	N-[5-(1-adamantyl)-1,3,4-thiadiazol-2-yl]-N'-phenylurea	49

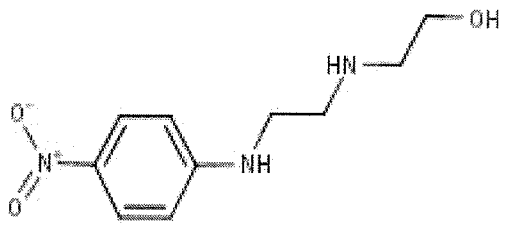
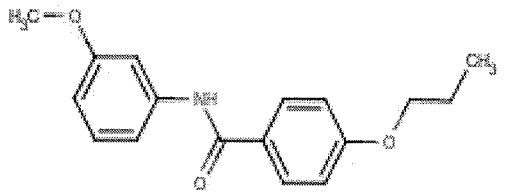
	409,3	C17 H14 Cl2 N4 O2 S	N-(3,4-dichlorophenyl)-N'-{5-[(4-methylphenoxy)methyl]-1,3,4-thiadiazol-2-yl}urea	53
	335,4	C20 H17 N O4	N-(2,3-dihydro-1,4-benzodioxin-6-yl)-2-(1-naphthyloxy)acetamide	10

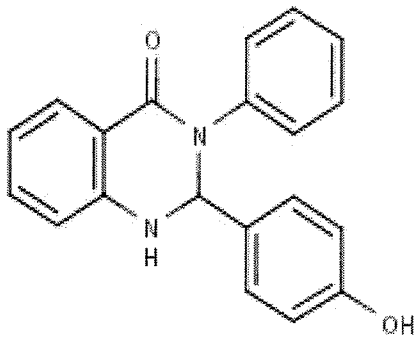
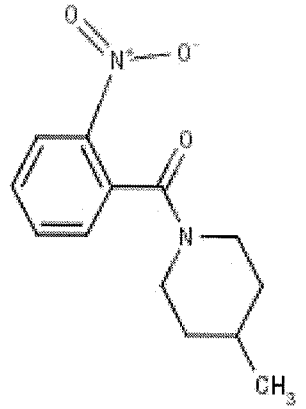
	412,4	C21 H24 N4 O5	N-[4-(4-acetyl-1-piperazinyl)phenyl]-4-ethoxy-3-nitrobenzamide	27
	275,7	C15 H11 Cl F N O	N-(2-chlorophenyl)-3-(4-fluorophenyl)acrylamide	40

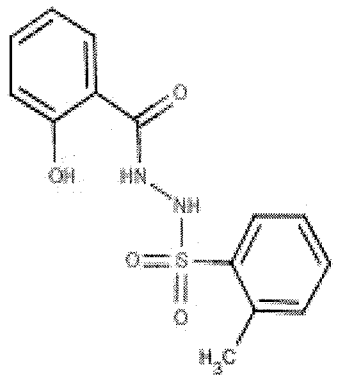
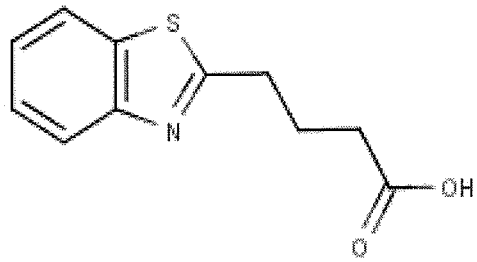
	228,3	C9 H12 N2 O3 S	1-[(dimethyl-lambda~4~-sulfanylidene)amino]-2-methoxy-4-nitrobenzene	43
	326,7	C17 H11 Cl N2 O3	5-benzylidene-1-(2-chlorophenyl)-2,4,6(1H,3H,5H)-pyrimidinetrione	48

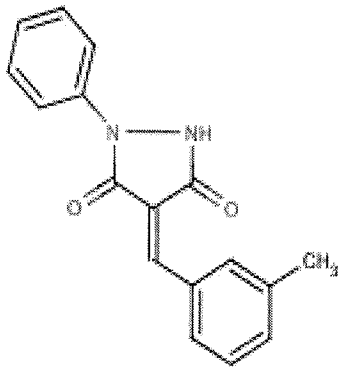
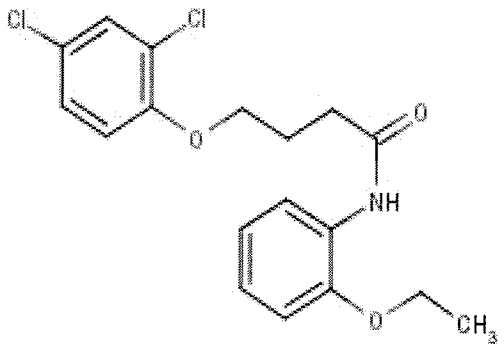
	212,3	C14 H16 N2	4-ethyl-5,6-dimethyl-2-phenylpyrimidine	50
	268,7	C16 H9 Cl O2	2-(3-chlorobenzylidene)-1H-indene-1,3(2H)-dione	1

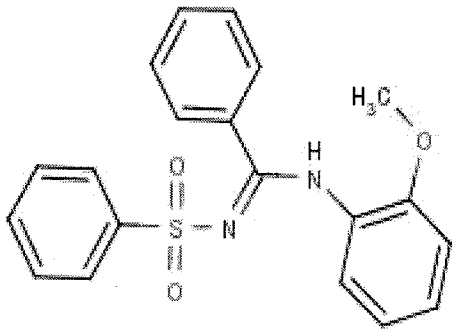
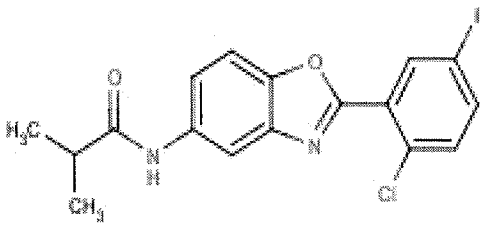
	397,4	C23 H15 N3 O4	5-{5-[(3-methyl-5-oxo-1-phenyl-1,5-dihydro-4H-pyrazol-4-ylidene)methyl]-2-furyl}-1H-isoindole-1,3(2H)-dione	7
	281,4	C18 H19 N O2	N-(2,5-dimethylphenyl)-3-(4-methoxyphenyl)acrylamide	8

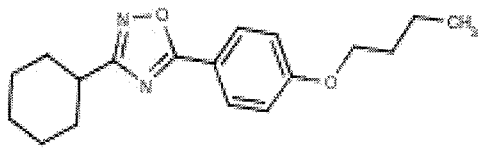
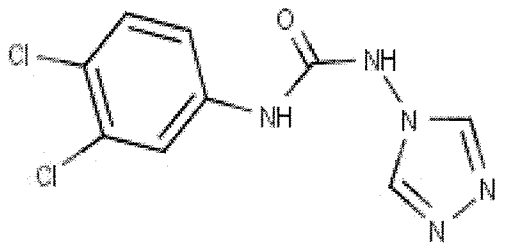
	225,2	C10 H15 N3 O3	2-({2-[(4-nitrophenyl)amino]ethyl}amino)ethanol	15
	285,3	C17 H19 N O3	N-(3-methoxyphenyl)-4-propoxybenzamide	17

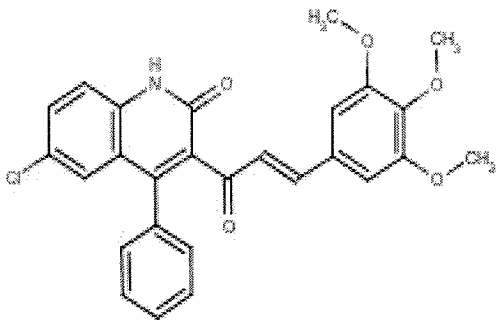
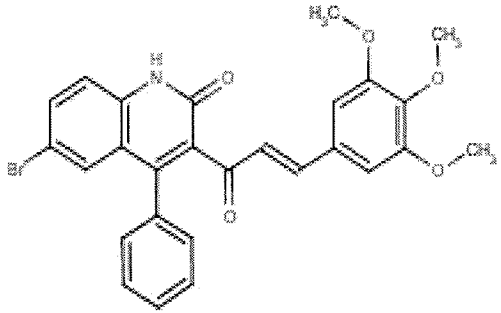
	316,4	C20 H16 N2 O2	2-(4-hydroxyphenyl)-3-phenyl-2,3-dihydro-4(1H)-quinazolinone	19
	248,3	C13 H16 N2 O3	4-methyl-1-(2-nitrobenzoyl)piperidine	28

	306,3	C14 H14 N2 O4 S	2-hydroxy-N'-[(2-methylphenyl)sulfonyl]benzohydrazide	31
	221,3	C11 H11 N O2 S	4-(1,3-benzothiazol-2-yl)butanoic acid	39

	278,3	C17 H14 N2 O2	4-(3-methylbenzylidene)-1-phenyl-3,5-pyrazolidinedione	41
	368,3	C18 H19 Cl2 N O3	4-(2,4-dichlorophenoxy)-N-(2-ethoxyphenyl)butanamide	2

	366,4	C20 H18 N2 O3 S	N-(2-methoxyphenyl)-N'-(phenylsulfonyl)benzenecarboximidamide	9
	440,7	C17 H14 Cl I N2 O2	N-[2-(2-chloro-5-iodophenyl)-1,3-benzoxazol-5-yl]-2-methylpropanamide	32

	300,4	C18 H24 N2 O2	5-(4-butoxyphenyl)-3-cyclohexyl-1,2,4-oxadiazole	38
	272,1	C9 H7 Cl2 N5 O	N-(3,4-dichlorophenyl)-N'-4H-1,2,4-triazol-4-ylurea	52

	475,9	C27 H22 Cl N O5	6-chloro-4-phenyl-3-[3-(3,4,5-trimethoxyphenyl)acryloyl]-2(1H)-quinolinone	29
	520,4	C27 H22 Br N O5	6-bromo-4-phenyl-3-[3-(3,4,5-trimethoxyphenyl)acryloyl]-2(1H)-quinolinone	36

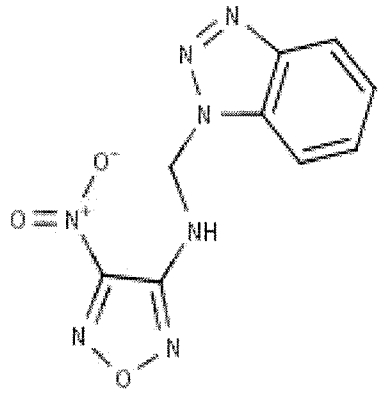
	261,2	C9 H7 N7 O3	N-(1H-1,2,3-benzotriazol-1-ylmethyl)-4-nitro-1,2,5-oxadiazol-3-amine	45

Table 2

Mol Name	Compound N°	Concentration µM	Survival at 72 hours	Resorption
4-[5-(4-bromophenyl)-3-(4-nitrophenyl)-4,5-dihydro-1H-pyrazol-1-yl]-4-oxobutanoic acid	4	100µM	-	-

2,2,2-trichloro-N-(1,1-dioxido-2,3-dihydro-3-thienyl)-N-(4-methylphenyl)acetamide	5	100μM	-	-
3-(3-chlorophenyl)-7-methyl-4-methylene-3,4-dihydro-2(1H)-quinazolinone	11	100μM	-	-
3-[4-(3-bromobenzylidene)-3-methyl-5-oxo-4,5-dihydro-1H-pyrazol-1-yl]benzoic acid	18	100μM	-	-
N-2,1,3-benzothiadiazol-4-yl-5-bromo-2-furamide	20	100μM	-	-
1-acetyl-4-(2-chloro-4-nitrophenyl)-2-methylpiperazine	22	100μM	-	-
3-(3-methoxybenzylidene)-5-(4-methylphenyl)-2(3H)-furanone	23	100μM	-	-
3-[5-(3,4-dichlorophenyl)-2-furyl]acrylic acid	24	100μM	-	-
(2-chloro-4-{[5-(2-chlorophenyl)-6-(ethoxycarbonyl)-7-methyl-3-oxo-5H-[1,3]thiazolo[3,2-a]pyrimidin-2(3H)-	25	25μM	-	-

ylidene]methyl}-6-methoxyphenoxy)acetic acid				
4-{[4-(diphenylmethyl)-1-piperazinyl]sulfonyl}-2,1,3-benzothiadiazole	26	100μM	-	-
4-[4-phenyl-5-(2-thienyl)-1H-imidazol-2-yl]-1,2-benzenediol	34	10μM	-	-
N-(3,4-dimethoxyphenyl)-4-[methyl(phenylsulfonyl)amino]benzamide	37	50μM	-	-
1-[(2-hydroxyphenyl)carbonothioyl]-3-phenyl-5-(trifluoromethyl)-4,5-dihydro-1H-pyrazol-5-ol	42	25μM	-	-
2-methoxyethyl 4-[(4-tert-butylbenzoyl)amino]benzoate	44	10μM	-	-
N-(2,3-dichlorophenyl)-3-(5-methyl-2-furyl)acrylamide	47	100μM	-	-
N-(4-fluorophenyl)-3-[3-(trifluoromethyl)phenyl]acrylamide	54	50μM	-	-

3-(2-furylmethyl)-2-(2-hydroxyphenyl)-2,3-dihydro-4(1H)-quinazolinone	55	100μM	-	-
2,6-di-tert-butyl-4-(2,3-dihydro-1H-perimidin-2-yl)phenol	30	100μM	-	+/-
3-benzyl-2-(2,6-dichlorophenyl)-2,3-dihydro-4(1H)-quinazolinone	33	100μM	-	+/-
1-(3,4-dichlorobenzyl)-1H-indole-3-carbaldehyde	35	50μM	-	+/-
N-(4-ethoxyphenyl)-2-{[5-(4-methoxyphenyl)-1,3,4-oxadiazol-2-yl]thio}acetamide	3	100μM	+/-	-
5-(4-nitrobenzylidene)-2-thioxo-3-[3-(trifluoromethyl)phenyl]-1,3-thiazolidin-4-one	16	100μM	+/-	-
(3,5-dichlorophenyl)[(phenylsulfonyl)carbonyl]amine	21	50μM	+/-	-
N-(2-bromophenyl)-3-(5-methyl-2-furyl)acrylamide	6	25μM	+	-

2-(2-chlorophenoxy)-N-[2-chloro-5-(trifluoromethyl)phenyl]acetamide	12	100μM	+	-
N-[4-(4-acetyl-1-piperazinyl)phenyl]propanamide	13	50μM	+	-
8-[(dimethylamino)methyl]-9-hydroxy-2-methyl-4H-pyrido[1,2-a]pyrimidin-4-one	14	100μM	+	-
4-tert-butyl-N-[1-{[(2-methoxyphenyl)amino]carbonyl}-2-(2-thienyl)vinyl]benzamide	46	100μM	+	-
2-chloro-N-(3-chloro-4-methoxyphenyl)benzamide	51	50μM	+	-
N-[5-(1-adamantyl)-1,3,4-thiadiazol-2-yl]-N'-phenylurea	49	50μM	+/-	+/-
N-(3,4-dichlorophenyl)-N'-{5-[(4-methylphenoxy)methyl]-1,3,4-thiadiazol-2-yl}urea	53	100μM	+/-	+/-
N-(2,3-dihydro-1,4-benzodioxin-6-yl)-2-(1-naphthyloxy)acetamide	10	50μM	+	+/-

N-[4-(4-acetyl-1-piperazinyl)phenyl]-4-ethoxy-3-nitrobenzamide	27	100μM	+	+/-
N-(2-chlorophenyl)-3-(4-fluorophenyl)acrylamide	40	100μM	+	+/-
1-[(dimethyl-lambda~4~-sulfanylidene)amino]-2-methoxy-4-nitrobenzene	43	100μM	+	+/-
5-benzylidene-1-(2-chlorophenyl)-2,4,6(1H,3H,5H)-pyrimidinetrione	48	100μM	+	+/-
4-ethyl-5,6-dimethyl-2-phenylpyrimidine	50	100μM	+	+/-

CLAIMS

1. A method for identifying a compound which inhibits the activation of RAC GTPase by DOCK5 protein comprising the steps of:

- coexpressing at least the DHR2 domain of DOCK5 and the RAC proteins in a cell, wherein said at least the DHR2 domain of DOCK5 protein induces the conversion of inactive RAC, which inactive RAC is bound to GDP, to active RAC, which active RAC is bound to GTP.

- contacting or not said cell with said compound,

- determining the conversion of inactive RAC to active RAC in the presence or absence of said compound, and

- selecting the compound inhibiting the conversion of inactive RAC to active RAC.

2. The method of claim 1, wherein the selected compound is useful for treating disease - associated bone loss.

3. The method of claim 2, wherein said disease associated with bone loss is selected in the group comprising osteoporosis, osteopenia due to bone metastases, periarticular erosions in rheumatoid arthritis, primary hyperparathyroidism, hypercalcemia of malignancy, Paget's disease of bone, periodontal disease, immobilization induced osteopenia, and glucocorticoid treatment.

4. The method of any one of claims 1 to 3, wherein said method further comprises the step of testing the inhibition of bone resorption by the selected compound.

5. The method of any one of claims 1 to 4, wherein said DOCK5 protein refers to a polypeptide comprising the DHR2 domain of the protein DOCK5 corresponding to the amino acid 1132 to 1661 of the DOCK5 protein from *Mus musculus* SEQ ID NO:1 and derivatives thereof.

6. The method of any one of claims 1 to 5, wherein said DOCK5 protein corresponds to SEQ ID NO:4 corresponding to *Homo sapiens* DOCK5 protein.

7. The method of any one of claims 1 to 6, wherein the RAC protein corresponds to SEQ ID NO:2 and derivatives thereof.

8. The method of any one of claims 1 to 7, wherein said method further comprises the expression of any protein capable to interact with the active RAC protein and not with the inactive RAC protein.

9. The method of claim 8, wherein said cell further comprises a reporter gene under the control of a promoter sequence, and said active RAC and protein interacting with are each fused either with a transactivation domain or with a DNA binding domain specific of said promoter sequence, wherein the interaction of active RAC with the interacting protein results in the induction of expression of the reporter gene.

10. The method of any of claims 8 and 9, wherein the protein interacting with active RAC protein is chosen in the group comprising PAK1 protein which corresponds to the SEQ ID NO:3 and derivatives thereof.

11. A method for the selection of compounds, which permit to decrease the level of expression of a DOCK5 gene in diseases associated with bone loss comprising the step of:

a) contacting a test compound with an host cell expressing a reporter nucleic acid comprising a nucleic acid sequence coding for a reporter placed under the control of a promoter, which promoter comprises all or part of the promoter sequence of DOCK5 gene or a derivative thereof, and

b) measuring the level of expression of the reporter.

12. A method for identifying a compound which inhibits the activation of RAC1/2 GTPase by inhibiting the binding of ELMO1 to the SH3 domain of DOCK5 comprising the steps of:

a) contacting a test compound with the ELMO1 protein or a derivative thereof;

b) determining the possible binding of said test compound to the ELMO1 protein or the derivative thereof; and optionally

c) selecting the compound inhibiting the conversion of inactive RAC1/2 to active RAC1/2.

13. A method for identifying a compound which inhibits the activation of RAC1/2 GTPase by inhibiting the binding of ELMO1 to the SH3 domain of DOCK5 comprising the steps of:

a) contacting a test compound with the ELMO1 protein or the derivative thereof and a polypeptide comprising at least the SH3 domain of DOCK5 or the derivative thereof;

b) measuring the binding between said ELMO1 protein and said polypeptide in the presence or in the absence of said compound; and optionally

c) selecting the compound inhibiting the conversion of inactive RAC1/2 to active RAC1/2.

14. A compound chosen in the group consisting in:

- 4-[5-(4-bromophenyl)-3-(4-nitrophenyl)-4,5-dihydro-1H-pyrazol-1-yl]-4-oxobutanoic acid;
- 2,2,2-trichloro-N-(1,1-dioxido-2,3-dihydro-3-thienyl)-N-(4-methylphenyl)acetamide;
- 3-(3-chlorophenyl)-7-methyl-4-methylene-3,4-dihydro-2(1H)-quinazolinone;
- 3-[4-(3-bromobenzylidene)-3-methyl-5-oxo-4,5-dihydro-1H-pyrazol-1-yl]benzoic acid;
- N-2,1,3-benzothiadiazol-4-yl-5-bromo-2-furamide;
- 1-acetyl-4-(2-chloro-4-nitrophenyl)-2-methylpiperazine;
- 3-(3-methoxybenzylidene)-5-(4-methylphenyl)-2(3H)-furanone;
- 3-[5-(3,4-dichlorophenyl)-2-furyl]acrylic acid;

- (2-chloro-4-{[5-(2-chlorophenyl)-6-(ethoxycarbonyl)-7-methyl-3-oxo-5H-[1,3]thiazolo[3,2-a]pyrimidin-2(3H)-ylidene]methyl}-6-methoxyphenoxy)acetic acid;

- 4-{[4-(diphenylmethyl)-1-piperazinyl]sulfonyl}-2,1,3-benzothiadiazole;

- 4-[4-phenyl-5-(2-thienyl)-1H-imidazol-2-yl]-1,2-benzenediol;

- N-(3,4-dimethoxyphenyl)-4-[methyl(phenylsulfonyl)amino]benzamide;

- 1-[(2-hydroxyphenyl)carbonothioyl]-3-phenyl-5-(trifluoromethyl)-4,5-dihydro-1H-pyrazol-5-ol;

- 2-methoxyethyl 4-[(4-tert-butylbenzoyl)amino]benzoate;

- N-(2,3-dichlorophenyl)-3-(5-methyl-2-furyl)acrylamide;

- N-(4-fluorophenyl)-3-[3-(trifluoromethyl)phenyl]acrylamide;

- 3-(2-furylmethyl)-2-(2-hydroxyphenyl)-2,3-dihydro-4(1H)-quinazolinone;

- N-(4-ethoxyphenyl)-2-{[5-(4-methoxyphenyl)-1,3,4-oxadiazol-2-yl]thio}acetamide;

- 5-(4-nitrobenzylidene)-2-thioxo-3-[3-(trifluoromethyl)phenyl]-1,3-thiazolidin-4-one;

- (3,5-dichlorophenyl)[(phenylsulfonyl)carbonyl]amine;

- N-(2-bromophenyl)-3-(5-methyl-2-furyl)acrylamide;

- 2-(2-chlorophenoxy)-N-[2-chloro-5-(trifluoromethyl)phenyl]acetamide;

- N-[4-(4-acetyl-1-piperazinyl)phenyl]propanamide;

- 8-[(dimethylamino)methyl]-9-hydroxy-2-methyl-4H-pyrido[1,2-a]pyrimidin-4-one;

- 4-tert-butyl-N-[1-{[(2-methoxyphenyl)amino]carbonyl}-2-(2-thienyl)vinyl]benzamide;
- 2-chloro-N-(3-chloro-4-methoxyphenyl)benzamide;
- 2,6-di-tert-butyl-4-(2,3-dihydro-1H-perimidin-2-yl)phenol;
- 3-benzyl-2-(2,6-dichlorophenyl)-2,3-dihydro-4(1H)-quinazolinone;
- 1-(3,4-dichlorobenzyl)-1H-indole-3-carbaldehyde;
- N-[5-(1-adamantyl)-1,3,4-thiadiazol-2-yl]-N'-phenylurea;
- N-(3,4-dichlorophenyl)-N'-{5-[(4-methylphenoxy)methyl]-1,3,4-thiadiazol-2-yl}urea;
- N-(2,3-dihydro-1,4-benzodioxin-6-yl)-2-(1-naphthyloxy)acetamide;
- N-[4-(4-acetyl-1-piperazinyl)phenyl]-4-ethoxy-3-nitrobenzamide;
- N-(2-chlorophenyl)-3-(4-fluorophenyl)acrylamide;
- 1-[(dimethyl-lambda~4~-sulfanylidene)amino]-2-methoxy-4-nitrobenzene;
- 5-benzylidene-1-(2-chlorophenyl)-2,4,6(1H,3H,5H)-pyrimidinetrione;
- 4-ethyl-5,6-dimethyl-2-phenylpyrimidine;
- 2-(3-chlorobenzylidene)-1H-indene-1,3(2H)-dione;
- 5-{5-[(3-methyl-5-oxo-1-phenyl-1,5-dihydro-4H-pyrazol-4-ylidene)methyl]-2-furyl}-1H-isoindole-1,3(2H)-dione;
- N-(2,5-dimethylphenyl)-3-(4-methoxyphenyl)acrylamide;
- 2-({2-[(4-nitrophenyl)amino]ethyl}amino)ethanol;
- N-(3-methoxyphenyl)-4-propoxybenzamide;
- 2-(4-hydroxyphenyl)-3-phenyl-2,3-dihydro-4(1H)-quinazolinone;

- 4-methyl-1-(2-nitrobenzoyl)piperidine;

- 2-hydroxy-N'-[(2-methylphenyl)sulfonyl]benzohydrazide;

- 4-(1,3-benzothiazol-2-yl)butanoic acid;

- 4-(3-methylbenzylidene)-1-phenyl-3,5-pyrazolidinedione;

- 4-(2,4-dichlorophenoxy)-N-(2-ethoxyphenyl)butanamide;

- N-(2-methoxyphenyl)-N'-(phenylsulfonyl)benzenecarboximidamide;

- N-[2-(2-chloro-5-iodophenyl)-1,3-benzoxazol-5-yl]-2-methylpropanamide;

- 5-(4-butoxyphenyl)-3-cyclohexyl-1,2,4-oxadiazole;

- N-(3,4-dichlorophenyl)-N'-4H-1,2,4-triazol-4-yl urea;

- 6-chloro-4-phenyl-3-[3-(3,4,5-trimethoxyphenyl)acryloyl]-2(1H)-quinolinone;

- 6-bromo-4-phenyl-3-[3-(3,4,5-trimethoxyphenyl)acryloyl]-2(1H)-quinolinone; and

- N-(1H-1,2,3-benzotriazol-1-ylmethyl)-4-nitro-1,2,5-oxadiazol-3-amine for treating and/or preventing bone loss diseases in a subject in need thereof.

15. A pharmaceutical composition comprising at least one compound chosen in the group consisting in - 4-[5-(4-bromophenyl)-3-(4-nitrophenyl)-4,5-dihydro-1H-pyrazol-1-yl]-4-oxobutanoic acid;

- 2,2,2-trichloro-N-(1,1-dioxido-2,3-dihydro-3-thienyl)-N-(4-methylphenyl)acetamide;

- 3-(3-chlorophenyl)-7-methyl-4-methylene-3,4-dihydro-2(1H)-quinazolinone;

- 3-[4-(3-bromobenzylidene)-3-methyl-5-oxo-4,5-dihydro-1H-pyrazol-1-yl]benzoic acid;

- N-2,1,3-benzothiadiazol-4-yl-5-bromo-2-furamide;

- 1-acetyl-4-(2-chloro-4-nitrophenyl)-2-methylpiperazine;

- 3-(3-methoxybenzylidene)-5-(4-methylphenyl)-2(3H)-furanone;

- 3-[5-(3,4-dichlorophenyl)-2-furyl]acrylic acid;

- (2-chloro-4-{[5-(2-chlorophenyl)-6-(ethoxycarbonyl)-7-methyl-3-oxo-5H-[1,3]thiazolo[3,2-a]pyrimidin-2(3H)-ylidene]methyl}-6-methoxyphenoxy)acetic acid;

- 4-{[4-(diphenylmethyl)-1-piperazinyl]sulfonyl}-2,1,3-benzothiadiazole;

- 4-[4-phenyl-5-(2-thienyl)-1H-imidazol-2-yl]-1,2-benzenediol;

- N-(3,4-dimethoxyphenyl)-4-[methyl(phenylsulfonyl)amino]benzamide;

- 1-[(2-hydroxyphenyl)carbonothioyl]-3-phenyl-5-(trifluoromethyl)-4,5-dihydro-1H-pyrazol-5-ol;

- 2-methoxyethyl 4-[(4-tert-butylbenzoyl)amino]benzoate;

- N-(2,3-dichlorophenyl)-3-(5-methyl-2-furyl)acrylamide;

- N-(4-fluorophenyl)-3-[3-(trifluoromethyl)phenyl]acrylamide;

- 3-(2-furylmethyl)-2-(2-hydroxyphenyl)-2,3-dihydro-4(1H)-quinazolinone;

- N-(4-ethoxyphenyl)-2-{[5-(4-methoxyphenyl)-1,3,4-oxadiazol-2-yl]thio}acetamide;

- 5-(4-nitrobenzylidene)-2-thioxo-3-[3-(trifluoromethyl)phenyl]-1,3-thiazolidin-4-one;

- (3,5-dichlorophenyl)[(phenylsulfonyl)carbonyl]amine;

- N-(2-bromophenyl)-3-(5-methyl-2-furyl)acrylamide;

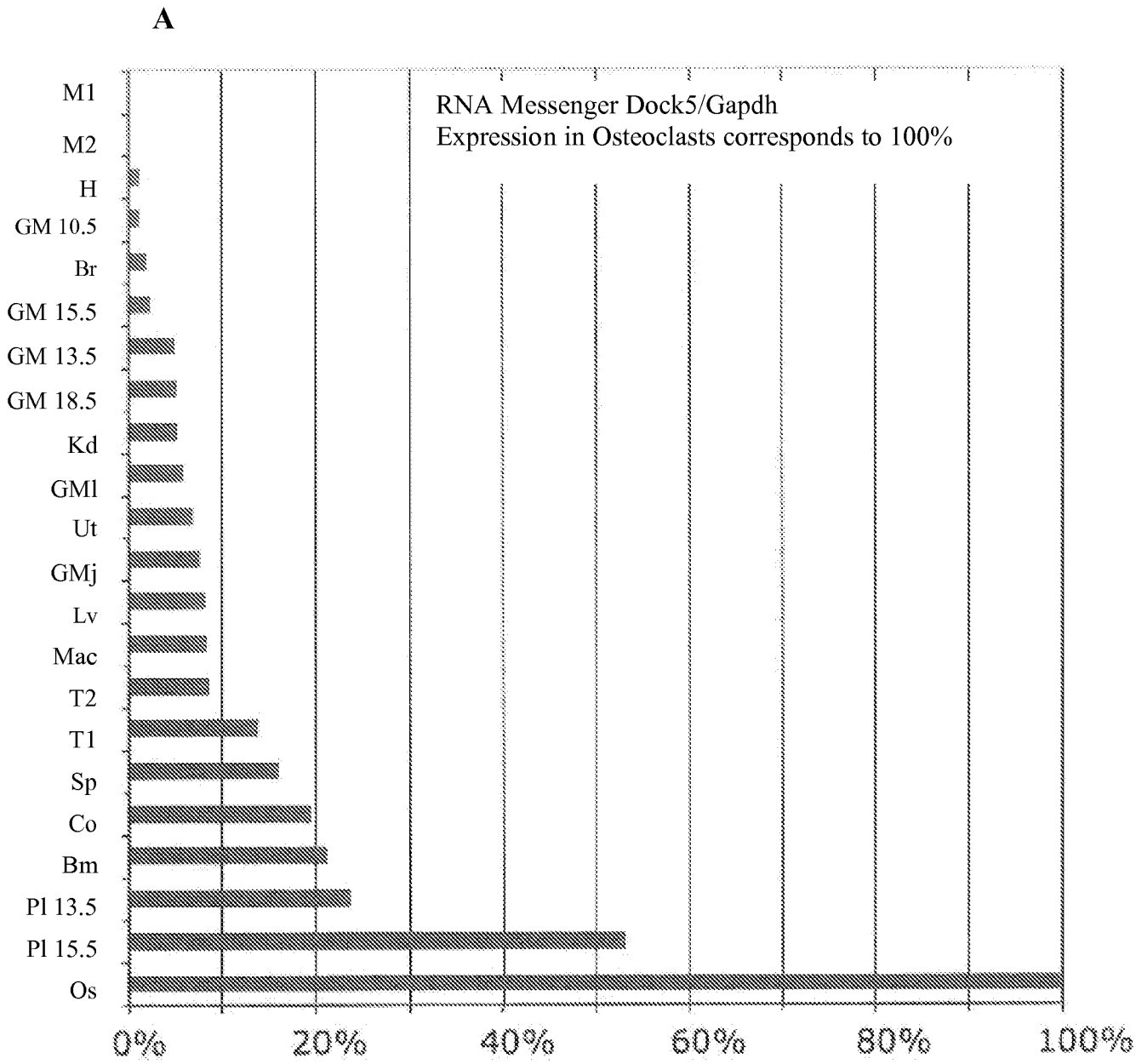
- 2-(2-chlorophenoxy)-N-[2-chloro-5-(trifluoromethyl)phenyl]acetamide;

- N-[4-(4-acetyl-1-piperazinyl)phenyl]propanamide;
- 8-[(dimethylamino)methyl]-9-hydroxy-2-methyl-4H-pyrido[1,2-a]pyrimidin-4-one;
- 4-tert-butyl-N-[1-{[(2-methoxyphenyl)amino]carbonyl}-2-(2-thienyl)vinyl]benzamide;
- 2-chloro-N-(3-chloro-4-methoxyphenyl)benzamide;
- 2,6-di-tert-butyl-4-(2,3-dihydro-1H-perimidin-2-yl)phenol;
- 3-benzyl-2-(2,6-dichlorophenyl)-2,3-dihydro-4(1H)-quinazolinone;
- 1-(3,4-dichlorobenzyl)-1H-indole-3-carbaldehyde;
- N-[5-(1-adamantyl)-1,3,4-thiadiazol-2-yl]-N'-phenylurea;
- N-(3,4-dichlorophenyl)-N'-{5-[(4-methylphenoxy)methyl]-1,3,4-thiadiazol-2-yl}urea;
- N-(2,3-dihydro-1,4-benzodioxin-6-yl)-2-(1-naphthyloxy)acetamide;
- N-[4-(4-acetyl-1-piperazinyl)phenyl]-4-ethoxy-3-nitrobenzamide;
- N-(2-chlorophenyl)-3-(4-fluorophenyl)acrylamide;
- 1-[(dimethyl-lambda~4~-sulfanylidene)amino]-2-methoxy-4-nitrobenzene;
- 5-benzylidene-1-(2-chlorophenyl)-2,4,6(1H,3H,5H)-pyrimidinetrione;
- 4-ethyl-5,6-dimethyl-2-phenylpyrimidine;
- 2-(3-chlorobenzylidene)-1H-indene-1,3(2H)-dione;
- 5-{5-[(3-methyl-5-oxo-1-phenyl-1,5-dihydro-4H-pyrazol-4-ylidene)methyl]-2-furyl}-1H-isoindole-1,3(2H)-dione;
- N-(2,5-dimethylphenyl)-3-(4-methoxyphenyl)acrylamide;

- 2-({2-[(4-nitrophenyl)amino]ethyl}amino)ethanol;
- N-(3-methoxyphenyl)-4-propoxybenzamide;
- 2-(4-hydroxyphenyl)-3-phenyl-2,3-dihydro-4(1H)-quinazolinone;
- 4-methyl-1-(2-nitrobenzoyl)piperidine;
- 2-hydroxy-N'-[(2-methylphenyl)sulfonyl]benzohydrazide;
- 4-(1,3-benzothiazol-2-yl)butanoic acid;
- 4-(3-methylbenzylidene)-1-phenyl-3,5-pyrazolidinedione;
- 4-(2,4-dichlorophenoxy)-N-(2-ethoxyphenyl)butanamide;
- N-(2-methoxyphenyl)-N'-(phenylsulfonyl)benzenecarboximidamide;
- N-[2-(2-chloro-5-iodophenyl)-1,3-benzoxazol-5-yl]-2-methylpropanamide;
- 5-(4-butoxyphenyl)-3-cyclohexyl-1,2,4-oxadiazole;
- N-(3,4-dichlorophenyl)-N'-4H-1,2,4-triazol-4-yl urea;
- 6-chloro-4-phenyl-3-[3-(3,4,5-trimethoxyphenyl)acryloyl]-2(1H)-quinolinone;
- 6-bromo-4-phenyl-3-[3-(3,4,5-trimethoxyphenyl)acryloyl]-2(1H)-quinolinone;

and

- N-(1H-1,2,3-benzotriazol-1-ylmethyl)-4-nitro-1,2,5-oxadiazol-3-amine and, optionally, a pharmaceutically acceptable support for treating and/or preventing bone loss diseases in a subject in need thereof.


A
RNA Messenger Dock5/Gapdh
Expression in Osteoclasts corresponds to 100%
M1
M2
H
GM 10.5
Br
GM 15.5
GM 13.5
GM 18.5
Kd
GMl
Ut
GMj
Lv
Mac
T2
T1
Sp
Co
Bm
Pl 13.5
Pl 15.5
Os
0%
20%
40%
60%
80%
100%

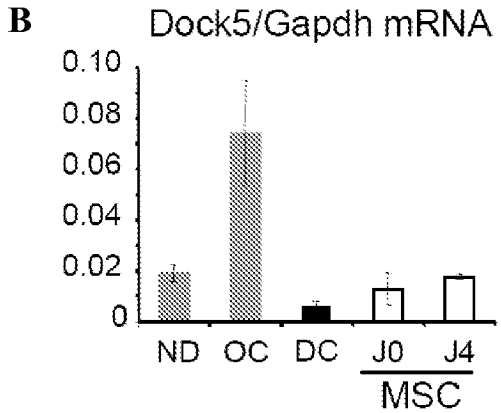

B
Dock5/Gapdh mRNA
0.10
0.08
0.06
0.04
0.02
0
ND
OC
DC
J0
J4
MSC

Figure 1

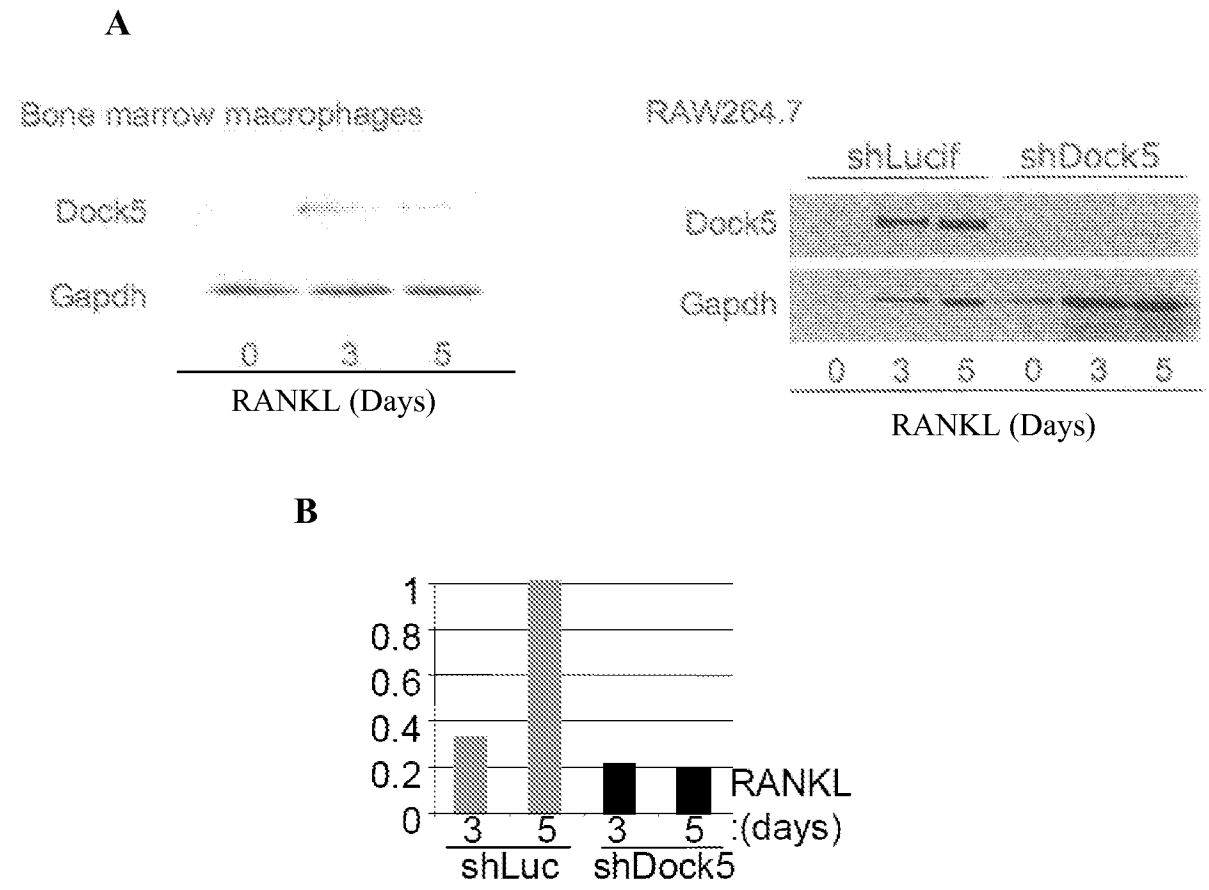

A
Bone marrow macrophages
Dock5
Gapdh
0 3 5
RANKL (Days)
RAW264.7
shLucif
shDock5
Dock5
Gapdh
0 3 5 0 3 5
RANKL (Days)
B
1
0.8
0.6
0.4
0.2
0
3 5 3 5
RANKL
(days)
shLuc shDock5
C
BMM
ND J3 J5 Ey Sp St Te Pl Lu Br He Li Ki Mu
Dock5
Tubulin

Figure 2

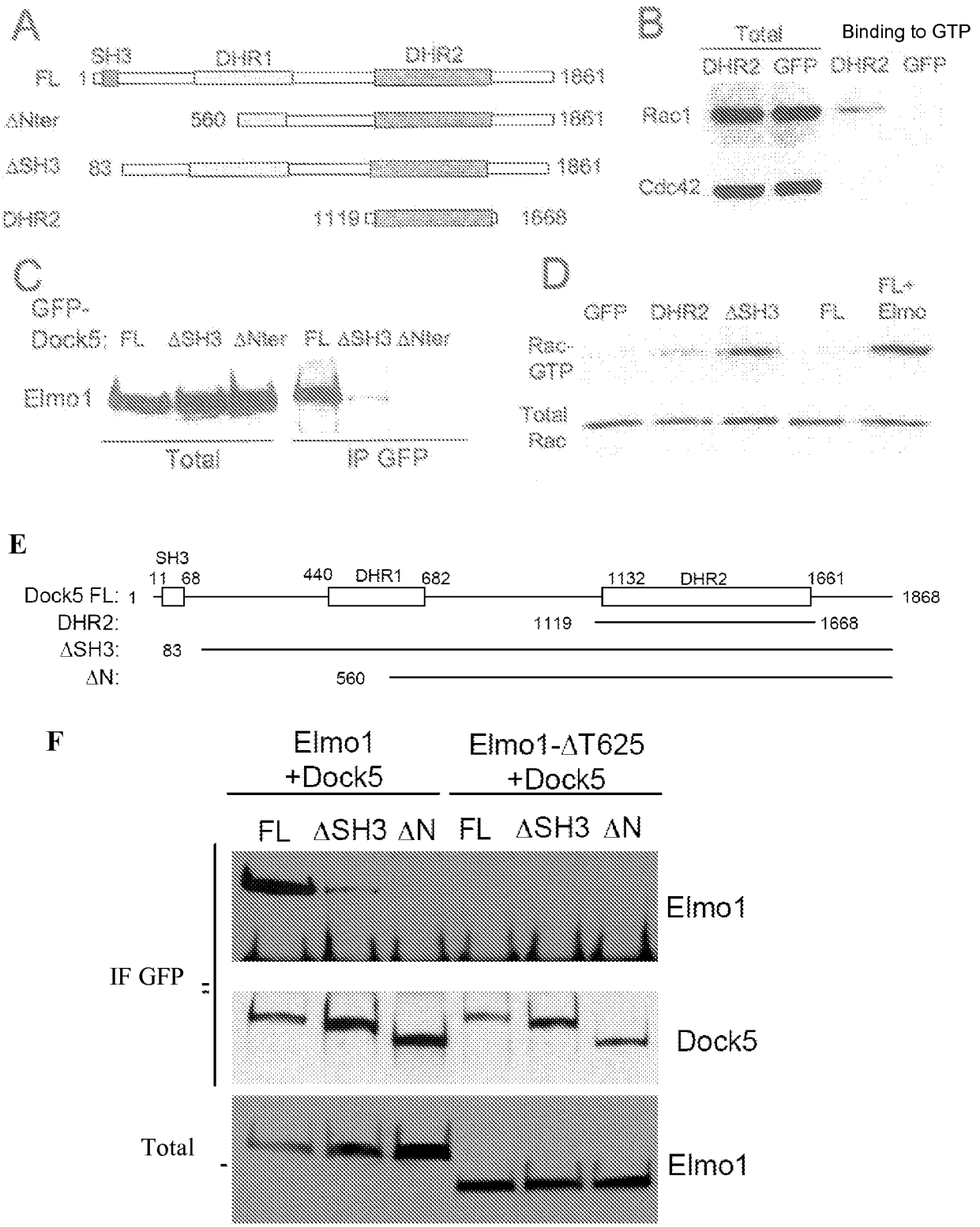
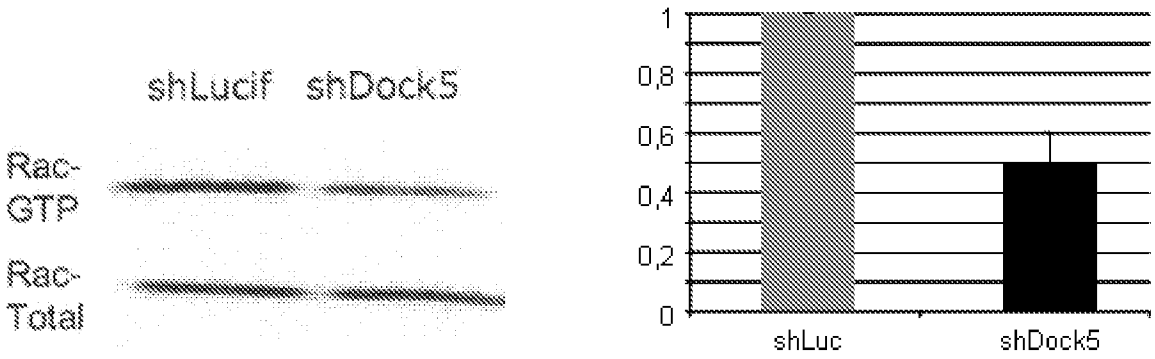

A
SH3
DHR1
DHR2
FL 1 1861
ΔNter 560 1861
ΔSH3 83 1861
DHR2 1119 1668
B
Total
Binding to GTP
DHR2 GFP DHR2 GFP
Rac1
Cdc42
C
GFP-
Dock5: FL ΔSH3 ΔNter FL ΔSH3 ΔNter
Elmo1
Total
IP GFP
D
GFP DHR2 ΔSH3 FL FL+ Elmo
Rac-GTP
Total Rac
E
SH3
11 68
440 DHR1 682
1132 DHR2 1661
Dock5 FL: 1 1868
DHR2: 1119 1668
ΔSH3: 83
ΔN: 560
F
Elmo1 +Dock5
Elmo1-ΔT625 +Dock5
FL ΔSH3 ΔN FL ΔSH3 ΔN
IF GFP
Elmo1
Dock5
Total
Elmo1

Figure 3

A


shLucif
shDock5
Rac-GTP
Rac-Total
1
0,8
0,6
0,4
0,2
0
shLuc
shDock5

B

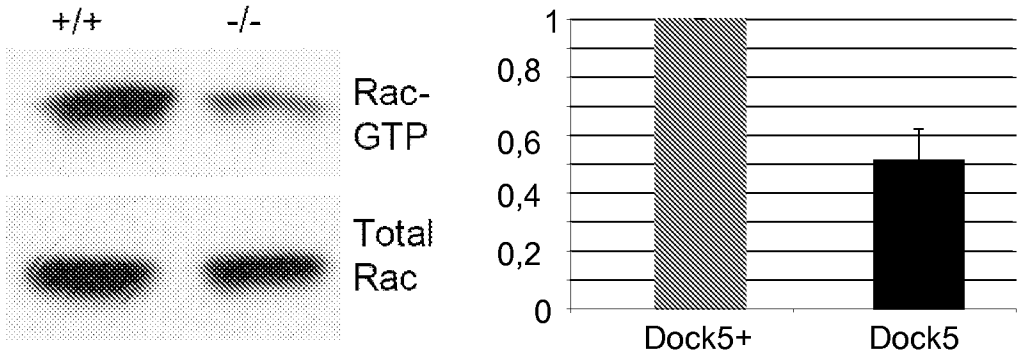

+/+
-/-
Rac-GTP
Total Rac
1
0,8
0,6
0,4
0,2
0
Dock5+
Dock5

Figure 4

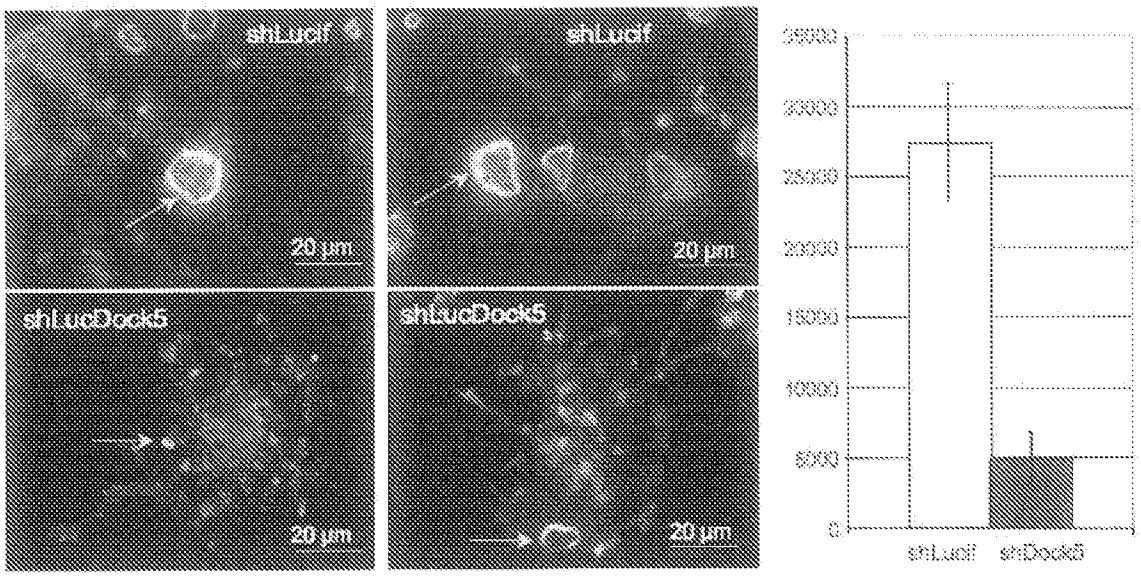


Figure 5

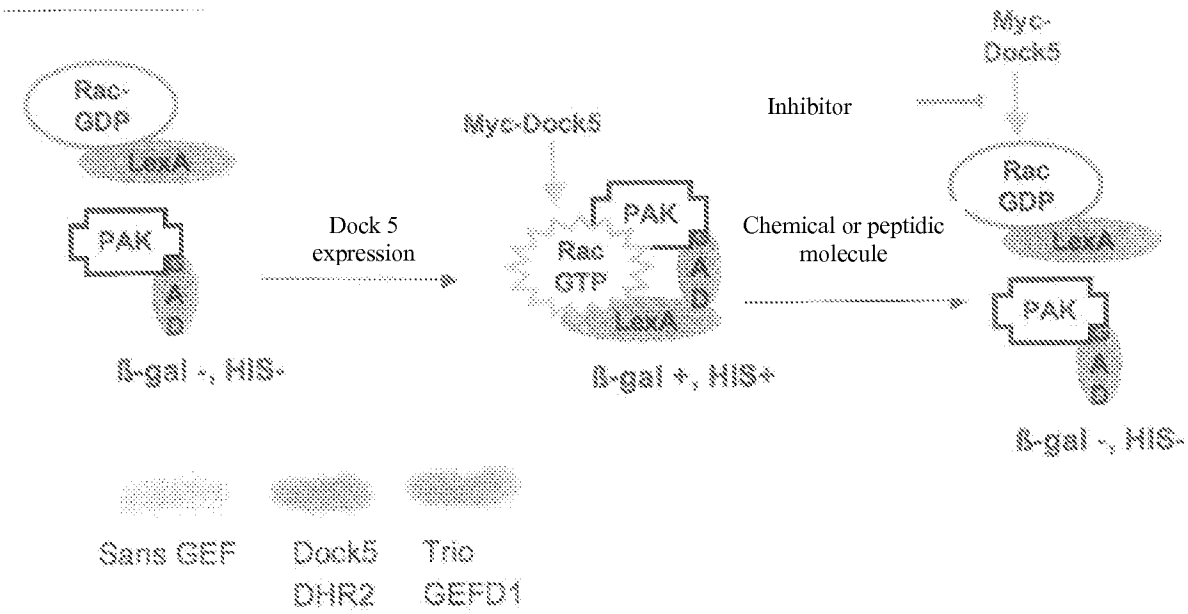


Figure 6

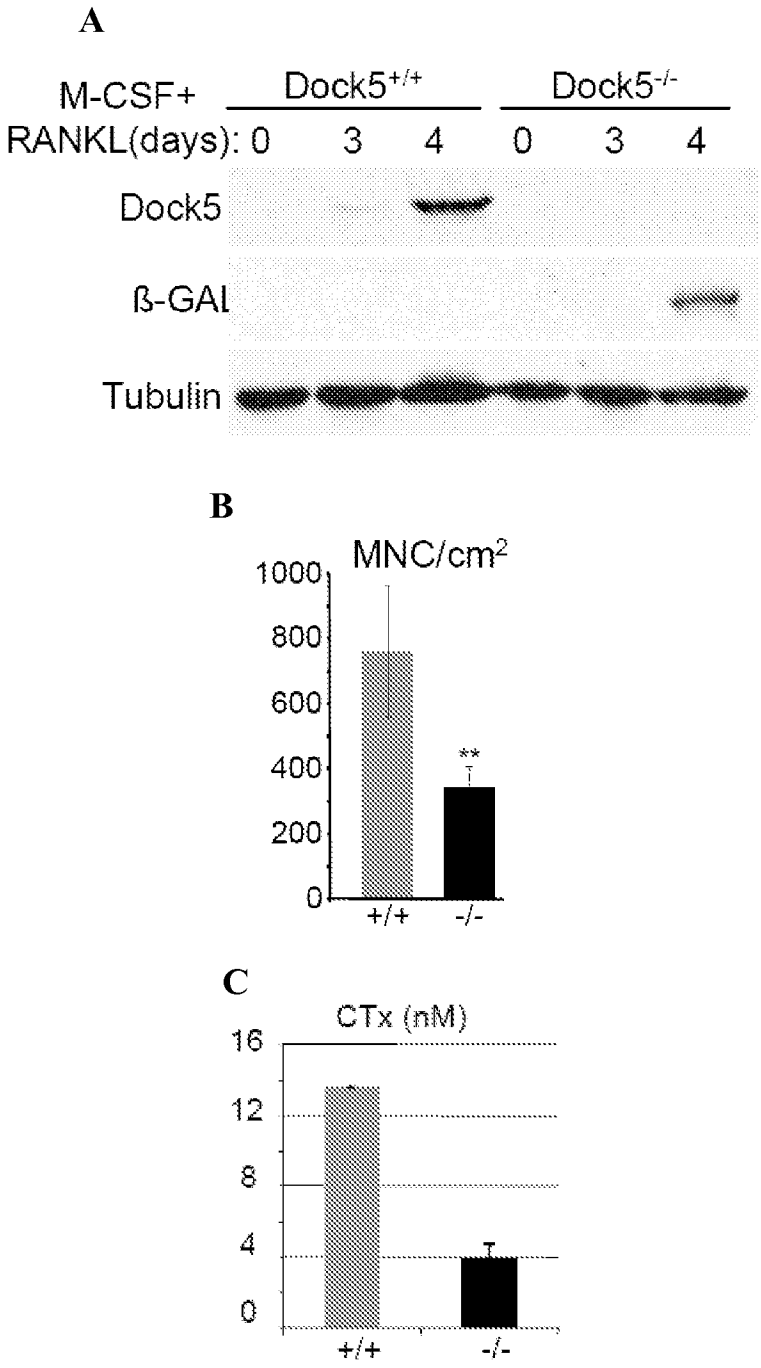
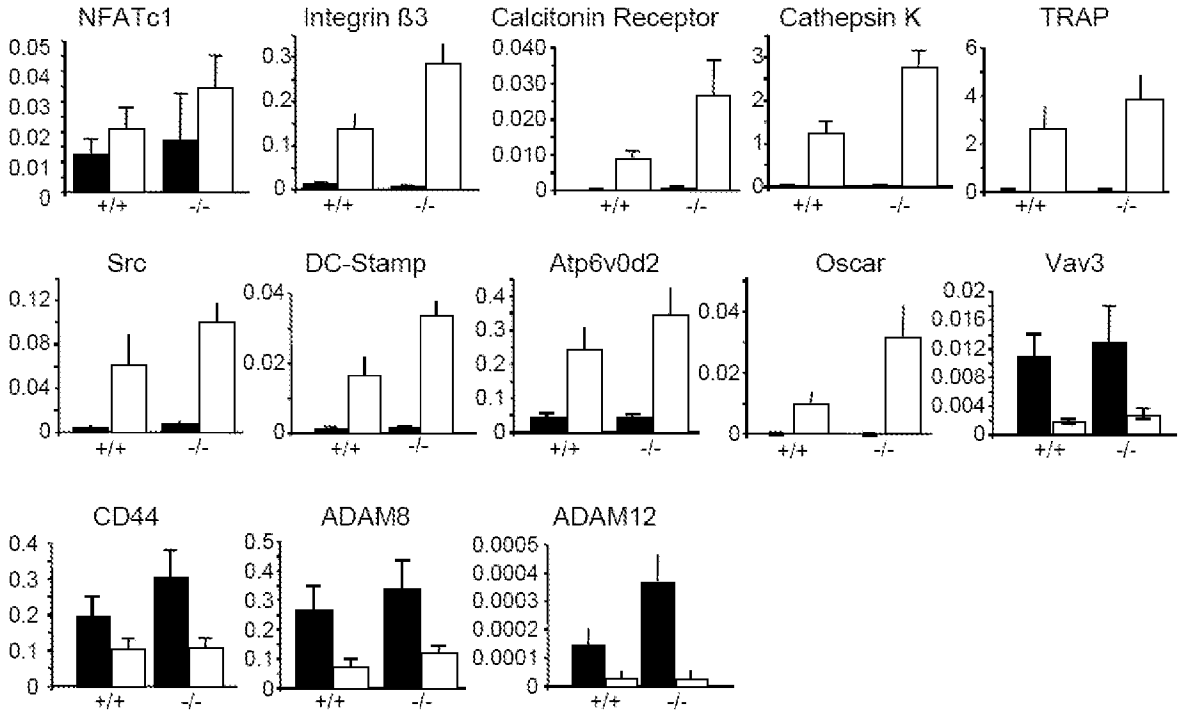
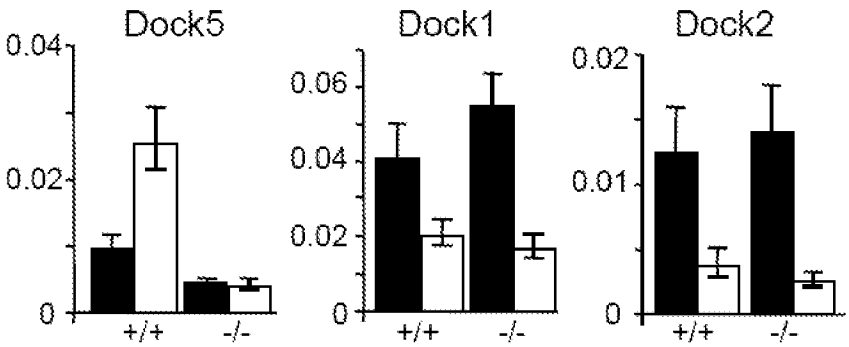

A
M-CSF+
Dock5+/+
Dock5-/-
RANKL(days): 0 3 4 0 3 4
Dock5
ß-GAL
Tubulin
B
MNC/cm2
1000
800
600
400
200
0
**
+/+
-/-
C
CTx (nM)
16
12
8
4
0
+/+
-/-

Figure 7

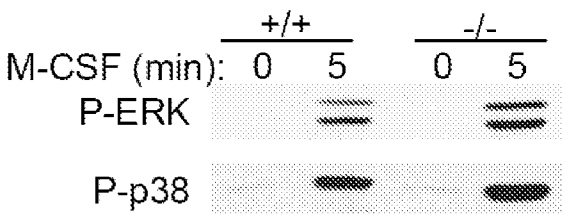
A


NFATc1
Integrin ß3
Calcitonin Receptor
Cathepsin K
TRAP
Src
DC-Stamp
Atp6v0d2
Oscar
Vav3
CD44
ADAM8
ADAM12
+/+
-/-

B


Dock5
Dock1
Dock2
+/+
-/-

C


+/+
-/-
M-CSF (min): 0 5 0 5
P-ERK
P-p38

D

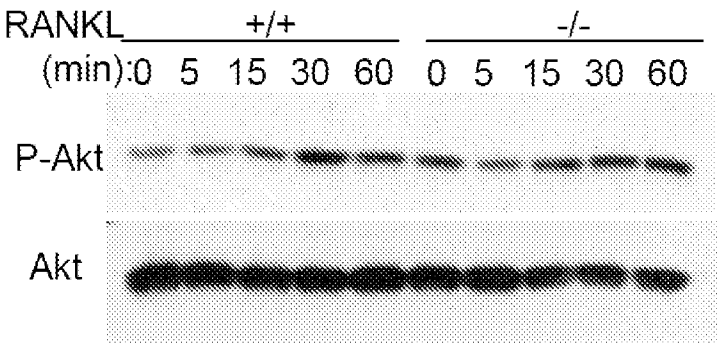


Figure 8