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(54) **Title**: USE OF ANASTROZOLE AND VITAMIN D ANALOGUE IN THE COMBINED THERAPY OF BREAST CANCER

(57) **Abstract**: The invention relates to the use of anastrozole and vitamin D analogues in combined therapy of breast cancer. Vitamin D analogues are selected from a group including calcipotriol, tacalcitol and (1S,3R,5E,7E,22E,24S)-24-cyclopropyl-9,10-secochola- 5,7,10(19),22-tetraen-l,3,24-triol (isomer 5,6-trans of calcipotriol).

USE OF ANASTROZOLE AND VITAMIN D ANALOGUE IN THE COMBINED THERAPY OF BREAST CANCER

Field of the invention

The present invention belongs to the field of breast cancer treatment. Specifically, the invention relates to the use of anastozole and vitamin D analogues in the combined therapy of breast cancer.

Background of the invention

Regardless constant progress in medicine, many women, especially at postmenopausal age, are still affected by breast cancer, for this reason this disease remains serious medical as well as social problem. In this target group of patients, the anti-estrogen tamoxifen is routinely used as the first-line chemotherapy treatment of advanced breast cancer. When hormonal secretion of ovaries has been ceased after menopause, adrenal gland becomes the main source of estrogen in woman blood serum. This estrogen secretion can be selectively suppressed by aromatase inhibitors, which have been recently introduced into medical practice. Primarily used unselective aromatase inhibitors have been lately substituted with the selective suppressors of estrogen biosynthesis. These drugs hamper the transformation of androstenedione into estrone, which results in decrease of the total concentration of estrogenes in blood serum. The estrogenes along with progesterone stimulate growth, differentiation and life span of breast cancerepithelial cells. Anastrozole is the selective, competitive, non-steroidal aromatase inhibitor of the new generation. This drug is used only in advanced breast cancer therapy of postmenopausal women. However, anastrozole based therapy becomes effective, if the cancer cells are characterized by the presence of estrogen and/or progestagen receptors on the membrane sites. Anastrozole is the only aromatase inhibitor approved for adjuvant cancer therapy of estrogen and/or progestagen receptor positive postmenopausal patients. Diminished cell number, fibrosis, nuclei pleomorphism and altered mitosis of cancer cells are the effects proved by histopatological imaging after anastrozole treatment of the locally advanced breast cancer. According to clinically relevant findings, positive response to anastrozole chemotherapy results in diminished size of tumor in majority of examined medical cases.

It is also known that active metabolites and cholecalciferol (vitamin D) analogues influence the calcium-phosphate balance and are used in treatment of meta-

bolic diseases and skeleton system disease, especially osteoporosis. Moreover, the main natural active form of vitamin D, 1α,25-dihydroxycholecalciferol (calcitriol) and its synthetic analogues show antiproliferative action and beneficially influence differentiation of cancer cells and epidermal keratinocytes. Due to this, cholecalciferol analogues are used in treatment of hyperproliferative diseases, especially skin diseases such as some forms of plaque psoriasis, ichtiosis and keratosis (Beckman M.J., DeLuca H.F., Modern view of vitamin D_3 and its medicinal uses, w: Ellis GP, Luscombe DK, Oxford AW, ed., Progress in Medicinal Chemistry. t.35, Amsterdam, Elsevier Science Publishers, 1998, p.1-56; Vitamin D, t. I, ed. II, edited by D. Feldman, Amsterdam, Elsevier Science Publishers, 2005).

Among cholecalciferol analogues 1,24(R)-dihydroxycholekalciferol is of special interest, this is a precursor of 1,24(R),25-trihydroxycholecalciferol, a natural calcitriol metabolite known under generic name tacalcitol (Peters D.C., Balfour J.A., Tacalcitol., *Drugs* 1997; 54:265-271). Contrary to other members of vitamin D analogues group, tacalcitol does not show calcium side effects, therefore it may be administered in other diseases in higher doses than in dermatology and moreover, it qualifies to general administration.

It has been revealed in the description of Polish patent application P-339112 that tacalcitol showed also a beneficial effect *in vitro* and *in vivo* on differentiation and inhibition of proliferation of some lines of human cancer cells. At the same time, activity of tacalcitol is statistically significantly higher than activity of its C-24 diastereomer (1α,24(S)-dihydroxycholecalciferol) and natural calcitriol.

Calcipotriol, (1S,3R,5Z,7E,22E,24S)-24-cyclopropyl-9,10-secochola-5,7,10(19),22-tetraeno-1,3,24-triol (M.J. Calverley, *Tetrahedron* 43, 4609 (1987)), also exerts a beneficial effect on inhibition of excessive proliferation of epidermal keratinocites (F.A.C.M. Castelijins i wsp. *Acta Derm. Venereol.* 79, 111 (1999)). Studies carried out in rats showed that it exerts 100-200-fold lesser influence on calcium metabolism than calcitriol (L. Binderup, E. Bramm, *Biochem. Pharmacol.* 37, 889 (1988)).

In the Polish patent application P-378586, use of structural analogues of calcipotriol for preparation of pharmaceutical of pharmaceutical agents of antineoplastic activity was claimed. The above mentioned analogues are selected from a group consisting of (1S,3R,5*Z*,7*E*,22*E*,24R)-24-cyclopropyl-9,10-secochol-5,7,10(19),22-tetraeno-1,3,24-triol ((24R)-isomer), (1S,3R,5*Z*,7*E*,22*Z*,24*S*)-24-

cyclopropyl-9,10-secochol-5,7,10(19),22-tetraeno-1,3,24-triol (22,23-cis isomer), (1R,3R,5*Z*,7*E*,22*E*,24*S*)-24-cyclopropyl-9,10-secochol-5,7,10(19),22-tetraen-1,3,24-triol (1β-OH-isomer) and (1S,3R,5E,7*E*,22*E*,24*S*)-24-cyclopropyl-9,10-secochol-5,7,10(19),22-tetraeno-1,3,24-triol (5,6-trans-isomer).

Now, the present Inventors have found on the basis of results of *in vitro* studies on selected cell lines of human breast cancer, that implementing of some vitamin D analogues, specifically tacalcitol and 5,6-trans isomer of calcipotriol, to the protocol of breast cancer treatment with aromatase inhibitor anastrozole, might result in unexpected, synergistic antiproliferative effect. Therefore, the combination of vitamin D analogues with anastrozole may be very effective, when applied in combined therapy of breast cancer.

Summary of the invention

The present invention relates to the use of anastozole and vitamin D analogues in the combined therapy of breast cancer.

The present invention is based on *in vitro* findings, that vitamin D analogues potentiate antiproliferative activity of aromatase inhibitors towards selected breast cancer cell lines. This is the prove of synergistic activity of selected vitamin D analogues and anastrozole against cancer cells.

MCF-7 and SKBR-3 cell lines were used to study *in vitro* the influence of compositions of therapeutic mixture on cell cycle phases and apoptosis. According to obtained results, cancer cells exposed to individual vitamin D analogue increased their number in G0/G1 phase, whereas combination of vitamin D analogue and anastrozole resulted in decrease of the cell number in G0/G1 phase. For MCF-7 and T47D apoptotic cell lines, the increase in cell number was noticed in comparison with anastrozole itself. When SKBR-3 cells were treated either with anastrozole alone or in combination with vitamin D analogue, increased number of cells in G2/M phase was observed.

The increased number of MCF-F and SKBR-3 cells in phase G2/M was also noticed, when culturing cells either with anastrozole or anastrozole in combination with vitamin D analogues.

In view of obtained results confirming the augmentation of anticancer activity of anastrozole by vitamin D analogues in MCF-7 model of human breast cancer, one

may conclude, combination of anastrozole with vitamin D analogues *in vivo* should also increase the antineoplastic activity of the former, comparing with anastrozole alone. The vitamin D analogues such as, tacalcitol and 5,6-trans isomer of calcipotriol show similar efficacy towards inhibition of tumor growth, when used in a combined therapy. In addition, for calcipotriol 5,6-trans isomer longer survival time of mice was also noticed.

Beneficial synergistic effect of anastrozole and vitamin D analogues is better observed in long time periods, which results from the mechanism of biological activity of the latter. It influences cancer cells differentiation, which can be detectable after certain period of incubation time (J. Wietrzyk at al.: Toxicity and antineoplastic effect of (24R)-1,24-dihydroxyvitamin D_3 (PRI-2191). *Steroids, 69/10: 629-635, **2004***; A. Opolski at al.: Biological activity *in vitro* of side-chain modified analogues of calcitriol. *Current Pharmaceutical Design,* 6: 755-765, **2000**.)

The toxicity of the combined therapy, which is evaluated on the basis of such parameters as body mass of mice and calcium level in plasma, is not the limiting factor of the potential possibility to administer this anticancer combined therapy to humans, according to the present invention. Body mass of mice treated with anastrozole in combination with vitamin D analogues does not differ significantly in comparison with weight of mice treated with anastrozole alone. In some cases the decrease of body mass was noticed, when mice were administered anastrozole in combination with tacalcitol. This fact is related to much higher activity of tacalcitol on calcium metabolism in comparison with that of calcipotriol 5,6-trans isomer. On the other hand, the influence on calcium metabolism and toxicity of tacalcitol are considerably lower than those exerted by calcitriol (J. Wietrzyk at al.: The antitumor effect of lowered doses of cytostatics combined with new analogs of vitamin D in mice. *Anticancer Res., 27: 3387-98, **2007**; J.* Wietrzyk at al.: Toxicity and antineoplastic effect of (24R)-1,24-dihydroxyvitamin D_3 (PRI-2191). *Steroids, 69/10: 629-635, **2004**.*).

Wherever vitamin D analogue is mentioned hereinafter it is to be understood as comprising two major forms thereof, ie. vitamin D_2 or ergocalciferol, and vitamin D_3 or cholecalciferol, known collectively as calciferol. The structural difference between vitamin D_2 and vitamin D_3 is in their side chains. The side chain of D_2 contains a double bond between carbons 22 and 23, and a methyl group on carbon 24, while vitamin D_3 has several forms, namely cholecalciferol which is an inactive,

unhydroxylated form of vitamin D_3, calcifediol (25-hydroxyvitamin D_3, 25(OH)D, and calcitriol (1,25-dihydroxyvitamin D_3), as well their semisynthetic analogues mentione above.

On the basis of the obtained results, in a preferred embodiment of the present invention vitamin D analogues are selected from a group comprising tacalcitol, calcipotriol and 5,6-trans-isomer of calcipotriol, ie. 1S,3R,5E,7*E*,22*E*,24*S*)-24-cyclopropyl-9,10-secochola-5,7,10(19),22-tetraen-1,3,24-triol.

Use, according to the present invention, in particular includes administration of combination of vitamin D analogue and anastrozole to inhibit growth of tumor, which is characterized by the expression of estrogen receptor, analogously to MCF 7 line cells

The components of the composition may be administered to the patient as a pharmaceutical formulation in a fixed dosage form comprising the therapeutically effective amounts of the active substances in combination with pharmaceutically acceptable carriers and/or excipients.

Pharmaceutical preparations, beside the active substance, may contain known pharmaceutically acceptable carriers and/or excipients appropriate for a given pharmaceutical form, not having their own pharmacological action and adverse reactions with the active substance.

Therapeutically effective dose of the active substance in treatment of and/or prevention from cancer may be established by a clinician, based on clinical trials and adjusted to the medical condition, age, body weight of the patient and the risk of methastasis as well as the route of administration and individual response to therapy.

The daily dose may be administered to the patient as a single unit dose once daily or divided into several daily doses in determined time intervals.

The effective daily dose of vitamin D analogue for adult human may very from 0,1 to 200 μg, preferably from 0,5 to 50 μg.

Effective daily dose of anastrozole is 1 mg, administered once daily.

The pharmaceutical combination according to the present invention, may be formulated in the pharmaceutical form acceptable for systemic administration, for example orally, such as tablets, capsules, film-coated tablets, enteric coated tablets; in the form acceptable for parenteral use, such as solutions, suspensions or lyophilisate for reconstitution *ex tempore*; or in the form for local administration. The selection and amount of carriers and excipients depends on the form and route of

administration of the agent. The appropriate drug form may be formulated with use of techniques well known to those skilled in the art, using any pharmaceutically carriers, solvents, fillers and other excipients.

A pharmaceutical preparation for oral administration may specially be in the form of capsules. In this case, the active substance is combined with a carrier and gelatin capsules are filled with the obtained composition. Capsule filling is in the form of oil solution, suspension or emulsion. Appropriate carriers include, for example castor, coconut, olive, palm, corn, peanut oil, synthetic and natural triglycerides of fatty acids, unsaturated medium-chain fatty acids, modified long-chain fatty acids, glycol esters, polyethylene glycols and others. Appropriate excipients are tensides, for example lecithine, mono- and diglycerides and esters of polyoxyethylenesorbitan.

Capsules may be soft and hard gelatin capsules, differing by composition of gelatin shell for its preparation. Gelatin shell in case of soft capsules include plastisizers, such as glycerol, sorbitol; preservatives, such as benzoic acid and its salts, alkyl hydroxybenzoates; colourants and flavourings.

Pharmaceutical formulation for parenteral administration may be in the form of suspension ready to use, lyophilisate form for reconstitution *ex tempore* or a concentrate for preparation of intravenous infusions. Carriers appropriate for intravenous pharmaceutical formulations include, for example, sterile aqueous solutions, such as saline solution, carbohydrate solution, for example glucose, mannitol, dextrose lactose and aqueous solutions of buffers, for example phosphate buffer. Moreover, the agent may contain other excipients, conventionally used in order to ensure osmolarity, antioxidants, preservatives and others.

BIOLOGICAL ACTIVITY

In a further description, the following codes of vitamin D analogues are used:

PRI-2191 - tacalcitol

PRI-2201 – calcipotriol

PRI-2205 – 5,6-trans isomer of calcipotriol

In the *in vitro* experiments calcitriol (1,25-dihydroxycholecalciferol) was also used, as the naturally occurring biologically active form of vitamin D.

I. *IN VITRO* STUDIES

1. Antiproliferative activity of vitamin D analogues towards human breast cancer cells studies *in vitro*

Antiproliferative activity of the combination of vitamin D analogues *in vitro* was studied, as well as its influence on anastrozole cytostatic activity towards selected human cancer cell lines.

After 24 h incubation, the cells of MCF-7 human breast cancer, SKBR-3, and T47D lines were exposed to vitamin D analogues: PRI-2191, PRI-2205 and calcitriol as the reference, for 120 h. The cells suspended in culture medium 10^5 cells/ml were seeded, each 10^4 cells in 100 µl into single wells. To control cells culture medium 100 µl/well was added, tested compounds in medium of appropriate concentration were added to the other wells. The final concentration of vitamin D analogues was 100 nM (or 10 nM for T47D cell line) in 200 µl/well. Each experiment was repeated 3-4 times. SRB assay was performed [Skehan et al., J. Natl. Cancer Inst., 82:1107-1112, 1990], according to which inhibition of target cells proliferation was determined, after 120 h incubation.

SRB colorimetric assay:

When incubation was over, to each well cold trichloroacetic acid (Sigma) was added. The plate was incubated at 4°C for 1 h, then it was washed 5 times under running water, dried and 0,4% sulfrodamine B (Sigma) was added. The plate was left at room temperature for 30 minutes, it was than washed 4 times with 1% acetic acid. The plate was dried again and TRIS buffer (Sigma) was added. After 30 minutes absorbance was measured with Multiscan RM spectrophotometer (Labsystems, Helsinki, Finlandia) at 540 nm wave length. The absorbance values were proportional to the number of living cells present in each well. Percent of inhibition of cell proliferation was calculated using obtained values according to the following formula:

$$\text{Inhibition of proliferation}\,(\%) = \left(\left(\frac{A_p - A_m}{A_k - A_m}\right) * 100\right) - 100$$

wherein:

- A_m – absorbance value obtained for culture medium (blank control);
- A_k - absorbance value obtained for cells in culture medium (cell control);

- A_p - absorbance value obtained for cells treated with tested compounds;

Table 1. *Inhibition of cell proliferation of human breast cancer cells after 120 h treatment with calcitriol, PRI-2191 or PRI-2205*

Vitamin D analogue	Percent of inhibition of cell proliferation [%]		
100nM	**MCF-7**	**SKBR-3**	**T47D***
CALCITRIOL	47,67±2,8	20,17±5,18	53,09±0,3
PRI-2191	49,4±5,2	24,28±4,1	58,6±1,2
PRI-2205	21,4±9	7,43±2,8	26,9±5,4

* for T47D cell line the concentration of vitamin was 10 nM

1. **The influence of vitamin D analogues on anastrozole antiproliferative activity towards human breast cancers cells studies *in vitro***

The influence of vitamin D analogue: PRI-2191 and PRI-2205 on anastrozole antiproliferative activity was studied *in vitro*.

After 24 h incubation, the cells were treated simultaneously with calcitriol or vitamin D analogue and anastrozole for 120 h. Final concentration of vitamin D analogues was 100 nM, and anastrozole 100 μg/ml. Statistical analysis of obtained results of proliferation inhibition was carried out by analysis of percent of proliferation inhibition in particular combinations of tested compounds. Hypothetical value of inhibition of cell proliferation by combination of vitamin D analogue and anastrozole (%H) was calculated according to the following formula:

%H = 100-[(100 - %cyt) * (100 - %wit)] /100

wherein:

%H – hypothetical inhibition of proliferation by the combination of compounds [%];

%cyt – inhibition of proliferation by the cytostatic alone [%];

%wit – inhibition of proliferation by vitamine D analogue alone [%].

The obtained results were assigned the following values:

- antagonistic effect,

\+ sub-additive effect,

++ additive effect,

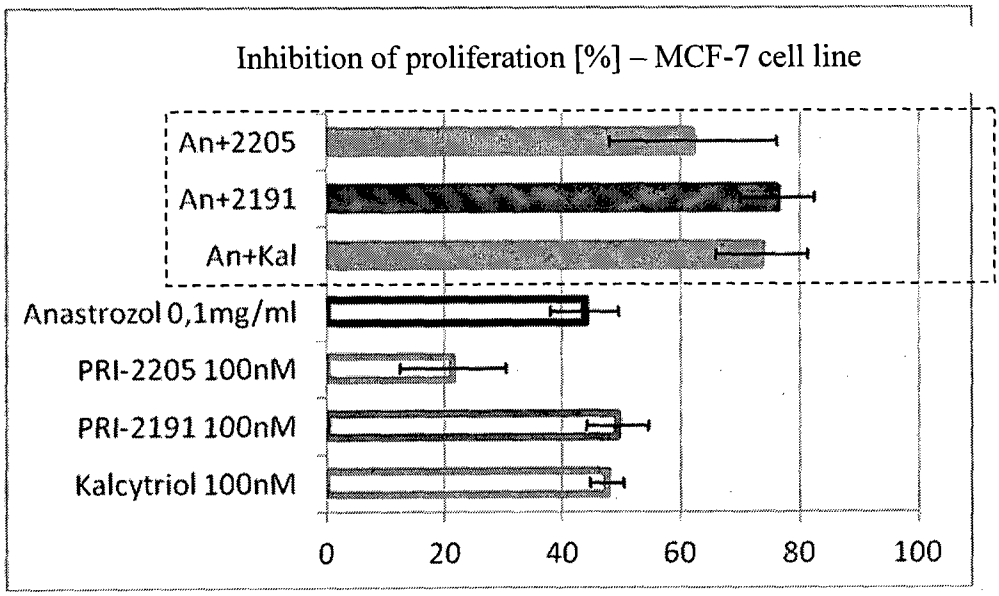
+++ synergistic effect.

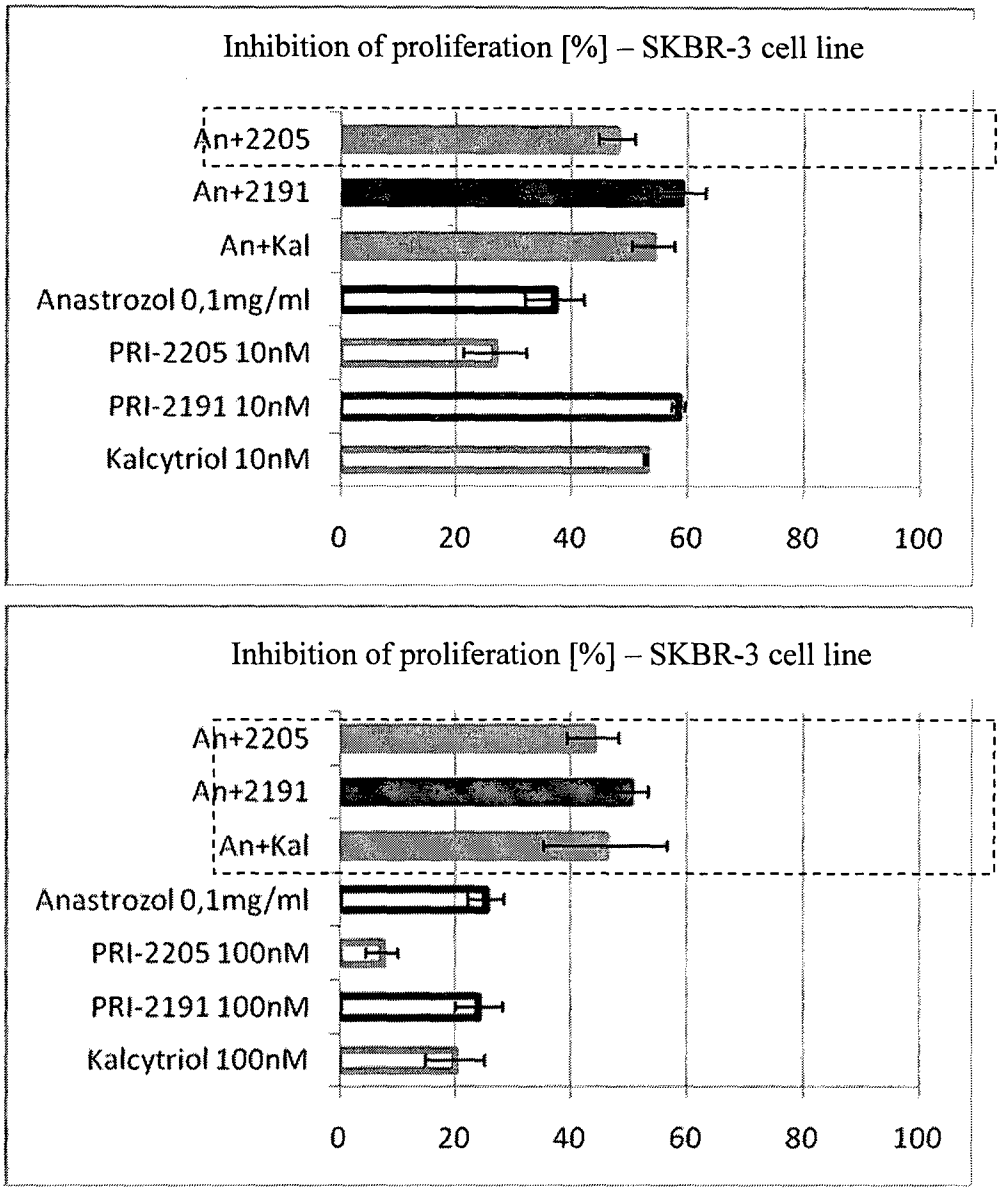
The hypothetical values below experimental ones, determined for combination of compounds prove the synergism of action of the compounds. Additive effect occurs when the experimental and hypothetical value are similar, sub-additive effect occurs when experimental value is lower than hypothetical value but above value for a cytostatic alone, and antagonistic action – when a value determined for a cytostatic used alone is above experimental value for combination of both compounds. [Peters G.J. i wsp.: Pharmacol Ther 2000, 87, 227-253]

Table 2. *Type of interaction of vitamin D analogues and anastrozole after 120 h incubation of human breast cancer cells with tested compounds*

Vitamin D analogue **100nM**	Concentration of anastrozole **100 µg/ml**		
	MCF-7	**SKBR-3**	**T47D**(10nM wit)**
CALCITRIOL	+++	+++	+
PRI-2191	+++	+++	+
PRI-2205	+++	+++	+++

Fig.1. *Inhibition of proliferation of human breast cancer cells after 120 h incubation with calcitriol, PRI-2191 or PRI-2205 in combination with anastrozole*





** Data in a frame indicate synergistic activity*

120 h Incubation of MCF-7 human breast cancer cell model and SKBR-3 cells with tested compounds demonstrated synergistic effect, when cancer cells were concurrently exposed to vitamin D derivatives and anastozole (Table 2, Fig. 1). After T47D cell line culturing with the combination of calcitriol and PRI-1291 analogue with anastrozole, subadditive effect was observed, but the combination of PRI-2205 analogue with anastrozole showed synergistic effect.

2. **Influence of PRI-2191 and PRI-2205 analogues used in combination with anastrozole on cell cycle phases and apoptosis of human breast cancer cells *in vitro* studies.**

The following assay was performed to estimate the influence of combination of vitamin D analogue and anastrozole on cell cycle phases and cancer cell apoptosis. The cells were suspended in culture medium 10 x 10^5 cells/ml, the cell monolayer was generated by seeding 1 ml of the suspension into single wells of a 24-well flat bottom plate (Corning, NY, USA). After 24 h incubation, tested compounds in culture medium or 0,01% ethanol as the control were added to each well (1 ml/well). The stocks of tested compounds and ethanol were prepared at four times higher concentrations then the final concentrations reached in the wells. At the beginning vitamin D analogues and calcitriol (vitamin control) were added at 100 nM or 10 nM final concentration (for T4TD cell line, Fig. 4). Next, anastrozole in culture medium at 100 μg/ml final concentration was added. The cells were incubated with tested compounds for 120 h. The experiment was repeated 4-5 times.

Protocol for preparation of cells for cell cycle phases and apoptosis analysis

On the basis of the results of proliferation inhibition assay, MCF-7, SKBR-3 and T47D cell lines were chosen to study cell cycle phases.

The cells were incubated with tested compounds in a 24-well format (Sarstedt). 1 ml of the cell suspension in culture medium, 2,5x10^4 cell/ml (T47D) and 0,5x10^5 cell/ml (MCF-7, SKBR-3), was transferred into each well. To the wells, 500 μl of vitamin solution at following concentrations: 10 nM (T47D cells) and 100 nM (MCF-7, SKBR-3 cells), as well as 500 μl of anastrozole solution at 0,1 mg/ml concentration were added. After 120 h incubation with vitamin analogues and anastrozole (MCF-7 and SKBR-3), the cells were tripsinized, transferred in their supernatants into the tubes and centrifuged at 1300 rpm for 5 min at 4°C.

The supernatant was discarded, the cell pellet was resuspended in 1 ml of phosphate-buffered saline (PBS) supplemented with 2% FBS.

The cells (5 x 10^5) were suspended in 1 ml of ice cold 70% ethanol and they were frozen at -18°C for at least 24 h. They were suspended in PBS, centrifugated to remove ethanol (4°C, 10 min., 2000 rpm), transferred into FACS tubes and

centrifuged (4°C, 5 min., 2000 rpm). The cell pellet was resuspended in RNAse buffer (5 x 10^2 cells in 500 µl of RNAse solution). After incubation with RNAse buffer at 37°C for 60 min. each cell sample was treated with 50 µl (50 mg/ml) of propidium iodide and incubated at 4°C for 30 min. The cells prepared according to the described protocol were subject to analysis.

Obtained data were analyzed using WinMDI 2.9. program. Experiments were repeated four times. The results are presented as mean value with standard deviation.

Fig. 2. *Interaction of vitamin D analogues PRI-2191 and PRI-2205 with anastrozole. The influence of combination of the compounds on cell cycle phases and apoptosis of MCF-7 human breast cancer cells. Results are represented as mean values of the results obtained in four repetitions of experiments.*

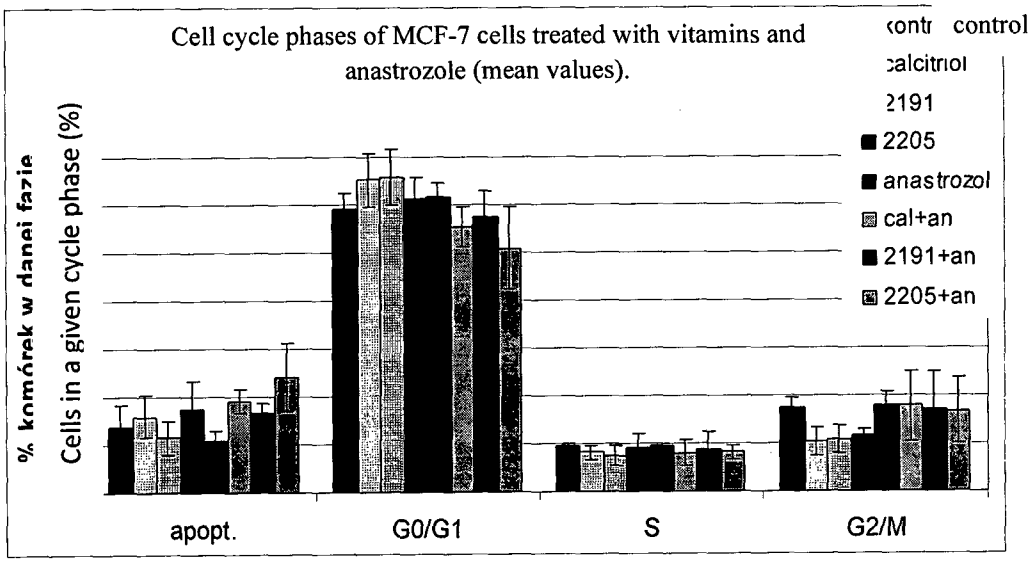


Fig. 3. *Interaction of vitamin D analogues PRI-2191 and PRI-2205 with anastrozole. The influence of combination of the compounds on cell cycle phases and apoptosis of SKBR-3 human breast cancer cells. Results are represented as mean values of the results obtained in fife repetitions of experiments.*

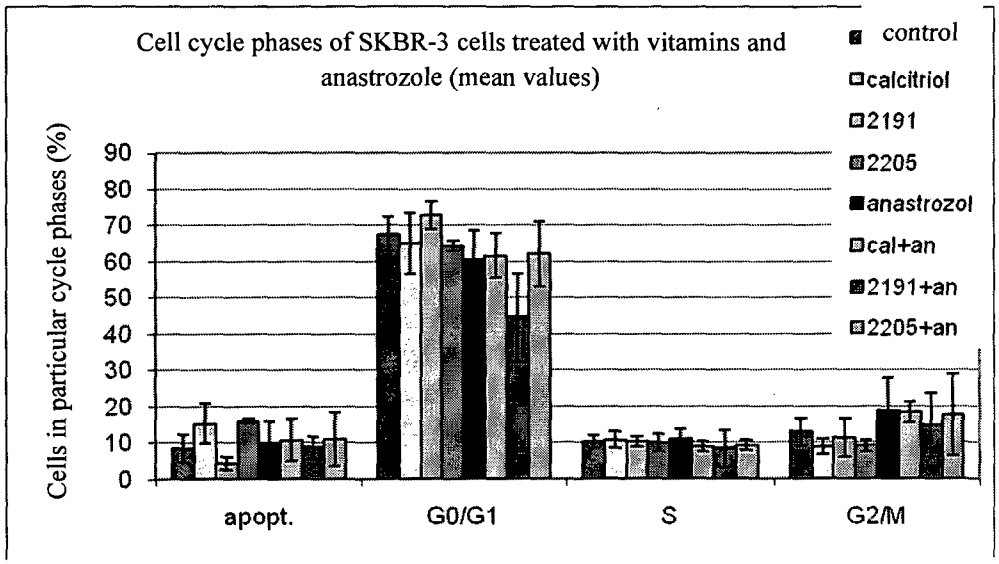
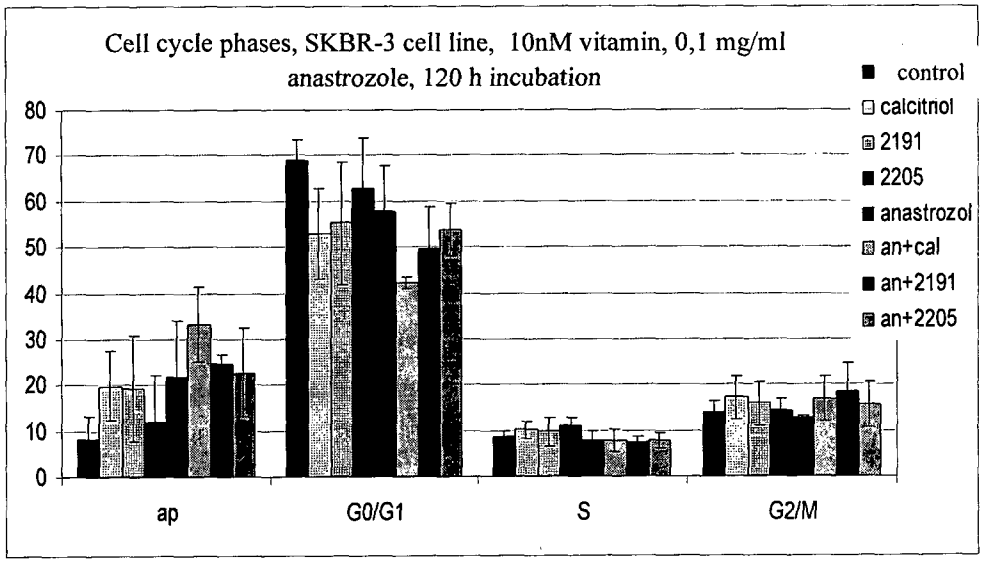


Fig. 4. *Interaction of vitamin D analogues PRI-2191 and PRI-2205 with anastrozole. The influence of combination of the compounds on cell cycle phases and apoptosis of T47D human breast cancer cells. Results are represented as mean values of the results obtained in four repetitions of experiments.*



It was observed that calcitriol and vitamin D analogues in combination with anastrozole increase the amount of apoptotic cells in comparison with anastrozole used alone in MCF-7 cell line culture (Fig. 2).

When all human breast cancercell lines were treated with combination of anastrozole and calcitriol or vitamin D analogues, decreased percent of cells in G0/G1 cell cycle phase was detected.

In addition, when MCF-7 and SKBR-3 cell lines were incubated either with combination of anastrozole and vitamin D analogues or anastrozole alone, increased cell number in G2/M phase was measured.

For T47D cell line, combination of calcitriol and vitamin D analogues with anastrozole cause the increase of apoptotic cell number in comparison with anastrozole used alone in cancer cell cultures (Fig. 4).

Summarizing, obtained results prove, vitamin D analogues enhance beneficial antineoplastic activity of anastrozole, therefore application of these compounds in combined therapy of breast cancer is reasonable.

IN VIVO **STUDIES**

1. Influence of PRI-2191 and PRI-2205 analogues on anastrozole antineoplastic activity in MCF-7 human breast cancer model

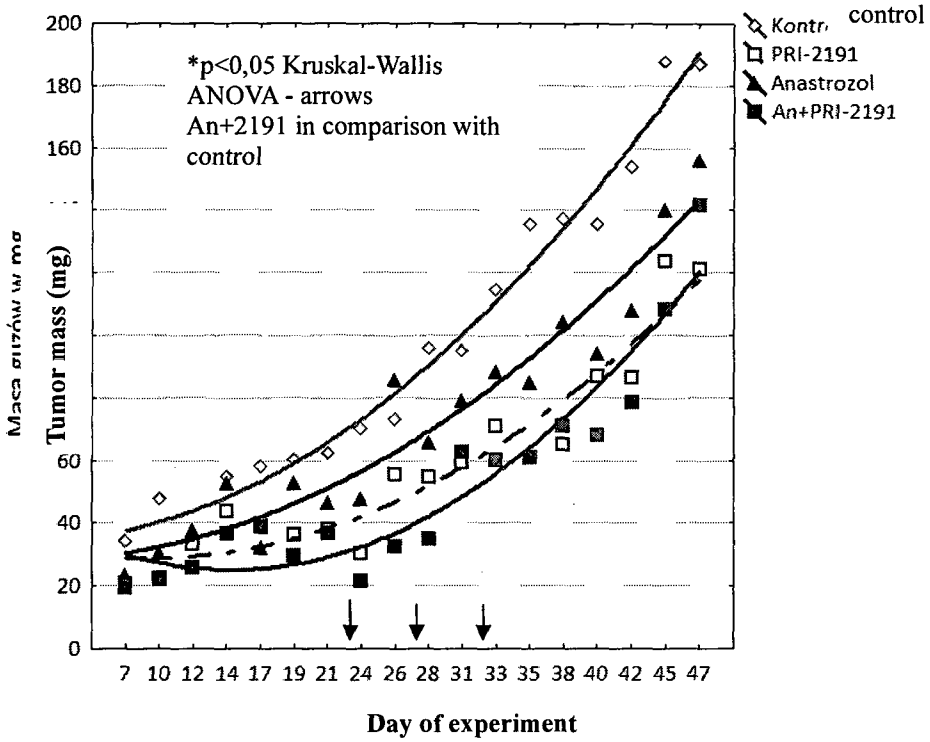
- NOD/SCID Mice strain (females) were inoculated subcutaneously with breast cancer cells (5 x 10^6 cells/0,25 ml/mice).
- Number of mice in experimental groups: control – 6; PRI-2191 – 6; PRI-2205 – 6; anastrozole (An) – 7; An + PRI-2191 – 7; An + PRI-2205 – 7.
- Mice treatment started on day **5** of experiment.
- Anastrozole: dosage **200µg/kg/day, subcutaneously,** every day (days 5-28 and day 31, 38).
- **PRI-2191**: dosage **1 mg/kg/day**, subcutaneously, once every two weeks, multiple administration, **3x** a week (days: 10, 12, 14, 17, 19, 21, 24, 26, 28, 31, 33, 35, 38, 40, 42, 45, 47).
- **PRI-2205**: dosage **10 mg/kg/day**, subcutaneously, once every two weeks, multiple administration, **3x** a week (days: 10, 12, 14, 17, 19, 21, 24, 26, 28, 31, 33, 35, 38, 40, 42, 45, 47).

On day **48** the experiment was completed, blood and organs were taken.
Sizes of subcutaneous tumors were measured during experiment.

Statistical analysis of obtained data was performed using Kruskal-Wallis ANOVA test for multiple comparisons and group comparisons was based on Mann-Whitney U test.

Fig. 5. *Masses of MCF-7 breast cancertumors in mice, treated with combination of anastrozole (200μg/kg/day) and vitamin D analogues.*

5. A- Anastrozole and PRI-2191 analogue.



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5.B- Anastrozole and PRI-2205 analogue

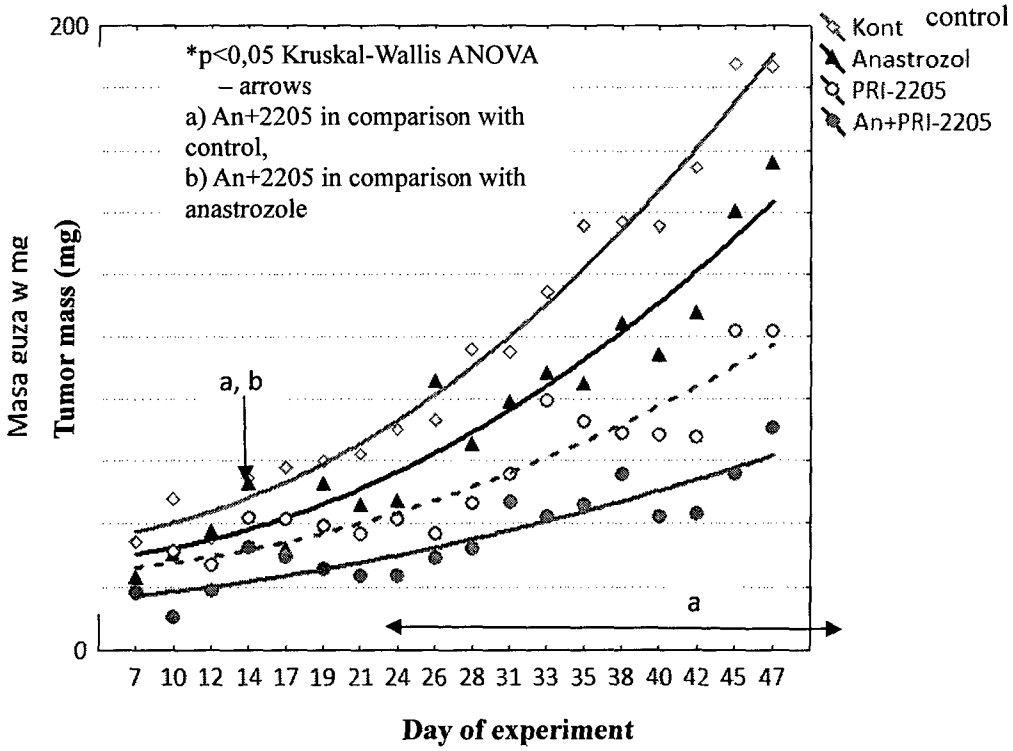


Table 3. *Days of experiment, when differences of tumor sizes among experimental groups were of statistical significance (p<0,05), on the basis of Kruskal-Wallis ANOVA and Mann-Whitney U tests.*

Control Group	***Reference Group***	****p<0.05 Kruskal-Wallis ANOVA test***	****p<0.05 Mann-Whitney U test***
PRI-2191	Control	Day:38	Day:10, 19, 24, 35, 38, 42
PRI-2205	Control	-	Day: 19, 21, 26, 28, 35-47
Anastrozole + PRI-2191	Control	Day: 19, 24, 28	Day: 10, 19-28, 33-42
	Anastrozole	-	Day: 10, 12, 24
Anastrozole + PRI-2205	Control	Day: 10, 19-47	Day: 10, 14-47
	Anastrozole	-	Day: 10, 12, 19, 24, 26, 45
	PRI-2205	-	Day: 10, 19, 21, 24, 28, 33, 40, 42

Table 4. *Influence of vitamin D analogues on tumor growth in mice inoculated with MCF-7 breast cancer cells and treated with anastrozole.*

* p<0.05 Mann-Whitney U test as control reference

N – number of mice in a group

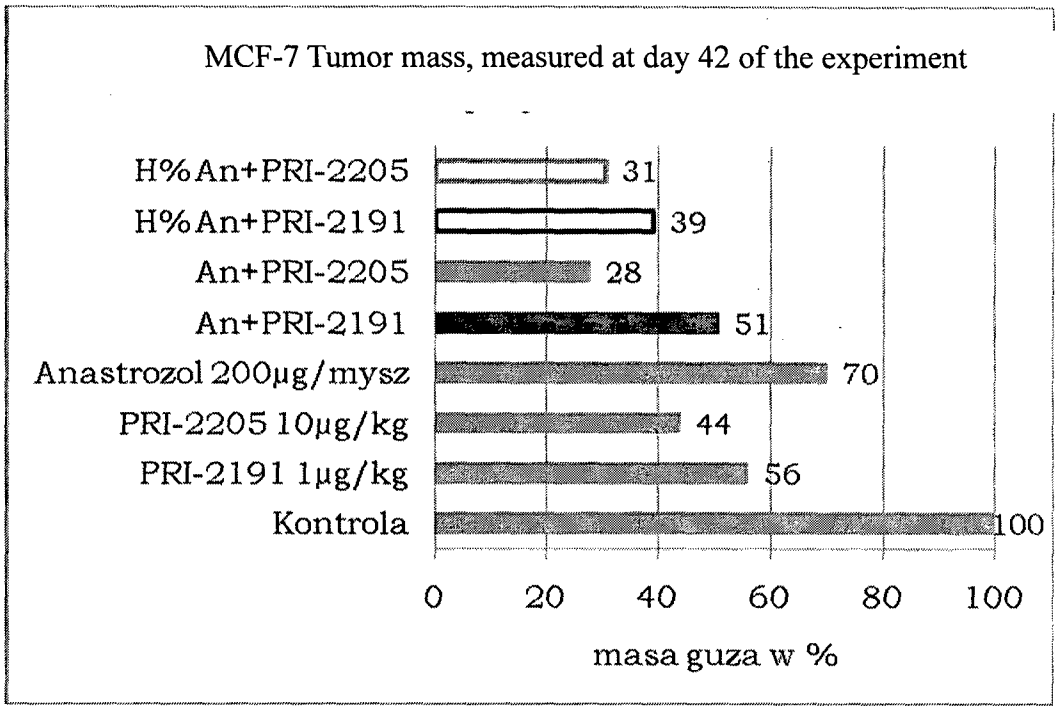
TGI [%] calculated according to the equation:

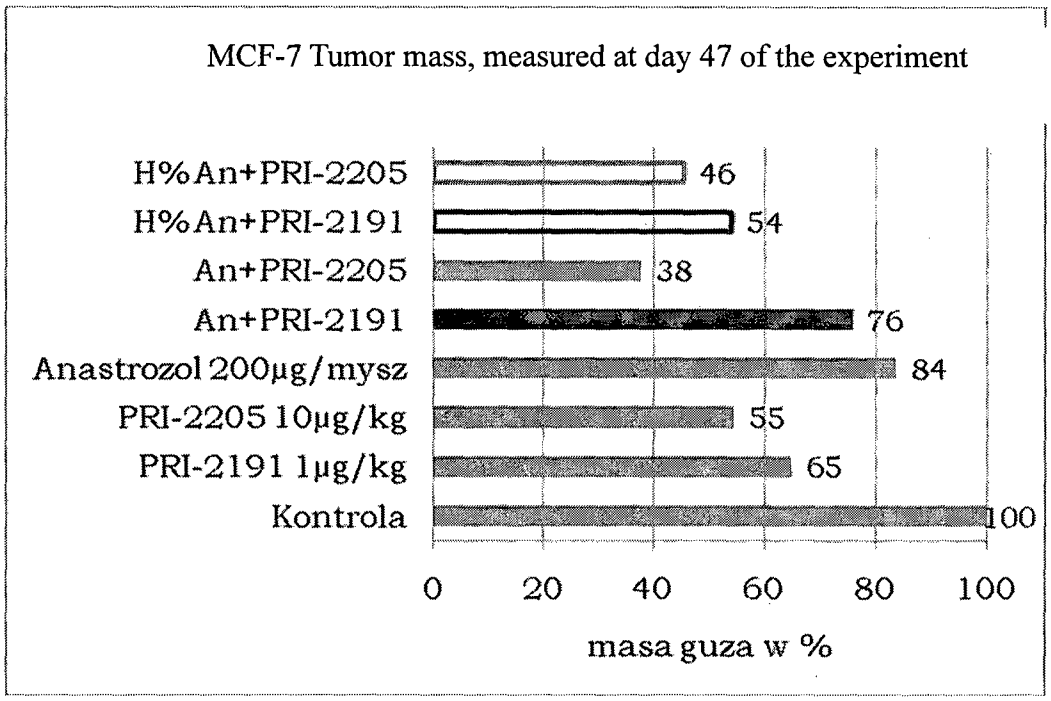
TGI=100-[(average mass of tumor in a treated group/average mass of tumor in a control) x 100)

TGI hip. Calculated according to the equation:

%H=100-[(100-TGI An) x (100-TGI vit. D analogue)/100]

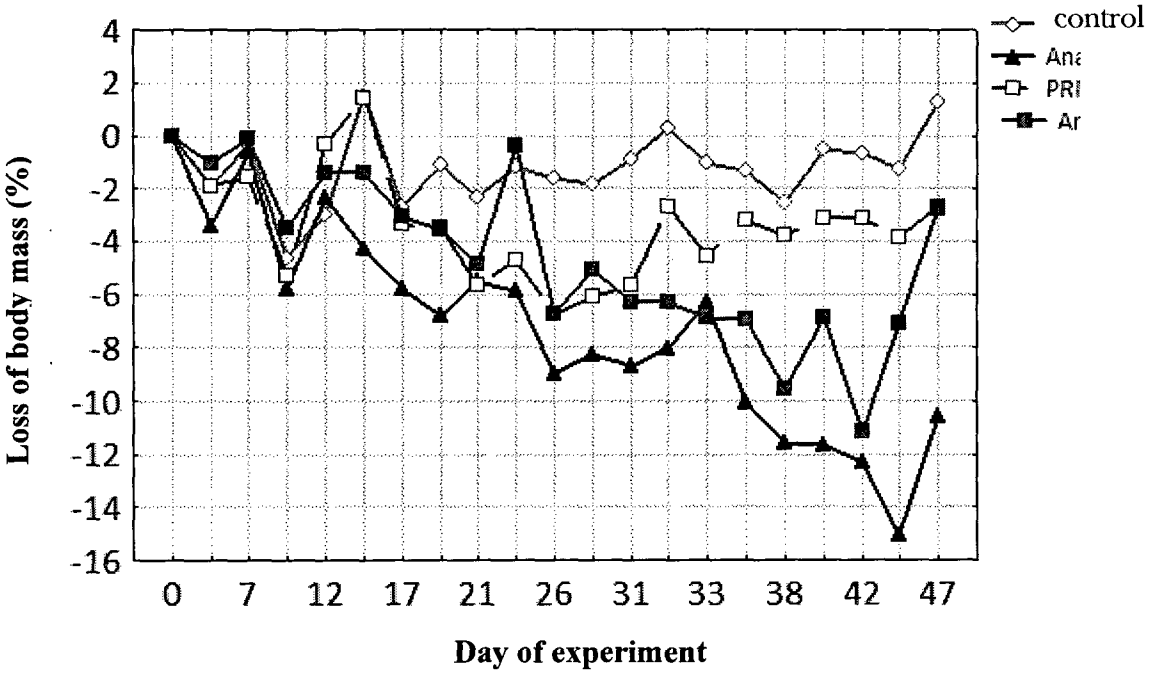
Fig. 6. *Masses of tumors in mice treated with combined therapy of anastrozole and vitamin D analogues and hypothetical masses of tumors for the combination – day 42 and 47. Masses of tumors are represented as a % of tumor masses in the control group.*

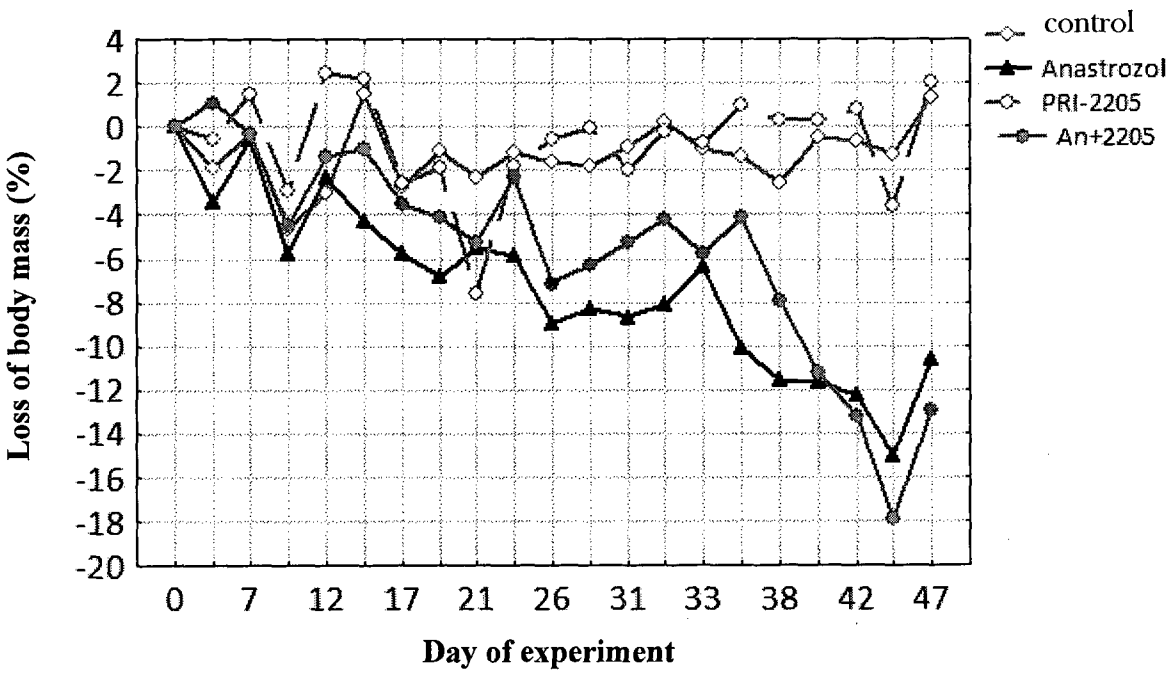




%H=100-[(100-TGI An) x (100-TGI vit. D analogue)/100]

Fig. 7. *Body mass changes of mice with MCF-7 breast cancer (cancer cells were inoculated subcutaneously), treated with anastrozole (**200µg/kg/day**, administered subcutaneously) in combination with vitamin D analogues administered subcutaneously 3x a week (PRI-2191:* **1 mg/kg/day**, PRI-2205: **10 mg/kg/day).**





Results:

Anastrozole in combination with PRI-2191 analogue decreases MCF-7 tumor masses at statistically significant level, when comparing with the control group, starting measuring from day 19 to 42.

Similarly, combination of anastrozole with PRI-2205 analogue causes statistically significant reduction of tumor growth in comparison with control, beginning from day 10 to last day of tumor size measurements, until day 47. The PRI-2205 analogue used alone considerably inhibits tumor growth. In comparison with the control group, significant tumor growth inhibition was observed on days: 19, 21, 26, 28 and from day 35 to the last day of experiment. Analogue PRI-2191 when used alone causes the decrease of MCF-7 tumor growth, but statistically relevant inhibition was observed on days: 10, 19, 24, 35, 38, and 42.

When PRI-2191 analogue as well as PRI-2205 in combination with anastrozole were administrated, substantial reduction of tumor masses at statistically significant level, in comparison with anastrozole (An) alone, was also observed (Fig. 5-6).

Mathematical TGI (tumor growth inhibition) analysis, enabled to assess types of interactions within tested compounds in combinations and proved, that hypothetical TGI values are similar or slightly lower than the results obtained in all experimental days. Considering the experiment as a whole, this result suggests additive effect of action, but in particular days of experiment synergistic effect against MCF-7 human breast cancertumor growth inhibition was observed .

The invention is illustrated by the following examples, which should not be constructed to limit its scope in any way.

Examples

Example 1

Soft gelatin capsules containing 0,50 μg of vitamin D analogue.

Composition of capsule content:

5,6-trans isomer of calcipotriol	0,50 μg
BHA	0,02240 mg
Citric acid	0,01680 mg
Ethanol absolute	1,29560 mg

Soy oil	78,66470 mg

The weighed amount of oil (about 80% of the total) was mixed for 15 minutes in nitrogen atmosphere. To the oil, the active substance dissolved in anhydrous ethanol with BHA and citric acid was added. It was stirred for 20 minutes in nitrogen atmosphere to the complete dissolution of ethanol phase in the oil phase, then the remaining part of the oil was added and stirred for 15 minutes.

Gelatin shell was prepared of the following composition:

gelatin	67,13%
glycerol	24,06%
water	8,78%
Aseptine A	0,01%
Cochineal E120	0,02%

The ingredients were dissolved in hot water, degassed, stirred for 30 minutes at the temperature of 65°C. The whole together with the filling was transferred to capsulating apparatus, where at the temperature of about 60°C, it was dropped to paraffin cooled to about 0°C. The capsules were then rinsed with tetrachloroethyelene and dried for 48 hours at the temperature of about 30°C.

Mean mass of a single capsule was 0.1275 g ±10%.

Mean mass of filling of the single capsule was about 0.080 g ± 10%.

Capsules were packed to orange glass jars and closed with polyethylene stoppers.

Example 2

Tablets containing 1 mg of anastrozole.

Composition of tablets:

Anastrozole 1 mg

Lactose

Hydroxypropylmethylcelulose

Polypropylene glycol

Povidon

Sodium starch glycolate

Magnesium stearate

Titanium dioxide

Claims

1. The use of anastrozole and vitamin D analogue in the combined therapy of breast cancer.

2. The use according to claim 1, wherein vitamin D analogue is selected from a group comprisng calcipotriol, tacalcitol and (1S,3R,5E,7*E*,22*E*,24*S*)-24-cyclopropyl-9,10-secochola-5,7,10(19),22-tetraen-1,3,24-triol.

3. The use according to claim 1 or 2, wherein therapeutically effective amounts of anastrozole and vitamin D analogue is administered to the patient.

4. The use according to claim 1 - 3, wherein therapeutically effective amounts of anastrozole and vitamin D analogue is administered to the patient orally.

INTERNATIONAL SEARCH REPORT

International application No
PCT/PL2012/000012

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/4196 A61K31/593 A61K45/06 A61P35/00
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DATABASE MEDLINE [Online] US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US; July 2006 (2006-07), PELCZYNSKA MARZENA ET AL: "Antiproliferative activity of vitamin D compounds in combination with cytostatics.", XP009161120, Database accession no. NLM16886680 abstract & ANTICANCER RESEARCH 2006 JUL-AUG LNKD-PUBMED:16886680, vol. 26, no. 4A, July 2006 (2006-07), pages 2701-2705, ISSN: 0250-7005 ----- -/--	1-4

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
12 July 2012	19/07/2012

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/PL2012/000012

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FILIP B ET AL: "Antitumor properties of (5E,7E) analogs of vitamin D3", JOURNAL OF STEROID BIOCHEMISTRY AND MOLECULAR BIOLOGY, ELSEVIER SCIENCE LTD., OXFORD, GB, vol. 121, no. 1-2, 1 July 2010 (2010-07-01), pages 399-402, XP027142251, ISSN: 0960-0760 [retrieved on 2010-07-01] abstract -----	1-4
Y	WO 2009/137104 A1 (RADIUS HEALTH INC [US]; LYTTLE C RICHARD [US]; HATTERSLEY GARY [US]; O) 12 November 2009 (2009-11-12) claims 1,4,20,35 -----	1-4
Y	WO 2010/143986 A1 (INST FARMACEUTYCZNY [PL]; WIETRZYK JOANNA [PL]; MILCZAREK MAGDALENA [P) 16 December 2010 (2010-12-16) the whole document -----	1-4

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/PL2012/000012

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		WO 2009137104 A1	12-11-2009
WO 2010143986 A1	16-12-2010	US 2012101057 A1	26-04-2012
		WO 2010143986 A1	16-12-2010