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USE OF 4-(3,4-DICHLORO-PHENYL)-1,2,3,4-TETRAHYDRO-NAPHTHALEN-1-YLAMINE FOR TREATING CANCER

The present invention relates to the use of 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine or one of its enantiomers or any combination of its enantiomers for preparing pharmaceutical compositions for treating cancer, as well as treatment and prevention of metastases.

The annual number of new cancer cases has grown considerably over the last twenty years. Cancer treatment is therefore a veritable public health problem. Irrespective of the type of cancer, chemotherapy treatment is generally recommended, if only as additional treatment. There is therefore an ongoing need to develop novel molecules for chemotherapy.

The inventors studied 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and its enantiomers or any combination of its enantiomers, and showed that these compounds have antiproliferative activity on numerous types of tumor cells, and they can be used in the prevention or treatment of cancer.

The present invention therefore relates to the use of 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine or one of its enantiomers selected from ((1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine, (1S,4R) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine, (1R,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine, and (1R,4R) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine) or any combination of its enantiomers, for preparing a pharmaceutical composition for treating cancer, as well as treatment and prevention of

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metastases. Treatment of cancer or metastases is understood according to the present invention to essentially mean the capacity for a compound to selectively destroy the tumor cells without substantially affecting healthy cells, given that this selectivity can vary as a function of the condition of the patient and the type cancer being treated.

As is evident from the examples hereinbelow, the compounds according to the present invention have a remarkable capacity to destroy tumor cells. The results prove to be particularly impressive as to the capacity of these products to affect tumor cells coming from a large number of different types of cancer.

According to the present invention, said cancer or said metastases have come from cancer selected from the group made up of cancer of the prostate, osteosarcoma, cancer of the lung, cancer of the lung not with small cells, breast cancer, cancer of the endometrium or cancer of the colon, chronic or acute leukaemia, solid tumors in children, lymphocitary lymphomas, cancer of the pancreas, skin cancer, cutaneous or intraocular melanoma, cancer of the head and neck, cancer of the uterus, ovarian cancer, gynaecological tumors, Hodgkin's disease, bone cancer, cancer of the rectum, cancer of the anal region, stomach cancer, cancer of the oesophagus, cancer of the small intestine, cancer of the endocrine system, soft-tissue sarcomas, cancer of the urethra, cancer of the penis, cancer of the bladder, kidney cancer, cancer of the ureter, malignant paediatric tumors and tumors of the central nervous system, in particular cerebral tumors. Said cancer or said metastases have preferably originated from breast cancer. Otherwise, said cancer is leukaemia.

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Further, the inventors showed that 4-(3,4-dichloro-phenyl)- 1,2,3,4-tetrahydro-naphthalen-1-ylamine or one of its enantiomers or any combination of its enantiomers, administered in combination with at least one alkaloid, preferably a vinca alkaloid, for example vincristine or vinblastine, has a synergy of cytotoxic activity specific to finding tumor cells.

Another aspect of the invention therefore relates to the use of 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine or one of its enantiomers or any combination of its enantiomers and at least one alkaloid for preparing a pharmaceutical composition for treating cancer, as well as treating and preventing metastases. The alkaloid is preferably a vinca alkaloid. The vinca alkaloid is more preferably selected in the group constituted by: vinblastine, vincristine, vindesine, and vinorelbine, preferably vincristine and/or vinblastine.

According to the present invention vinca alkaloid is understood to designate vinca alkaloids, Vinca rosea, and their derivatives obtained by chemical modification. Their goal is the tubuline of the achromatic spindle of the mitosis whereof they inhibit the polymerisation. Since this tubuline also constitutes nerve fibres, vinca alkaloids have a nerve toxicity, especially sensitive, and induce a depressive tendency.

Another aspect of the invention concerns products comprising 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine or one of its enantiomers or any combination of its enantiomers and at least an alkaloid, as a combination product for simultaneous use, separate or staggered over time in anti-cancer therapy.

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The selected alkaloid is preferably a vinca alkaloid. The vinca alkaloid is more preferably selected in the group constituted by: vinblastine, vincristine, vindesine, and vinorelbine, preferably vincristine and/or vinblastine.

The pharmaceutical compositions and/or the combination products according to the present invention can be administered orally, intravenously, enterally, parenterally, intramuscularly, intranasally, subcutaneously, sublingually or topically. In general, oral or by default injectable administration is preferred, especially for treatment of tumors. Remarkably, the inventors showed that the products of the present invention can be efficacious orally, with the exception of numerous anti-tumoral products currently available for patients (Goodman & Gilman's Pharmacological Basis of Therapeutics, Mc Graw Hill Editor).

Daily dosages should take into account the state of the patient, but also the cancer being treated, its stage and progression.

The examples hereinbelow are mentioned only by way of illustration and cannot be considered as limiting the idea of the present description.

FIGURES

Figure 1: Study of the synergistic effect of combinations of cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and vincristine on the inhibition of the proliferation of cells of leukaemic U937 type

Figure 2: Study of the synergistic effect of combinations of cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and vinblastine on the

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inhibition of the proliferation of cells of leukaemic U937 type

Figure 3: Study of the synergistic effect of combinations of cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and vincristine on the inhibition of the proliferation of breast cancer cells of MDA-MB231 type

Figure 4: Study of the synergistic effect of combinations of cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and vinblastine on the inhibition of the proliferation of breast cancer cells of MDA-MB231 type

Figure 5: Evolution throughout treatment of the median volume of tumors implanted in SCID mice after chronic administration of Cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine intraperitoneally

Figure 6: Evolution throughout treatment of the median volume of tumors implanted in SCID mice after chronic administration of (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine intraperitoneally

EXAMPLES:

"Cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine" is understood to mean the racemic mixture of the two enantiomers (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and (1R,4R) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine.

"Trans 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine ", is understood to mean the racemic mixture of the two enantiomers (1S,4R) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-

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ylamine and (1R,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine.

"Cis/Trans 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine", is understood to mean the racemic mixture of the above four enantiomers.

EXAMPLES 1 to 3:

In these examples the inventors unexpectedly point out the antitumoral effect of 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and its enantiomers or any combination of its enantiomers on cellular tumoral lines of various origins.

Example 1: Determination of CI_{50} on cells of leukaemic U937 type

The method described hereinbelow was applied to determine the CI_{50} values of the following compounds on the U937 cells:

- Cis/Trans 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine
- Cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine
- Trans 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine
- (IS, 4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine
- (IS, 4R) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine
- (IR, 4R) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine
- (IR, 4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine

The CI_{50} values are summarised in the table hereinbelow (Table 1)

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Table 1

Compound tested	CI50 on U937
Cis/Trans 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydronaphthalen-1-ylamine	4.80 µM
Cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine	3.80 µM
Trans 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine	4.00 µM
(1S, 4 S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydronaphthalen-1-ylamine	7.85 µM
(1S, 4R) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydronaphthalen-1-ylamine	6.24 µM
(1R, 4R) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydronaphthalen-1-ylamine	8.17 µM
(1R, 4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydronaphthalen-1-ylamine	12.0 µM

These results show that 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine, the combinations of its enantiomers and all its enantiomers have the property of inhibiting proliferation of cells of leukaemic U937 type and consequently antitumoral activity. The antitumoral activities of 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine, the combinations of its enantiomers and all its enantiomers are equivalent to the extent where the CI_{50} values obtained are all between 3 µM and 12 µM. As a consequence, any combination of enantiomers of 4-

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(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine has the property of inhibiting the proliferation of cells of leukaemic U937 type and therefore antitumoral activity.

Example 2: Determination of CI_{50} on breast cancer cells MDA- MB231

The method described hereinbelow was applied to determine the CI_{50} values of the following compounds on the MDA-MB231 cells:

- Cis/Trans 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine
- Cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine
- Trans 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine

The CI_{50} values are summarised in the table hereinbelow (Table 2)

Table 2

Compound tested	CI_{50} on MDA-MB231
Cis/Trans 4-(3,4-dichloro-phenyl)-1,2,3,4- tetrahydro-naphthalen-1-ylamine	6.20 µM
Cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine	4.48 µM
Trans 4-(3,4-dichloro-phenyl)-1,2,3,4- tetrahydro-naphthalen-1-ylamine	6.00 µM

These results show that 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and

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combinations of its enantiomers have the property of inhibiting the proliferation of breast cancer cells of MDA-MB231 type and consequently antitumoral activity. The antitumoral activities of 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and the combinations of its enantiomers are equivalent to the extent where the values of the CI_{50} obtained are all between 4 µM and 7 µM. As a consequence, all the enantiomers of 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and even any combination of these enantiomers have the property of inhibiting the proliferation of breast cancer cells of MDA-MB231 type and therefore antitumoral activity

Example 3: Determination of CI_{50} of (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine on 30 cellular tumoral lines

The method described hereinbelow was applied to determine the CI_{50} values of (1S,4S)-4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine on 30 cellular lines derived from breast cancer, colon, lung, ovary, prostate, pancreas, skin, brain and blood.

Table 3 summarises the CI_{50} values of (1S,4S)-4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine on 30 different tumoral lines coming from haematological tumors and highly varied solids.

In this example the inventors demonstrate unexpectedly that (1S-4S)-4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine has a large anti-tumoral range of action and is capable of inhibiting the proliferation of tumor cells originating from all cancers tested.

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Table 3

ORGAN	LINE	CANCER	CI_{50} (.1.M)
Breast	MCF-7	Invasive ductal carcinoma, metastases with pleural effusion	7.45
	ZR-75-1	Invasive ductal carcinoma, metastases	5.09
Colon	HCT 116	Carcinoma	13
	HT-29	Adenocarcinomas	6.65
	LoVo	Adenocarcinomas, metastases of left	24.50
Lung	Calu-6	Anaplasic carcinomas	2.81
	NCI-H460	Carcinomas, metastases with pleural effusion (large cell)	9.09
Ovary	NIH:OVCAR-3	Adenocarcinomas, metastases with ascites	7.45
	SK-OV-3	Adenocarcinomas, metastases with ascites	22.00
Prostate	PC-3	Adenocarcinomas	6.80
	MDA PCa 2b	Adenocarcinomas, bone	6.55
	DU 145	Carcinomas, cerebral	14.60
Pancreas	BxPC-3	Adenocarcinomas	20.70
	PANC-1		7.00
	MIA PaCa-2		6.91
Skin	CMEL-5	Melanomas	6.24
Brain	CGL-3	Glioblastomas	7.15
	CGL-9	Glioblastomas	17.20
	U-87 MG	Glioblastomas	7.00
Blood	Jurkat J6	Acute lymphoblastic leukaemia, T cells	9.84
	CCRF-CEM	Acute lymphoblastic leukaemia	6.59

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	MOLT-4	Acute lymphoblastic leukaemia	4.56
	HL-60	Acute promyelocytic leukaemia	8.17
	THP-1	Acute monocytic leukaemia	8.36
	OCI-AML2	Acute myelold leukaemia	9.40
	EHEB	Chronic leukaemia with B	7.27
	BV-173	Chronic myelold leukaemia	1.73
	K-562	Chronic myelold leukaemia	7.55
	NAMALWA	Burkitt lymphomas	3.78
	U937	Lymphomas	3.95

EXAMPLES 4 to 9:

In these examples the inventors show unexpectedly the synergistic antitumoral effect of 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and its enantiomers with Vinca alkaloids.

Example 4: Study of the synergistic effect of combinations of cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and vincristine on the inhibition of the proliferation of cells of leukaemic U937 type

The method described hereinbelow for evaluating a synergistic effect of combinations of two products on the inhibition of the proliferation of cells of leukaemic U937 type was applied with the following compounds:

- Cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine
- Vincristine

The final 6 concentrations selected for testing cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine were 25, 13, 6, 3, 3.1 and 1.6 micromolar (μM). The final 6 concentrations selected

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for testing vincristine were 4000, 1000, 250, 63, and 16 picomolar (pM).

Incubation of the cells U937 with different combinations of concentrations of cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and vincristine reveals the existence of synergy between the antiproliferative effects of two compounds.

The results are presented in figure 1.

The antiproliferative activity (inhibition percentage of the proliferation of 73%) of a combination of vincristine (250 pM) and cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine (3.1 µM) is clearly higher than the effect of the most active agent only (21% inhibition for vincristine at 250 pM). Also, the antiproliferative effect observed with this combination shows no simple additivity of the separate effects of the two products, given the positive value (+49) calculated for the excedent relative to the " Bliss " additivity.

In conclusion there is a synergistic effect of this combination of cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and vincristine.

Example 5: Study of the synergistic effect of combinations of cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and vinblastine on the inhibition of the proliferation of cells of leukaemic U937 type

The method described hereinbelow for evaluating a synergistic effect of combinations of two products on the inhibition of the proliferation of cells of leukaemic U937 type was applied with the following compounds:

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- Cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine

- Vinblastine

The final 6 concentrations selected for testing cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine were 25, 13, 6, 3, 3.1 and 1.6 micromolar (µM). The final 6 concentrations selected for testing vinblastine were 4000, 1000, 250, 63, and 16 picomolar (pM).

Incubation of the U937 cells with different combinations of concentrations of cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and vinblastine reveals the existence of synergy between the antiproliferative effects of two compounds. The results are presented in figure 2.

The antiproliferative activity (percentage of inhibition of the proliferation of 54%) of a combination of vinblastine (250 pM) and cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine (3.1 µM) is clearly higher than the effect of the most active agent only (19% of inhibition for the vinblastine at 250 pM). Also, the antiproliferative effect observed with this combination does not come under simple additivity of the separate effects of the two products, given the positive value (+31) calculated for the excedent relative to "Bliss" additivity. In conclusion, there is a synergistic effect of this combination of cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and vinblastine.

Example 6: Study of the synergistic effect of combinations of (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and vincristine on the inhibition of the proliferation of cells of leukaemic U937 type

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The method described hereinbelow for evaluating a synergistic effect of combinations of two products on the inhibition of the proliferation of cells of leukaemic U937 type was applied with the following compounds:

- (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine
- Vincristine

The incubation of U937 cells with different combinations of concentrations of (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and vincristine reveals the existence of synergy between the antiproliferative effects of two compounds. The antiproliferative effect observed of a combination of (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine (3.1 µM) and vincristine does not come under simple additivity of the separate effects of the two products, given the positive value (+8) calculated for the excedent relative to "Bliss" additivity. In conclusion, there is a synergistic effect of this combination of (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and vincristine.

Example 7: Study of the synergistic effect of combinations of (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and vinblastine on the inhibition of the proliferation of cells de leukaemic U937 type

The method described hereinbelow for evaluating a synergistic effect of combinations of two products on the inhibition of the proliferation of cells of leukaemic U937 type was applied with the following compounds:

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- (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine

- Vinblastine

The incubation of U937 cells with different combinations of concentrations of (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and vinblastine reveals the existence of synergy between the antiproliferative effects of two compounds. The antiproliferative effect observed in a combination of (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine (3.1 µM) and vinblastine does not come under simple additivity of the separate effects of the two products, given the positive value (+5) calculated for the excedent relative to "Bliss" additivity. In conclusion, there is a synergistic effect of this combination of (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and vinblastine.

Example 8: Study of the synergistic effect of combinations of cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and vincristine on the inhibition of the proliferation of breast cancer cells of MDA- MB231 type

The method described hereinbelow for evaluating a synergistic effect of combinations of two products on the inhibition of the proliferation of breast cancer cells of MDA-MB231 type was applied with the following compounds: - Cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine

- Vincristine

The final 6 concentrations selected for testing cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine were 25, 13, 6,3, 3.1 and 1.6 micromolar (µM). The final 6 concentrations selected

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for testing vincristine were 4000, 1000, 250, 63, and 16 picomolar (pM).

Incubation of the MDA-MB231 cells with different combinations of concentrations of cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and vincristine reveals the existence of synergy between the antiproliferative effects of two compounds.

The results are presented in figure 3. The antiproliferative activity (percentage of inhibition of the proliferation of 59%) of a combination of vincristine (1000 pM) and cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine (3.1 µM) is clearly higher than the effect of the most active agent only (25% of inhibition for the vincristine at 1000 pM). Also, the antiproliferative effect observed with this combination does not come under simple additivity of the separate effects of the two products, given the positive value (+28) calculated for the excedent relative to "Bliss" additivity. In conclusion, there is a synergistic effect of this combination of cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and vincristine.

Example 9: Study of the synergistic effect of combinations of cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and vinblastine on the inhibition of the proliferation of breast cancer cells of MDA- MB231 type

The method described hereinbelow for evaluating a synergistic effect of combinations of two products on the inhibition of the proliferation of cells of leukaemic U937 type was applied with the following compounds:

- Cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine

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- Vinblastine

The final 6 concentrations selected for testing the cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine were 25, 13, 6, 3, 3.1 and 1.6 micromolar (µM). The final 6 concentrations selected for testing vinblastine were 4000, 1000, 250, 63, and 16 picomolar (pM).

Incubation of the cells MDA-MB231 with different combinations of concentrations of cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and vinblastine reveals the existence of synergy between the antiproliferative effects of two compounds.

The results are presented in figure 4.

The antiproliferative activity (percentage of inhibition of the proliferation of 61%) of a combination of vinblastine (1000 pM) and cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine (1,6 µM) is clearly higher than the effect of the most active agent only (33% of inhibition for the vinblastine at 1000 pM). Also, the antiproliferative effect observed with this combination does not come under simple additivity of the separate effects of the two products, given the positive value (+27) calculated for the excedent relative to "Bliss" additivity. In conclusion, there is a synergistic effect of this combination of cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and vinblastine. Similarly, the antiproliferative activity (percentage of inhibition of the proliferation of 87%) of a combination of vinblastine (1000 pM) and cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine (3.1 µM) is clearly higher than the effect of the most active agent only (33% of inhibition for vinblastine at 1000 pM).

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Also, the antiproliferative effect observed with this combination does not come under simple additivity of the separate effects of the two products, given the positive value (+47) calculated for the excedent relative to "Bliss" additivity. In conclusion, there is a synergistic effect of this combination of cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and vinblastine.

EXAMPLES 10 and 11:

In these examples the inventors unexpectedly show the antitumoral effect of 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and its enantiomers administered in animals with a tumor.

Example 10: Study of the antitumoral effect in mice of cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine after intraperitoneal chronic administration

In this example the inventors tested the antitumoral effect in mice of Cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine, by applying the method described hereinbelow. So as to test this effect the inventors administered Cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine to mice on which tumor cells had earlier been grafted. They compared the growth of the tumors in animals treated with Cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine to that of tumors in controlled non-treated animals or those treated solely with the vehicle of the substance test. The inventors were able to remarkably show that intraperitoneal administration of Cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine

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significantly slowed tumoral growth in mice thus treated.

The inventors analysed the average size of tumors in mice with a subcutaneous tumor and treated with Cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and compared it to that of " control " mice with these tumors and treated solely with the vehicle of the substance test.

Figure 5 represents the average volume of subcutaneous tumors in mice (in mm^3) treated by Cis 4-(3,4-dichloro-phenyl)- 1,2,3,4-tetrahydro-naphthalen-1-ylamine as a function of time elapsed (in days) since the first day of treatment (50 mg/kg intraperitoneally), compared to the average volume of tumors in mice treated by the vehicle.

The volume of tumors in mice treated with the compound of the invention is clearly less than that of non-treated mice, which reveals an anti-tumoral effect manifesting Cis 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine.

Example 11: Study of the antitumoral effect in mice of (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine after intraperitoneal chronic administration

In this example the inventors tested the antitumoral effect in mice of (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine, by applying the method described hereinbelow. So as to test this effect the inventors administered (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine to mice on which tumor cells had earlier been grafted. They compared the growth of tumors in animals treated with (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine to that of tumors in

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controlled non-treated animals or those treated solely with the vehicle of the substance test. The inventors were able to remarkably show that intraperitoneal administration of (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine significantly slowed tumoral growth in mice thus treated.

The inventors analysed the average size of the tumors in mice with subcutaneous tumor and treated with (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and compared it to that of the " control " mice with these tumors and treated solely with the vehicle of the substance test.

Figure 6 represents the average volume of subcutaneous tumors in mice (in mm^3) treated by (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine as a function of time elapsed (in days) since the first day of treatment (15 mg/kg twice per day intraperitoneally), compared to the average volume of tumors in mice treated by the vehicle.

The volume of tumors in mice treated with the compound of the invention is clearly less than that of the non-treated mice, which reveals an anti-tumoral effect manifesting (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine.

EXAMPLES 12 and 13:

In these examples the inventors demonstrate remarkably that 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine and its enantiomers have properties intrinsic to their oral therapeutic use.

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Example 12: Study of the parameters of pharmacokinetics (half-life, bioavailability) of (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine in mice

Pharmacokinetic studies were conducted with (1S,4S)-4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine on rodents (CD1 mice). This compound was administered to the animals intravenously at a dose of 1 mg/kg and orally at doses of 5, 10 and 20 mg/kg. The circulating rates of (1S,4S)-4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine in the blood of the mice were measured by liquid chromatography coupled to mass spectrometry, at different times of between 5 and 480 minutes after intravenous administration and between 15 and 1440 minutes after oral administration. These measurements showed significant circulating doses of (1S,4S)-4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine in the animals treated.

The results of the pharmacokinetic parameters of (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine in mice are summarised in Tables 4a and 4b hereinbelow.

Table 4a

Administration method	Subject	Dose (mg/kg)	T1/2 (min)	Cl (mL/min/kg)	AUCall (min*ng/mL)	AUCING (min*ng/mL)
iv	Mice	1	161	35	25057	28302

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Table 4b

Administration method	Subject	Dose (mg/kg)	Tmax (min)	Cmax (ng/mL)	T1/2 (min)	AUCall (min*ng/mL)	AUCING (min*ng/mL)
per os	Mice	5	120	49	379	30070	32255
per os	Mice	10	120	131	275	65769	67755
per os	Mice	20	120	254	316	151002	157847

With:

- T 1/2 = elimination at half-life
- AUCall = area under the curve from the instant of administration (1=0) to the time of last observation
- AUCESfF = zone under the curve from the instant of administration (1=0) extrapolated to infinity.
- Tmax = Maximum concentration time observed
- Cmax = Concentration corresponding to Tmax
- Cl = Total clearance

Also, the ratio of areas under the (AUC) curve obtained during oral and intravenous administration of (1S,4S)-4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine allows measuring of a bioavailability factor, whereof the value according to the concentrations is between 24 % and 30 %.

These experiences show that when (1S,4S)-4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine is administered orally, significant doses of this compound are discovered in the circulation, with a significant bioavailability factor, showing that it is therefore well adapted to oral treatment.

Example 13: Study of the effect antitumoral in mice of (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine after chronic oral administration

In this example the inventors tested the antitumoral effect in mice of (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine, by

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applying the method described hereinbelow. So as to test this effect the inventors administered (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine to mice on which tumor cells had earlier been grafted. They compared the growth of tumors in animals treated with (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine to that of tumors in non-treated control animals or those treated solely with the vehicle of the substance test. The inventors were able to remarkably show that oral administration of (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine significantly slowed tumoral growth in mice thus treated.

Table 5 summarises the median volumes (in mm^3) of subcutaneous tumors in SCID mice treated by (1S-4S)-4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine as a function of time elapsed (in days) from the first day of treatment (administration of 80 mg/kg per day and orally) and the median volumes of tumors in mice treated by the vehicle on the same days. Days D15 and D18 correspond respectively to the 15th and 18th day after the initial day of treatment.

Table 5 hereinbelow presents the median volumes of tumors implanted in SCID mice after chronic oral administration of (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine

Table 5

Median volume of tumors (mm^3)	D15	D18
Vehicle	1536	2670
(1S,4S)-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine:80	836	1536

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The median volume of tumors in the mice treated with the compound of the invention is significantly less (close to half) than the median volume of tumors in mice treated by the vehicle.

Consequently, (1S,4S)-4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine administered orally has an anti-tumoral effect in animals.

Example 14: Study of cerebral penetration of (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine in mice

The activity of anti-tumorals on tumors of the nervous system is often limited by their incapacity to pass through the haematoencephalic barrier and therefore to penetrate into the brain.

As indicated in Table 3, (1S,4S)-4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine is active in vitro on tumoral lines derived from glioblastomas. Interestingly, the experiments reported hereinbelow show that (1S,4S)-4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine has the capacity to pass through the haematoencephalic barrier significantly, which represents an important advantage for this compound for treatment of cerebral tumors.

The (1S,4S)-4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine was injected intravenously in a group of CD1 mice, at the rate of 1 mg/kg. The levels of (1S,4S)-4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine in the blood and brain were analysed by liquid chromatography coupled with mass spectrometry.

The results of this study on mice of the cerebral penetration of (1S,4S) 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine are summarised in Table 6 hereinbelow.

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Table 6

Time (minutes)	Brain concentration	Plasma Concentration	Brain/Plasma ratio
21	96	47	2
34	217	67	3
50	275	34	8
65	149	32	5
120	486	37	13
360	295	15	20
480	155	7	23
600	209	11	19

These experiments show that concentrations of (1S,4S)-4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine in the brain are more significant than in the plasma and that the concentration ratio of this compound between the brain and the blood increases over time (over the interval analysed), thus showing the capacity for cerebral accumulation of this compound.

Consequently, (1S,4S)-4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalen-1-ylamine has a major interest for the treatment of cerebral tumors.

MATERIALS AND METHODS

I. Procedure for determining CI_{50} on leukaemic U937 type cells

The compound to be tested is placed in solution in DMSO (100%) at a concentration 1.10^{-2} M (mother solution), then 8 samples of 8 different concentrations are prepared by successive dilution with the complete medium of cellular culture (RPMI 1640 Cambrex + 10% FBS

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+ 1% L-Glutamine + 40µg/ml of Gentamycine). For each sample, the dilution volume is adjusted to produce a concentration equal to twice the final Cx concentration selected. By way of example, 1.10^{-4} M, 5.10^{-5} M, $2,5.10^{-5}$ M, $1,3.10^{-5}$ M, $6,3.10^{-6}$ M, $3,1.10^{-6}$ M, $1,6.10^{-6}$ M and $7,8.10^{-7}$ M is a standard range of 8 final concentrations. At the same time, 8 controlled samples are prepared by successive dilution of a solution of DMSO (100 %) with the same cellular culture medium (RPMI1640 Cambrex + 10% FBS + 1% L-Glutamine + 40µg/ml of Gentamycine), the dilution volumes selected being strictly identical to those used for preparing samples of the compound to be tested.

In a white plaque of 96 wells, non transparent (fluoronunc 96F Nunclon Delta), 100 µL of a suspension of cells U937 at a concentration of 2,5 10^4 cells/ml in a medium of RPMI1640 Cambrex + 10% FBS + 1% L-Glutamine + 40µg/ml of Gentamycine are deposited in each well, or 2500 cells per well. 100 µL of a sample at a concentration twice Cx are added (one sample per well) and the medium is homogenised. After 144h of incubation at 37°C in an atmosphere (CO^2 5%, humidity 90 - 95%), quantification of the viability of cells in culture is done by measuring the ATP levels using the CellTiter-Glo(TM) luminescent analysis test supplied by Promega Corporation, by following the instructions of the supplier. The luminescence in each well is measured using a counter, VICTOR2 1420 (Wallac, PerkinElmer, Courtaboeuf, France). For each final Cx concentration of the compound to be tested, the percentage of inhibition of the cellular proliferation (%Inh. (Cx)) is obtained by applying the following formula:

$$\%\,\text{Inh.(Cx)} = 100 \times \left[\frac{\text{Lum.(ech. } Cx \text{ compound)} - \text{Lum.(White)}}{\text{Lum.(ech. } Cx \text{ control)} - \text{Lum.(White)}} \right]$$

in which

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- Lum. (ech. Compose Cx) = average of luminescence measurements of three wells of the sample to be tested at a final Cx concentration.

- Lum. (ech. Control Cx) = average of luminescence measurements of three wells of the corresponding control sample

- Lum. (white) = average of luminescence measurements of eight wells comprising solely 200 µL of cellular culture medium (RPMI 1640 Cambrex + 10% FBS + 1% L-Glutamine + 40µg/ml of Gentamycine)

The CI_{50} is the concentration of the compound necessary to obtain inhibition of 50 % of the cellular proliferation; these values are calculated with the XLfit 3 software (ID Business Solutions Ltd., UK) from semi-logarithmic curves.

II. Procedure for determining CI_{50} on MDA-MB231 breast cancer cells

The compound to be tested is placed in solution in DMSO (100%) at a concentration 1.10^{-2} M (mother solution) then 8 samples of 8 different concentrations are prepared by successive dilution with the complete medium of cellular culture (E-MEM Cambrex + 10% FBS, + 1% of non-essential amino acids + 1% Napyruvate + 1% L-Glutamine + 40µg/ml of Gentamycine). For each sample, the dilution volume is adjusted so as to produce concentration equal to twice the final Cx concentration selected. By way of example, 1.10^{-4} M, 5.10^{-5} M, $2,5.10^{-5}$ M, $1,3.10^{-5}$ M, $6,3.10^{-6}$ M, $3,1.10^{-6}$ M, $1,6.10^{-6}$ M and $7,8.10^{-7}$ M is a standard range of 8 final concentrations. At the same time, 8 control samples are prepared by dilution of a solution of DMSO (100 %) with the cellular culture medium (E-MEM Cambrex + 10% FBS, + 1% of non-essential amino acids + 1% Napyruvate + 1% L-Glutamine + 40µg/ml of Gentamycine), the selected

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dilution volumes being strictly identical to those used for preparing samples of the compound to be tested.

In a white plaque of 96 wells, non transparent (fluoronunc 96F Nunclon Delta), 100 µL of a suspension of MDA-MB231 cells at a concentration of 1.25 10^4 cells/ml in a E-MEM Cambrex medium + 10% FBS, + 1% of non-essential amino acids + 1% Napyruvate + 1% L-Glutamine + 40µg/ml of Gentamycine are deposited in each well, or 2500 cells per well. 100 µL of a sample at a concentration twice Cx are added (one sample per well) and the medium is homogenised. After 144h of incubation at 37°C under an atmosphere (CO_2 5%, humidity 90 - 95%), quantification of the viability of the cells in culture is carried out by measuring the ATP levels using the luminescent analysis test CellTiter-Glo(TM) supplied by Promega Corporation, following the instructions of the supplier. The luminescence in each well is measured using a counter, VICTOR2 1420 (Wallac, PerkinElmer, Courtaboeuf, France).

For each final Cx concentration of the compound to be tested, the percentage of inhibition of the cellular proliferation (%Inh. (Cx)) is obtained by applying the following formula:

$$\%\,\text{Inh.(Cx)} = 100 \times \left[\frac{\text{Lum.(ech. Cx compound)} - \text{Lum.(White)}}{\text{Lum.(ech. Cx control)} - \text{Lum.(White)}} \right]$$

in which

- Lum. (ech. Compound Cx) = average of luminescence measurements of three wells of the sample to be tested at a final Cx concentration.
- average of luminescence measurements of three wells of the corresponding control sample
- Lum. (white) = average of luminescence measurements of eight wells comprising solely 200 µL of

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cellular culture medium (E-MEM Cambrex + 10% FBS + 1% L-Glutamine + 40µg/ml of Gentamycine)

The CI_{50} is the concentration of the compound necessary to produce inhibition of 50 % of the cellular proliferation; these values are calculated using XLfit 3 software (ID Business Solutions Ltd., UK) from semi-logarithmic curves.

III. Method for evaluating a synergistic effect of combinations of two products A and B on the inhibition of the proliferation of cells of leukaemic U937 type

Each product is placed in solution in DMSO (100%) at a concentration 1.10^{-2} M (mother solution) then, for each product, 6 samples of 6 different concentrations are prepared by successive dilution with the complete medium of cellular culture (RPMI 1640 Cambrex + 10% FBS + 1% L- Glutamine + 40 µg/ml of Gentamycine). For each sample, the dilution volume is adjusted to produce concentration equal to four times the final concentration selected.

A cellular suspension at $2,5.10^4$ cells/ml is prepared in complete medium, and 36 x 100 µl of this solution (or 2500 cells/well) are distributed in 36 wells of a plaque of 96 wells, white and non transparent (fluoronunc 96F Nunclon Delta). Added to each well containing 100 µl of cellular suspension are 50 µL of a sample of product A concentrated 4 times and 50 µL of a sample of product B concentrated 4 times to obtain a final volume of 200 µL.

Each sample of the product A (6 concentrations A1, A2, A3, A4, A5, A6) is combined with each sample of product B (6 concentrations B1, B2, B3, B4, B5, B6). The concentrations A6 and B6 correspond to a zero concentration of product A and product B respectively.

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The combination A6/B6 corresponds therefore to a well without drug or DMSO, serving as reference of growth cellular.

The content of the 36 well can be illustrated schematically as follows:

A1 / B6	A1 / B5	A1 / B4	A1 / B3	A1 / B2	A1 / B1
A2 / B6	A2 / B5	A2 / B4	A2 / B3	A2 / B2	A2 / B1
A3 / B6	A3 / B5	A3 / B4	A3 / B3	A3 / B2	A3 / B1
A4 / B6	A4 / B5	A4 / B4	A4 / B3	A4 / B2	A4 / B1
A5 /B6	A5 / B5	A5 / B4	A5 / B3	A5 / B2	A5 / B1
A6 / B6	A6 / B5	A6 / B4	A6 / B3	A6 / B2	A6 / B1

After 144h of incubation at 37°C under an atmosphere (CO_2 5%, humidity 90 - 95%), quantification of the viability of the cells in culture is done by measuring the ATP levels using the luminescent analysis test CellTiter-Glo(TM) supplied by Promega Corporation, by following the instructions of the supplier. The luminescence in each well is measured using a counter, VICTOR2 1420 (Wallac, PerkinElmer, Courtaboeuf, France).

For each combination An/Bm, the percentage of inhibition of cellular proliferation (%Inh. (An/Bm)) is obtained by applying the following formula:

$$\%\,\text{Inh.(An/Bm)} = 100 \times \left[1 - \frac{[\text{Lum.M(An/Bm)} - \text{Lum.M(White)}]}{[\text{Lum.M(A6/B6)} - \text{Lum.M(White)}]}\right]$$

in which

- Lum.M (An/Bm) = average of luminescence measurements of two wells comprising the combination of samples An and Bm
- Lum.M (A6/B6) = average of luminescence measurements of two wells comprising the combination of samples A6 and B6

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- Lum. (blanc) = age of luminescence measurements of 6 wells comprising solely 200 µL of cellular culture medium (RPMI 1640 Cambrex + 10% FBS + 1% L-Glutamine + 40 µg/ml of Gentamycine)

For each An/Bm combination the excedent relative to the most active agent only (Easlpa (An/Bm)) is calculated by applying the following formulas:

- If %Inh. (An/B6) > %Inh. (A6/Bm) thus E_{aslpa} (An/Bm) = %Inh. (An/Bm) - %Inh. (An/B6)
- If %Inh. (An/B6) < %Inh. (A6/Bm) thus E_{aslpa} (An/Bm) = %Inh. (An/Bm) - %Inh. (A6/Bm)

Finally, to differentiate the addition of the effects of synergy, the percentage of inhibition for each combination An/Bm is expressed as an excess relative to "Bliss" additivity. The " Bliss " model predicts a combined response C for two active agents having a given effect A and B according to the formula: C = A + B - AB with A and B expressed as a fraction between 0 and 1.

IV. Method for evaluating a synergistic effect of two products on the inhibition of the proliferation of MDA-MB231 breast cancer cells

The method utilised for MDA-MB231 breast cancer cells is similar to that used for U937 cells of leukaemic type, with the exception of the concentration of the cellular suspension ($1,25.10^4$ cells/ml for MDA-MB231 in place of $2,5.10^4$ cells/ml for U937). The composition of the complete medium is likewise modified: E-MEM Cambrex + 10% FBS, + 1% of non-essential amino acids + 1% Napyruvate + 1% L-Glutamine + 40 µg/ml of Gentamycine.

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V. Procedure to determine a CI_{50} on cellular lines originating from cancer of different type

The cells were placed in incubation in a final volume of 200 µl of a culture medium supplemented with 10 % of foetal bovine serum containing the compound tested at 37°C under atmosphere containing 5 % of CO_2.

The tumor cells were placed in incubation over 96 hours with 10 final concentrations of the tested compound (range of 1×10^{-4} to 4×10^{-10} M). Each final concentration was tested in quadriplicate. Control cells were treated with the vehicle of the tested compound.

At the end of the treatment period the antiproliferative activity was evaluated by a colorimetric test using tetrazolium salt MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3- carboxymethoxy phenyl)-2-(4 - sulfophenyl)-2H-tetrazolium, Baltrop J.A. et al, Bioorg. Med. Chem. Lett. 1991, 1:611-614).

On completion of the cell treatment 40 µl of a freshly filtered solution at 0.22 µm of MTS (20 ml at 2 mg/ml, Ref Gl 111 , Promega, Charbonnieres, France) and PMS (1 ml at 0.92 mg/ml, Ref P9625, Sigma) in a saline solution buffered with Dulbecco phosphate (DPBS, Ref 17-513F, Cambrex) were added to each well. The culture plaques were incubated over 2 hours at 37°C. The absorbency each well was measured at 490 nm using a multimark counter, VICTOR3(TM) 1420 (Wallac, PerkinElmer, Courtaboeuf, France). The absorbency values correspond to the average of 4 experimental measurements.

For each finale Cx concentration of the compound to be tested, the percentage of inhibition of the cellular proliferation (%Inh. (Cx)) is obtained by applying the following formula:

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$$\%\,Inh.(Cx) = 100\,x\left[1 - \frac{[Abs\,Compound\,(Cx)]}{[Abs.\,Control]}\right]$$

in which

- Abs. Compound (Cx) = average of absorbency measurements of the four wells of the sample to be tested at a final Cx concentration.

- Abs. Control = average of absorbency measurements of the four control wells (absence of compound)

The CI_{50} is the concentration of the compound necessary to produce inhibition of 50 % of the cellular proliferation; these values are calculated using XLfit 3 software (ID Business Solutions Ltd., UK) from semi-logarithmic curves.

VI. Procedure for determining the in-vivo antitumoral effect of products tested

Tumoral induction

Viable U937 cells in a volume of 200 µl of RPMI culture medium are injected subcutaneously into the right flank of a SCID mouse. The injection of the cells is done 24 hours after complete irradiation of the body with a γ source (1.8 Gy, Co^{60}, INRA BRETENIERE, Dijon, France).

Treatment plan

Treatment is initiated when the tumors have reached an average volume of around 50 mm^3. The mice are distributed randomly in groups of 10 mice such that the average of the tumoral volume of each group is not different statistically to the other groups variance (analysis).

For each dose of test compound, an appropriate quantity of product is placed in suspension in Tween-20 (Ref P7949, Sigma). The suspensions are then diluted in

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water to reach the desired concentration of the test compound and a final concentration of Tween-20 of 1% (v/v).

The mice receive repeated intraperitoneal injections (IP) of the vehicle (Tween-20 1% (v/v) or of the test compound at a dose and in keeping with the selected treatment plan (once per day or twice per day over 30 days). For oral treatments the products and the vehicle are administered by booster pump.

Determination of the tumoral volume

The length and width of the tumor are measured twice per week using a callipers and the volume of the tumor is estimated by the formula: tumoral volume = ($width^2$ x length)/2.

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Amended Claims

1. Use of 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalene-1-ylamine, or any combination of its enantiomers or one of its enantiomers as active ingredients only, or in association with at least one alkaloid, for preparing a pharmaceutical composition for treating cancer, as well as treating and preventing metastases.

2. Use as claimed in Claim 1, characterised in that the alkaloid is a vinca alkaloid.

3. Use as claimed in Claim 2, characterised in that the vinca alkaloid is selected in the group constituted by : vinblastine, vincristine, vindesine, vinorelbine, or their mixtures, preferably vincristine and/or vinblastine.

4. Utilisation as claimed in any one of Claims 1 to 3, characterised in that said cancer or said metastases originates from cancer selected in the group constituted by prostate cancer, osteosarcomas, lung cancer, lung cancer not in small cells, breast cancer, cancer of the endometrium or colon cancer, chronic or acute leukaemia, solid tumors in children, lymphocytary lymphomas, cancer of the pancreas, skin cancer, cutaneous or intraocular melanoma, cancer of the head and neck, cancer of the uterus, ovarian cancer, gynaecological tumors, Hodgkin's disease, bone cancer, rectal cancer, cancer of the anal region, stomach cancer, cancer of the oesophagus, cancer of the small intestine, cancer of the endocrine system, soft tissue sarcomas, urethra cancer, cancer of the penis, cancer of the bladder, kidney cancer, cancer of the ureter, malignant paediatric tumors, and tumors of the central nervous system.

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5. Use as claimed in Claim 4, characterised in that said cancer is breast cancer, or said metastases originate from breast cancer.

6. Use as claimed in Claim 4, characterised in that said cancer is leukaemia.

7. Utilisation as claimed in any one of Claims 1 to 6, characterised in that the pharmaceutical composition is administered orally, intravenously, enterally, parenterally, intramuscularly, intranasally, sub-cutaneously or sublingually.

8. A product comprising 4-(3,4-dichloro-phenyl)-1,2,3,4-tetrahydro-naphthalene-1-ylamine or at least one of its enantiomers or any combination of its enantiomers and at least an alkaloid, as a combination product for simultaneous, separate or staggered use over time in anti-cancer therapy.

9. The product as claimed in Claim 8, characterised in that the alkaloid is a vinca alkaloid.

10. The product as claimed in Claim 9, characterised in that the vinca alkaloid is selected in the group constituted by vinblastine, vincristine, vindesine, vinorelbine, or their mixtures, preferably vincristine and/or vinblastine.