Platelet Aggregation Inhibiting and Anticoagulant Effects of Oligoamines, XVI: Cytostatic and Cytotoxic Side Effects of Oligoamines [+)]

Klaus Rehse[a)*], Birgit Noack, Rainer Maurer, and Peter Hilgard[b)]

[a)] Institut für Pharmazie der Freien Universität Berlin, Königin-Luise-Str. 2+4, D-1000 Berlin 33
[b)] ASTA Pharma AG, Bielefeld

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Selected synthetic oligoamines were able to inhibit (IC_{50}) the growth of leukemic L 1210 cells in concentrations between 4-10 µmol/L. The essential structural features were at least two basic nitrogen functions in suitable distance, substituted with arylalkyl or alkyl groups. The favorable chain length is about eight carbon atoms. The cytostatic effect is complete after 30 min and cannot be washed out with buffer. Viability measurements showed that the leukemic cells were killed in a time dependent manner. As no influence on the cell nucleus could be observed this is most probably due to interaction with the cell membrane. When high local concentrations are applied *in vivo*, the oligoamines are toxic because of cytolytic properties. This toxicity can be overcome by administration of suitable prodrugs (LD_{50} > 1000 mg/kg).

Antiaggregatorische und anticoagulante Eigenschaften von Oligoaminen, 16. Mitt.: Cytostatische und cytotoxische Nebenwirkungen von Oligoaminen

Geeignet sturkturierte Oligoamine hemmen in Kultur das Wachstum von Leukämiezellen des Typs L 1210 in Konzentrationen zwischen 4-10 µmol/L halbmaximal. Die essentiellen Strukturmerkmale sind mindestens zwei basische Stickstoffunktionen in geeignetem Abstand voneinander, die mit Arylalkyl- oder Alkylresten substituiert sind. Eine Kettenlänge von etwa acht Kohlenstoffatomen ist optimal. Der cytostatische Effekt ist nach bereits 30 min vollständig und kann nicht ausgewaschen werden. Viabilitätsuntersuchungen zeigten, daß Oligoamine die Leukämiezellen in zeitabhängiger Weise abtöteten. Da kein Einfluß auf den Zellkern beobachtet wurde, ist dies höchstwahrscheinlich auf Membraneffekte zurückzuführen. Bei hohen lokalen Konzentrationen *in vivo* sind die untersuchten Oligoamine aufgrund cytolytischer Eigenschaften toxisch. Werden diese durch Verabreichung in Form von Prodrugs vermieden, verschwindet die Toxizität (LD_{50} > 1000 mg/kg).

In a series of fifteen papers we have elucidated the structure activity relationships concerning the antiplatelet, anticoagulant and antithrombotic properties of oligoamines. The results obtained strongly suggest that these effects are mediated by interaction of the oligoamines with phospholipids especially those of cell membranes. We therefore have investigated the influence of selected oligoamines on the growth of isolated cells in culture. The formula of the compounds tested are summarized in fig. 1. Criteria for the selection of these compounds were their antiplatelet activity, as much variation in structure as possible and their solubility in water. The latter property was most important for the *in vitro* tests.

The influence of oligoamines on the cell growth *in vitro* was investigated using the microclonogenic assay described by *Maurer* and *Ali-Osman*[1)]. The results obtained for the leukemic cell line L 1210 are summed up in the second column of table 1.

Table 1: Antiplatelet, cytostatic and toxic effects of thirteen oligoamines (n.t. = not tested; n.tb. = not testable)

Compound	Platelet IC_{50} [µmol/L]	L 1210 IC_{50} [µmol/L]	T-Lymphocytes IC_{50} [µmol/L]	L 1210 washed after 30′ IC_{50} [µmol/L]	180′ IC_{50} [µmol/L]	Toxicity LD_{50} mice mg/kg i.p.	mg/kg p.o.
RE 1369	20	n.t.	n.t.	n.t.	n.t.	46.4	n.t.
RE 1410	50	60	30	n.t.	n.t.	n.t.	n.t.
RE 1475	5	5	n.t.	n.t.	n.t.	n.t.	n.t.
RE 1492	4	4	3	3	2	21.5	n.t.
RE 1537	5	4	n.t.	n.t.	n.t.	46.4	n.t.
RE 1583	36	40	n.t.	n.t.	n.t.	n.t.	n.t.
RE 1618	4	4	n.t.	n.t.	n.t.	n.t.	n.t.
RE 1629	4	4	3	n.t.	n.t.	n.t.	n.t.
RE 1663	30	30	20	n.t.	n.t.	n.t.	n.t.
RE 1677	4	4	n.t.	8	2	n.t.	n.t.
RE 1762	8	n.t.	n.t.	n.t.	n.t.	11	>31.6
RE 1770	8	8	n.t.	n.t.	n.t.	n.t.	n.t.
RE 1492 C	n.tb.	n.tb.	n.tb.	n.tb.	n.tb.	>1000	>1000

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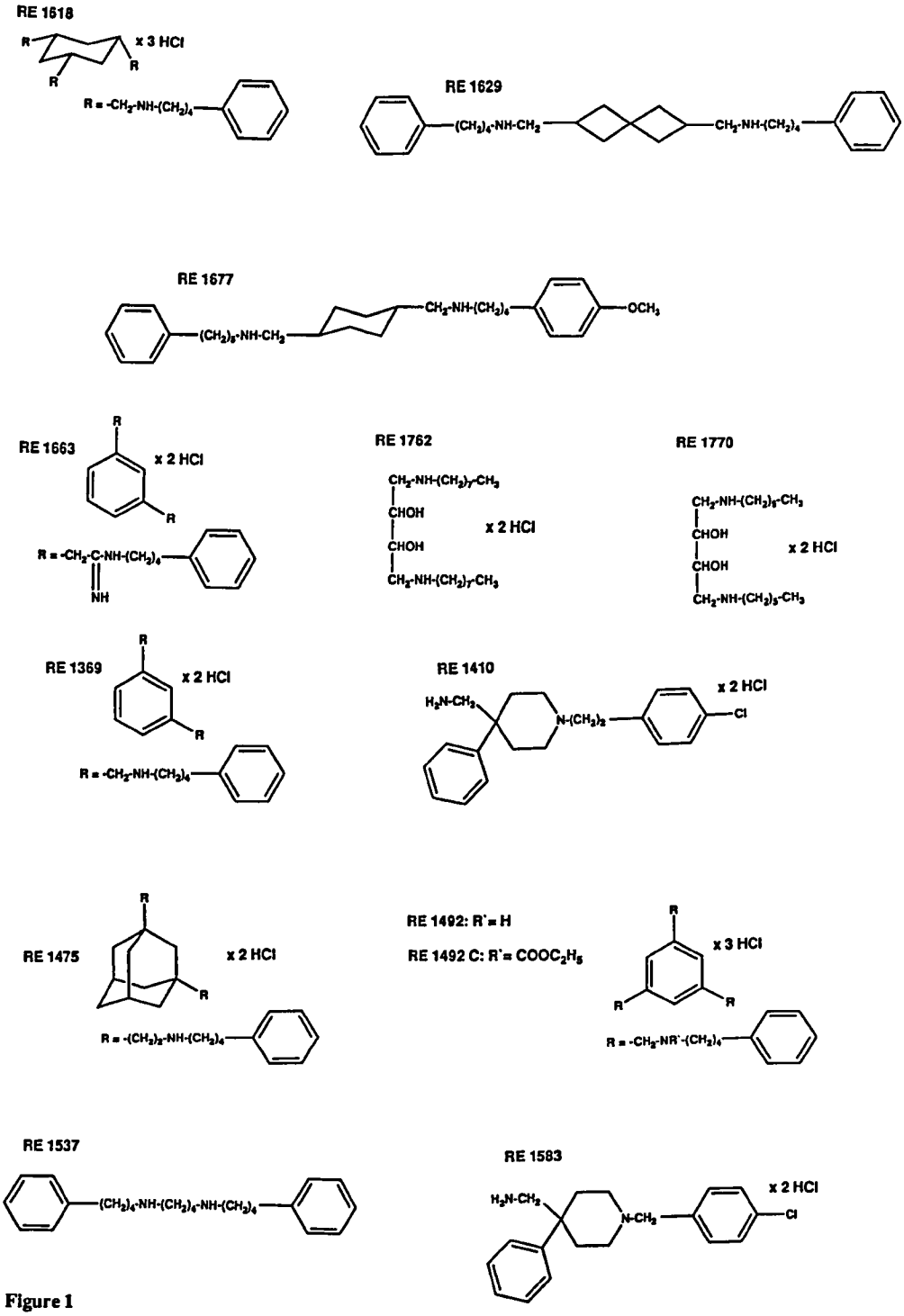

Figure 1

All oligoamines tested were able to reduce the growth of leukemic cells by 50% in concentrations between 4-60 µmol/L. Seven of them exerted this effect in concentrations below 10 µmol/L and are therefore interesting concerning therapeutic purposes. Among the most active compounds are diamines (RE 1475, RE 1537, RE 1629, RE 1677, RE 1770) as well as triamines (RE 1492, RE 1618) of different structures. The only common feature is rather a high lipophilicity and the presence of several basic nitrogen functions. Comparison of the second column with the first column of table 1 suggests a strong correlation between the antiplatelet activity and the inhibition of the cell growth. However the conclusion that this points out a common mechanism of action must be regarded with care as the antiplatelet activity is tested in human (platelet rich) plasma while the cell growth is investigated in an artificial medium containing much less proteins.

To characterize the specificity of growth inhibition a second cell type namely T-lymphocytes was treated with two potent (RE 1429, RE 1629) and two less potent (RE 1410,

RE 1663) oligoamines. The observed inhibition (see 3rd column) runs parallel with the influence on L 1210 cells or platelets indicating a lack of specificity and supporting the membrane hypothesis formulated above. The binding of the oligoamines to the (L 1210) cells appears to be strong as the effect cannot be washed out neither after 180 min nor even after 30 min of incubation (see table 1, column 4 for RE 1492 and RE 1677).

In order to differentiate cytostatic from cytotoxic effects the viability of L 1210 cells after incubation with RE 1492 was checked by the trypan blue method[2]. The results are summarized in table 2.

In concentrations above 1 µmol/L RE 1492 develops cytotoxic properties. This effect is time dependent in the range between 2-8 µmol/L. This suggests that at first cytostatic properties are exhibited which, however, become cytotoxic with prolonged incubation periods. After 48 hours all cells are either dead or alive only depending from the concentration of the drug.

To differentiate membrane effects from actions on the cell nucleus the sister chromatide exchange was measured using the method of Obe and Beek[3]. To investigate the possible influence of metabolites an assay with S9 mix is run in parallel. The results are compiled in table 3. Comparison of RE 1492 and RE 1677 with compounds which are mutagenic themselves (Trenimon®) or after metabolism (cyclophosphamide) gives no hint for mutagenic influences of the oligoamines on the cell nucleus.

At first glance it seems contradictory to investigate the influence of oligoamines on cell growth and mutagenic properties at concentrations where the viability measurements suggest that the cells are already dead.

However, one has to keep in mind that different test systems are used and the overall concentrations applied to each system tell nothing about the drug concentrations really existing at the site of action as there is no information on the distribution of the drug in the test system. The same is particularly true for the situation *in vivo*. When the drug is administered by intraperitoneal injection high local concentrations are achieved. As we could show that RE 1492 in concentrations above 100 µmol/L has cytolytic properties[4] it is not surprising that strong toxic effects are observed when the *i.p.* injection mode is used. This difficulty cannot be overcome by *p.o.* administration as oligoamines are poorly ab-

Table 2: Viability [%] of L 1210 cells after incubation with RE 1492 after 3-96 h (n = 4; x ± σ)

[µmol/L]	3 h	6 h	24 h	48 h	72 h	96 h
control	98.6±1.3	96.8±2.4	97.6±1.7	98.3±0.8	91.9±3,0	49.9±1.7
100	0	0	0	0	0	0
10	0	0	0	0	0	0
8	5.6±3.6	2.1±2.2	2.3±2.1	0	0	0
6	10.3±4.7	3.6±2.5	2.0±2.4	0	0	0
4	14.9±3.8	7.6±2.8	5.3±3.5	0	0	0
2	94.8±3.4	86.2±7.5	6.4±6.0	0	0	0
1	94.9±1.6	93.2±2.4	96.2±1.1	97.3±1.3	92.7±1.2	46.1±1.1
0.1	92.6±2.4	91.5±1.0	98.8±0.8	97.9±1.0	89.9±1.5	46.7±0.6
0.01	93.2±9.2	91.1±0.9	96.3±0.8	96.3±1.0	94.3±1.5	44.0±1.5

Table 3: Occurrence of SCE after incubation of human T-lymphocytes with RE 1492, RE 1677, Trenimon® or cyclophosphamide

Compound	[µmol/L]	S9-mix added	SCE rate
control	-	-	4.48 ± 0.45
	-	+	3.08 ± 0.36
RE 1492	10	-	7.60 ± 0.72
	10	+	5.23 ± 0.45
	1	-	4.20 ± 0.49
	1	+	3.52 ± 0.32
RE 1677	10	-	4.96 ± 0.54
	10	+	5.60 ± 0.47
	1	-	6.00 ± 0.48
	1	+	3.75 ± 0.35
Trenimon®	1	-	39.32 ± 2.38
	0.1	-	15.36 ± 1.53
cyclophosphamide	10	+	53.64 ± 2.50
	1	+	14.40 ± 0.79
	0.1	+	5.60 ± 0.32

Table 4: Effect of several oligoamines on the growth of different tumors (DMBA = dimethylbenzoanthracene)

Compound	tumor model	dose [mg/kg]	route of adm.	frequency of adm.	effect
RE 1369	P 388	14.7	i.p.	1	none
		4.64	i.p.	4	none
RE 1492	P 388	6.81	i.p.	1	still toxic
		2.15	i.p.	4	none
RE 1537	P 388	14.7	i.p.	1	none
		4.64	i.p.	4	none
RE 1762	Lewis lung	2.15	i.v.	3	none
RE 1492 C	DMBA	100	p.o.	4	none

sorbed from the gastrointestinal tract[5]. The infusion of the drug certainly would improve the situation. The most elegant way, however, to perform a smooth application was the design of a suitable prodrug. The tricarbamate derivative of RE 1492 ≡ RE 1492 C is readily absorbed from the gastrointestinal tract, then cleaved enzymatically and exerting strong antithrombotic effects in a 30 mg/kg oral dose[5]. Column 5 in table 1 shows that the toxicity can be abolished by this trick. Table 4 however shows that even a 100 mg/kg dose of RE 1492 C cannot inhibit the growth of a DMBA induced tumor. This suggests an odd distribution of RE 1492 which probably is located mainly in the blood vessel system, when the prodrug is given.

These findings may explain why the cytotoxic effect seen *in vitro* cannot be used for the inhibition of tumor growth *in vivo* when the doses documented in table 4 are administered.

Experimental Part

The syntheses of all compounds tested except for RE 1663 have been described in former papers of this series: RE 1369[6], 1410[7], 1475[8], 1492[9], 1492C[5], 1537[10], 1583[7], 1618[8], 1629[8], 1677[8], 1762[11], 1770[11].

N^{l},$N^{l'}$-Bis-4-phenylbutyl-benzene-1,3-diacetamidine (RE 1633[+])

40 mmol dry, powdered $AlCl_3$ is given quickly under vigorous stirring to a mixture of 3.0 g (20 mmol) 1,3-benzene-diacetonitrile and 5.7 g (40 mmol) 4-phenylbutanamine. The temp. rises to 200°C and is kept at 165-175°C for 30 min. The mixture is cooled to 130°C, poured with caution into 100 ml H_2O and adjusted to pH 11 by NaOH. After extraction with $CHCl_3$, drying and removal of the solvent *i. vac.* the residue is recrystallized from ethanol/water.- Crystals, mp. 121°, yield 50%.- $C_{30}H_{38}N_4 \cdot 0.5\ H_2O$ (463.7) Calc. C 77.8 H 8.42 N 12.1 Found C 77.9 H 8.48 N 11.9.- IR (KBr): 3205; 3040; 3020; 2920; 2850; 1610; 1570; 1490; 1470; 1450; 1420; 1390; 1360; 1340; 1230; 1190; 1160; 1090; 1060; 1030; 920; 820; 790; 740; 700 cm^{-1}.- ^{1}H-NMR (250 MHz/$CDCl_3$): δ (ppm) = 7.36-7.10 (m, 14H, aromat.), 4.8-4.3 (bs, 4H, NH, exchange with D_2O), 3.47 (s, 4H, Ar-CH_2-C=N), 3.17 (t, J = 7 Hz, N-CH_2), 2.61 (t, J = 7 Hz, 4H, Ph-CH_2), 1.68-1.48 (m, 8H, N-CH_2-CH_2-CH_2).- MS (70 eV, 150°C): m/z = 454 (7%, M^+), 306 (10), 280 (20), 214 (44), 201 (19), 187 (19), 130 (23), 105 (10), 104 (22), 103 (11), 91 (100), 86 (11), 78 (19), 77 (17), 65 (15), 45 (31).

1. Colony assays
1.1 Liquid media

RPMI 1640 (*R*oswell *P*ark *M*emorial *I*nstitute)

10.0 g RPMI 1640 dry medium with L-glutamine and without $NaHCO_3$; 2.0 g $NaHCO_3$; 10^5 I.U. penicillin G, Na-salt; 1.0 g streptomycin sulfate; aqua bidest. ad 1000 ml.

HBSS (*H*ank's *B*alanced *S*alt *S*olution)

201 mg $MgSO_4 \cdot 7\ H_2O$; 60 mg KH_2PO_4; 47 mg NaH_2PO_4, 186 mg $CaCl_2 \cdot H_2O$; 1000 mg dextrose; 400 mg KCl; 8000 mg NaCl; 350 mg $NaHCO_3$; aqua bidest. ad 1000 ml.

DMEM (*D*ulbeccos *M*odification of *E*agles *M*edium)

2.4 g DMEM dry powder with 1 g/ml dextrose and glutamine; 0.952 g $NaHCO_3$; 25000 I.U. penicillin; 0.025 streptomycine; aqua bidest. ad 250 ml.

PBS (*P*hosphate *B*uffered *S*aline)

3.5 g Na_2HPO_4; 3.4 g KH_2PO_4 ; 9.0 g NaCl; aqua bidest. ad 1000 ml. All media were sterilized by filtration (Sartorius membrane filters 0.22 µm)

1.2 Microcloning of L 1210 cells in Agar-containing glass capillaries

The test assay was prepared in 1.5 ml sterile Eppendorf tubes. Each tube contained 41 µl RPMI 1640, 100 µl test compound in RPMI 1640, 45 µl horse serum and 60 µl of suspension of 12000 L 1210 cells/ml RPMI 1640. This mixture was kept at 37°C. Then 54 µl of a mixture of one part agar solution (3%) with two parts RPMI 1640 was added. It was thoroughly mixed and in portions of 30 µl transferred to sterile glass capillaries (Boehringer - Mannheim 126 x 1,38 mm). Now gelation was performed on a cold plate and the capillaries put into a special holder[12] and incubated for 7 d at 37°C in an atmosphere of 7.5% CO_2. The relative humidity was ≥ 100%. For evaluation the colonies were counted by means of a stereo microscope (Nissho Seimitsu, Kogaku, Japan) in indirect light. A colony was assumed when an aggregate of at least fifty cells was seen. In controls 50-60 colonies in each capillary were observed. The mean colony number of six capilaries was determined. The error was calculated as the standard error of the means (SEM = σ^2/n). The IC_{50} was determined by the usual graphic procedure.

1.3 Cloning of T-lymphocytes[13]

20 ml venous blood of healthy donors were drawn by venipuncture under sterile conditions. For anticoagulation 500 I.U. heparin (Liquemin®, Hoffmann-La Roche) were added. The blood was diluted with 10 ml

[+] This compound was kindly supplied by *G. Claus*, Institut für Pharmazie, FU Berlin

HBSS. 8 ml of this mixture were placed on 6 ml Ficoll-Paque® (Pharmacia GmbH, Freiburg) and centrifuged at 400 x g (Labofuge II, Heraeus-Christ, Osterode; room temp.) for 40 min. After removal of the plasma-HBSS layer the lymphocytes were collected, washed twice with 10 ml DMEM and finally resuspended in 2 ml DMEM.

For prestimulation the cell concentration was adjusted to $1.25 \cdot 10^6 \cdot ml^{-1}$. Now 375 µl/ml autologous plasma, 12,5 µl/ml phytohemagglutinin M (Difco Laboratories, Detroit) and 10^7/ml red blood cells were added.

After incubation for 24 h at 37°C (see above) the prestimulated lymphocytes were washed twice with DMEM, resuspended and adjusted to $1.2 \cdot 10^6$ cells/ml with DMEM. 37 µl of this suspension were mixed with 2 µl phytohemagglutinin M solution, 63 µl autologous plasma, 23 µl DMEM, 100 µl test compound in saline and 75 µl DMEM agar medium (see above). The mixture was then transferred to the glass capillaries and handeled as described above. In control capillaries 30-40 colonies were counted. Evaluation of the results was performed as described for L 1210 cells.

1.4 Wash out assay with L 1210 cells

A 100 µl solution of the test compound in RPMI 1640, additional 140 µl RPMI medium and 60 µl cell suspension (10^6 cells/ml) were incubated for 30 min or 180 min at 37°C in black cap tubes. The cells were centrifuged, washed twice with 10 ml RPMI 1640 and adjusted to 12000 cells/ml. The cloning of the cells was performed as described above.

2. SCE (sister chromatide exchange) assay

Leukocytes were cultivated in a mixture of 4.0 ml RPMI 1640, 0.5 ml fetal calf serum, 0.12 ml phytohemagglutinin, 0.02 dihydrostreptomycin sulfate (1 ml = 50 mg) and penicillin (1 ml = 5000 I.U.) and 0.4 µl heparinized venous blood. After incubation in sterile one way centrifuge tubes for 24 h at 37°C 50 µl bromodesoxyuridine (BrdU 10^{-3} M) and 50 µl of a solution of the test compound were added. To avoid photolysis of BrdU-substituted DNA light was excluded. After incubation for 67.5 h at 37°C a solution of 0.2 ml colcemide was added for (concentration in culture: 0.08 µg/ml) accumulation of metaphases. After another incubation period of 4.5 h the vials were centrifuged at 900 rpm for 10 min. The top layer was removed. The sediment was resuspended at 37°C in 4 ml 0.074 M KCl solution and incubated for 12 min. After centrifugation and removal of the top layer the sediment was suspended in freshly prepared fixative solvent (CH_3OH/CH_3COOH 3:1) and centrifuged. This washing was repeated twice. At last the sediment was suspended in 500 µl of the fixative and dried on a infrared microscopic slide. Staining was performed by a modified fluorescence plus giemsa method[14]. The slides were dipped for 15 min into a solution of Hoechst 33258 (5 µg/ml). The excess of dye was removed with aqua dest. The slides were wetted with PBS buffer and irradiated with ultraviolett light (260 nm). Then the slides were shortly rinsed with water and dipped for 7 min into giemsa solution. They were washed twice with aqua dest. and dried at room temperature (1-2 h). For evaluation a Leitz microscope (100 x 10, oil immersion) was used.

The S9 mix was a gift from Dr. *M. Königstein*, prepared from livers of male Wistar rats treated with aroclor (500 mg/kg).

3. Animal experiments

3.1 Toxicity studies

The approximate LD_{50} in NMRI mice was calculated from the lethality following the intraperitoneal (i.p.) or oral (p.o.) administration of three logarithmically spaced escalating doses of the test compounds.

3.2 Tumor experiments

3.2.1. The P 388 lymphocytic leukemia was maintained in CD2F1 mice, and 10^5 cells were transplanted i.p. into test animals.

Treatment started the day after tumor transplantation and was given either once or on four consecutive days. The survival time of the animals was used as parameter for antitumor activity and compared to that of non-treated controls.

3.2.2. Suspensions of the Lewis lung carcinoma containing 5 x 10^5 viable tumor cells were injected intravenously into C 57 black mice. Treatment was initiated the following day for 3 consecutive days. The number of lung colonies ("metastases") was evaluated on day 18 after tumor application.

3.2.3. An autochthonous mammary carcinoma was induced by a single oral administration of dimethylbenzanthracene (DMBA). Treatment was given twice a week for two weeks, when the total tumor mass per animal had reached one gram. Tumor measurements were carried out according to the method previously described[15].

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