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Effect of a new de-*N*-acetyl-lysoglycosphingolipid on some tumour models

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Abstract

A new de-*N*-acetylated glycosphingolipid termed WILD20, a breakdown product of GM_1 obtained through alkaline hydrolysis, and characterized by nuclear magnetic resonance, mass spectrometry and elementary analysis, was found to inhibit phospholipase A_2 via phosphokinase C translocation blockade. The substance inhibited various tumour cell lines in vitro, in synergy with doxorubicin and cisplatin. In vivo, it showed an antitumoral effect when both the tumour cells and WILD20 were injected at the same site (peritoneal cavity). Tumour cells, incubated with WILD20, showed a dose-dependent decrease of oncogenicity without impairment of viability. WILD20 also down-regulated tumour cell adherence to laminin and fibronectin. When peritumorally administered, WILD20 impaired tumour growth and potentiated the peritumoral effects of recombinant interleukin 2. The results obtained merit exploration of the therapeutical possibilites of this agent in human cancer patients.

Keywords: Glycosphingolipid; Tumor; Recombinant interleukin-2; Adhesion; Peritumoral

1. Introduction

Glycosphingolipids are present on tumour cell surfaces and are involved in metastatic spreading by changing cell binding to various substrates. They act as antigen in some tumour cell membranes and are recognized as modulators of cell proliferation and adhesion (Inokuchi et al., 1990).

In the course of our laboratory studies carried out on glycosphingolipid breakdown products obtained through alkaline hydrolysis, one molecule in particular was isolated and characterized (Cavallo et al., 1993), defined through nuclear magnetic resonance (NMR) and mass spectrometry (MS) and elementary analysis, and found to possess the following structure:

de-*N*-acetyllysoGM$_1$, 113Neu114GalNGgOse3Sph;

$Gal\beta_1 \rightarrow 3GalN\beta_1 \rightarrow 4Gal[3 \leftarrow 2\alpha Neu]\beta_1 \rightarrow 4Glc\beta_1 1 \rightarrow Sph$

$C_{53}H_{97}N_3O_{28}$ MW = 1223

This substance (Fig. 1) is a lysoganglioside as it has lost a fatty acid chain, but it is also a de-*N*-acetylated ganglioside as it has lost two acetyl groups. It therefore shares the characteristics of both molecular families (Cavallo et al., 1993), and these profound structural

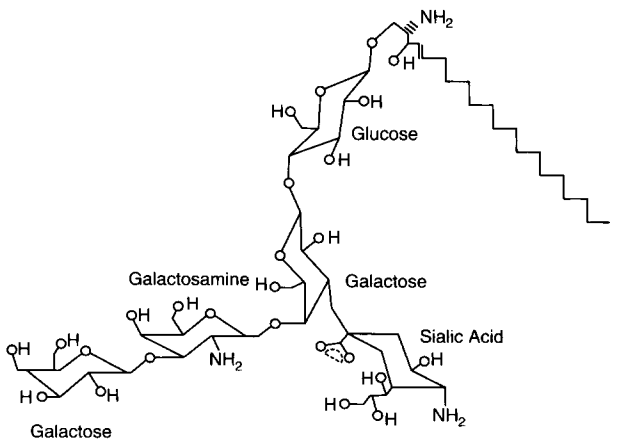

Fig. 1. Structure of the de-*N*-acetylated glycosphingolipid, WILD20.

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changes of the ganglioside GM_1 cause WILD20's water solubility, poor micelle formation and oral absorption (Tubaro et al., 1993a). WILD20 has immunodepressant activity by inhibiting cell proliferation, neutrophil chemotaxis, neutrophil oxidative processes and neutrophil adhesion to endothelial cells (Tubaro et al., 1994). It shows antiinflammatory activity, inhibition of leukocyte chemotaxis, and antiplatelet effects through blockade of the arachidonic acid cascade (Tubaro et al., 1993a).

This study was aimed at evaluation of whether WILD20 could influence tumour cell growth control in in vitro and in vivo models.

2. Materials and methods

2.1. Animals

Male CD1, CDF1 and C57BL6 mice were supplied by Ch. River, Calco, Como, Italy. They received standard laboratory chow and water ad libitum.

2.2. Tumour cell lines

Histiocytic leukemia P388, lymphoid leukemia L1210, lymphoid leukemia L5178Y, 3LLC Lewis lung carcinoma, sarcoma 180, HL60 and melanoma B16 from NCI Frederick Cancer Research, MD, USA and human bladder transitional cell carcinoma RT112, EJ or MGH-U1 (Evans et al., 1977; Marshal et al., 1977) were used. All tumour cells were received frozen and inoculated in vivo not less than 2–3 times before use.

2.3. Tumour cell proliferation

Cell culture medium was RPMI 1640 with 25 mM Hepes, L-glutamine (Gibco), supplemented with 10% (v/v) heat-inactivated foetal calf serum (Gibco) and antibiotics. Tumour cells were cultured in 0.2 ml cell culture medium in flat-bottom microtiter plates (Nunc) containing 5×10^4 tumour cells/well.

WILD20 (Wellcome Italia Research Labs.), BW755C (a dual inhibitor of cyclooxygenase and 5-lipoxygenase, Baran et al., 1994), BWA4C (a 5-lipoxygenase inhibitor, Hussey and Tisdale, 1994) (The Wellcome Research Labs., Beckenham, UK) and nordihydroguaiaretic acid (NDGA), a 5-lipoxygenase inhibitor, Bradley and Goetz, 1994) (Sigma), were solubilized in RPMI 1640 and added to the wells in a volume of 0.01 ml. 5-Lipoxygenase inhibitors were used as reference compounds due to their well-known activity in tumorigenesis (Kitagawa and Noguchi, 1994; Jiang et al., 1994) and metastatic spreading (Liu et al., 1994a,b; Tang et al., 1994). Cultures were maintained at 37°C in 5% CO_2 for 48 h. During the last 6 h, each well was pulsed with 1 μCi of [^{3}H]thymidine (2 Ci/mmol = 74 GBq/mmol; Amersham).

Cells were harvested onto filter paper using a semiautomatic Skatron 7031 cell harvester. The filters were dried and the incorporated radioactivity was determined by counting in 3 ml of Lipoluma scintillation fluid in a scintillation counter (LKB 1410 system).

Results were expressed as mean counts/min of six replicates for each experiment.

2.4. Tumour cell adhesion

Adhesive proteins and cell lines

Mouse laminin from murine Engelbreth-Holm-Swarm sarcoma, and human plasma fibronectin (Gibco) were used. These preparations were > 95% pure as checked by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The B16 melanoma cell line and human bladder transitional cell carcinoma lines RT112 and EJ, from different histological grade tumours (G2 and G3, respectively), were grown as monolayers in RPMI 1640 medium supplemented with 10% foetal calf serum, 2 mM glutamine, and 50 U/ml penicillin-50 μg/ml streptomycin (Flow Labs). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Single-cell suspensions for cell counting and cell seeding were prepared by culture trypsinization with 0.05% trypsin solution in 0.02% EDTA (Flow Labs.). Cell viability was checked by trypan blue exclusion test.

Binding assays

Both human plasma fibronectin and mouse laminin were diluted in phosphate-buffered saline (PBS), pH 7.4, to a final concentration of 10 and 20 μg/ml and 100 μl of this protein solution was pipetted into 96-well tissue culture flat-bottom plates (Costar). After overnight incubation at 4°C, the coated wells were washed 3 times with PBS to remove non-immobilized protein. To saturate the remaining protein binding sites, 200 μl of PBS supplemented with 1% heat-denatured bovine serum albumin was added and the plates were incubated at room temperature for 1 h. B16 melanoma, RT112 and EJ transitional carcinoma cells were labelled with ^{51}Cr (100 μCi/1 $\times 10^6$ cells) for 1 h at 37°C, washed twice, resuspended with RPMI 1640 and pipetted into polypropylene round-bottom test-tubes (1×10^6 cells/tube) (Falcon 2063).

WILD20 diluted in RPMI 1640 was added to a final concentration of 0.1–100 μg/ml; RPMI 1640 was added to control cell suspensions.

Tumour cells were then incubated at 37°C for 1 h with WILD20. Thereafter, they were washed twice and resuspended at 5×10^5 cells/ml in RPMI 1640 plus 1% bovine serum albumin (BSA). 5×10^4 tumour cells were allowed to adhere to the protein-coated surface

and incubated at 37°C. Incubation time was 10 min for B16 melanoma cells and 1 h for RT112 and EJ bladder cancer cells. After incubation, unattached cells were removed by washing with PBS and adherent cells were solubilized with 2% sodium dodecyl sulphate (SDS) (Sigma). ^{51}Cr released from these tumour cells was quantitated in a gamma counter. The percentage of cell adhesion was calculated as follows: % adhesion = {(cpm adherent cells)/(cpm adherent cells + cpm non-adherent cells)} × 100.

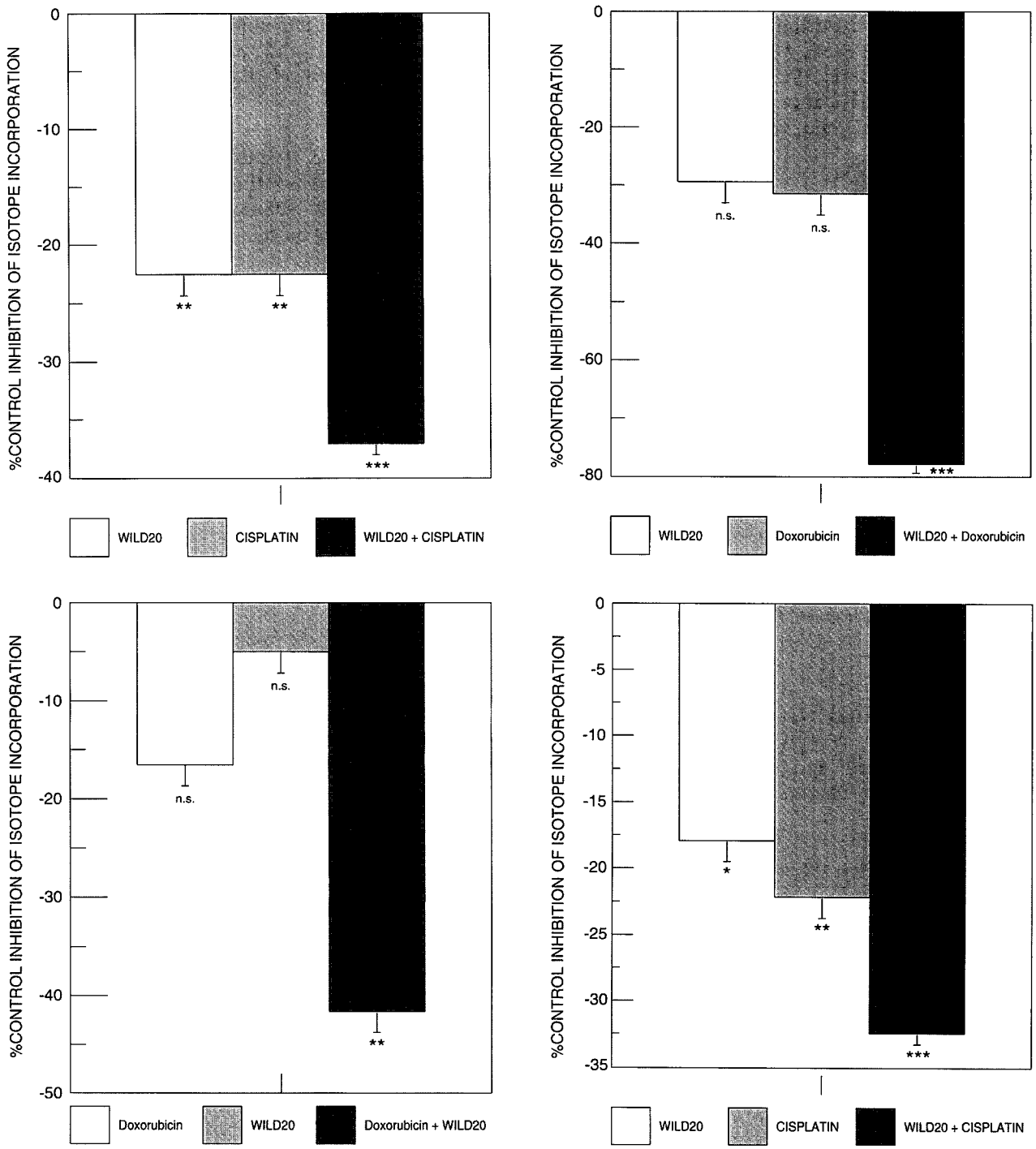

Fig. 2. Cystostatic agent-induced inhibition of tumour cell DNA synthesis; effect of WILD20. *Doxorubicin:* (a) Sarcoma 180: 5×10^4 cells/well. WILD20: 10 μM. Doxorubicin: 0.025 μg/ml. (b) HL60: 5×10^4 cells/well. WILD20: 80 μM. Doxorubicin: 0.025 μg/ml. *Cisplatin:* (c) Sarcoma 180: 5×10^4 cells/well. WILD20: 10 μM. Cisplatin: 0.2 μM. (d) HL60: 5×10^4 cells/well. WILD20: 40 μM. Cisplatin: 0.1 μM. Cells were incubated with drugs for 41 h. [^{3}H]Thymidine 1 μCi; incubation time: 6 h. Statistical evaluation: n.s. = not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

2.5. Systemic antitumoral effect with pre-incubated cells

This effect was studied in mice with both Lewis lung carcinoma and lymphoma L5178Y using C57BL6 and CD1 strains, respectively. Tumour cells were incubated with 40–120 μM solutions of WILD20 for 2 h at 37°C in the presence of 5% CO_2. Cell viability was verified through trypan blue exclusion test. 6.4×10^4 (3LLC) and 6.0×10^5 (L5178Y) cells were injected s.c. or i.p. in 0.1 ml of NaCl solution. Survival of the mice was established up to 50 days after inoculation. The results were statistically evaluated with the Mann-Whitney *U*-test.

2.6. Systemic antitumoral effect

WILD20 (0.01 and 0.001 mg/kg i.p.) was administered from day 3 to day 7 to CD1 mice using sarcoma 180 (1.5×10^5 cells i.p.) as challenge given at day 0. Mortality within 60 days from tumour cell inoculation was evaluated with the Mann-Whitney *U*-test. Synergy with cyclophosphamide was evaluated using WILD20 (1 mg/kg i.p.) and Cy (50 mg/kg i.p.) with both L1210 cells (5×10^4 i.p.) and P388 cells (5×10^4 i.p.). CDF1 mice were used for L1210 and C57BL6 for P388.

Mean survival time was calculated and evaluated with the Mann-Whitney *U*-test. The number of survivors at day 60 was also considered.

2.7. Effect of WILD20 and recombinant interleukin-2 peritumoral administration on 3LLC-implanted mice (intradermal)

Tumour

Lewis lung carcinoma was maintained in C57BL6 mice. Every 2–3 weeks 5×10^4 tumour cells were subcutaneously inoculated into the flank of the mice. Fifteen- to 18-day-old tumours were excised, cleared of debris and necrotic tissue and cut into fragments (1 mm^3). Tumour tissue was then placed in 0.5% trypsin (Sigma) in RPMI 1640 with gentamycin (50 μg/ml), penicillin (1000 U/ml), and streptomycin (200 μg/ml). After 30 min incubation at 37°C, trypsinization was halted by addition of complete medium containing foetal calf serum (final concentration 20% v/v). Cells were washed twice in complete medium, counted with a haemocytometer and resuspended in serum-free medium at a concentration of 1×10^6 cells/ml.

Drugs

WILD20 lot 495 (Wellcome Italy) and human recombinant interleukin-2 (Proleukin, Cetus Corp.) were dissolved in NaCl solution and injected s.c. (0.1 ml/mouse) starting on the day following tumour transplantation.

Dose of WILD20

2 μg/mouse/day into the tumour area. Recombinant interleukin-2: 1×10^4 U/mouse/day into the tumour area.

Administration regimen

Days 1, 2, and 3, then daily from day 6 to day 17. The mice were killed on day 20 and tumours were excised and weighed.

3. Results

Following up on previous experience with WILD20's antiproliferative effect on various cell lines (Tubaro et al., 1993b), an attempt was made to confirm this effect in four different tumour cell lines. Cytostatic drugs commonly used in tumour therapy, such as doxorubicin and cisplatin, were used as reference com-

Table 1
Antiproliferative effect of WILD20 and various lipoxygenase inhibitors on three tumour cell lines

Tumour	WILD20						BW755C					
	40 μM		60 μM		80 μM		10 μM		20 μM		40 μM	
	%	$P \leq$	%	$P \leq$	%	$P \leq$	%	$P \leq$	%	$P \leq$	%	$P \leq$
P388	−4 ± 1	n.s.	−18 ± 3	0.05	−43 ± 2	0.001	−6 ± 2	n.s.	+4 ± 2	n.s.	+10 ± 1	n.s.
L1210	−8 ± 1	n.s.	−9 ± 2	n.s.	−19 ± 3	n.s.	−16 ± 3	n.s.	−12 ± 3	n.s.	−14 ± 2	n.s.
L5178Y	−32 ± 2	0.01	−90 ± 2	0.001	−100 ± 3	0.001	−61 ± 0.5	0.001	−82 ± 2	0.001	−91 ± 2	0.001
Tumour	NDGA						BWA4C					
	5 μM		10 μM		20 μM		5 μM		10 μM		20 μM	
	%	$P \leq$	%	$P \leq$	%	$P \leq$	%	$P \leq$	%	$P \leq$	%	$P \leq$
P388	−29 ± 2	n.s.	−64 ± 3	0.001	−88 ± 2	0.001	−14 ± 2	n.s.	−16 ± 3	0.01	−44 ± 3	0.001
L1210	−20 ± 2	n.s.	−24 ± 3	n.s.	−40 ± 3	0.01	−10 ± 4	n.s.	−23 ± 2	n.s.	−56 ± 3	0.01
L5178Y	−42 ± 3	n.s.	−92 ± 1	0.001	−98 ± 2	0.001	−13 ± 4	n.s.	−24 ± 1	n.s.	−40 ± 1	0.01

Values are expressed as inhibition percentage ± S.E. of DNA synthesis evaluated as inhibition of [^{3}H]thymidine uptake compared to controls = 0. Drug-cell incubation time: 48 h. [^{3}H]Thymidine 6 h before test.

pounds/potentiating agents. Results obtained with sarcoma 180 as the representative of allogeneic and HL60 of syngeneic tumour lines, respectively, are reported in Fig. 2. There is a good degree of statistical evidence in favour of an antiproliferative effect of WILD20 and a synergy between drugs. The antiproliferative effect of WILD20 on P388, L1210, and L5178Y leukemia cells was investigated in comparison with three inhibitors of the lipoxygenase pathway of the arachidonic acid cascade, since previous research had established a WILD20-dependent inhibition of the arachidonic acid cascade, probably due to phosphokinase C translocation through phospholipase A_2 blockade. It is known that depressors of arachidonic acid metabolism inhibit tumour cell proliferation (Gati et al., 1990; Fulton et al., 1991). In particular, lipoxygenase inhibitors induce inhibition of proliferation and differentiation of tumour cells (Wilson et al., 1990). Results are reported in Table 1, where WILD20's inhibiting properties appear comparable with those of the lipoxygenase pathway inhibitors BWA4C, BW755C and NDGA.

The inhibition of phosphokinase C translocaton obtained with WILD20 (Tubaro et al., 1993a) led to investigation of a possible relationship between WILD20 and adhesive processes, on the basis of the knowledge that the activation of phosphokinase C is one of the mechanisms underlying spontaneous metastasis in murine adenocarcinoma cells (Korczak et al., 1989). Activators of phosphokinase C, in fact, increase tumour cell adhesion to endothelial cells (Herbert and Maffrand, 1991). The phosphokinase C inhibitor staurosporine inhibits the invasive behaviour of carcinoma cells (Schwartz et al., 1990). Moreover, sphingoid bases, which share part of the WILD20 molecule, are known to inhibit tumour cell adhesive function through phosphokinase C inhibition (Merrill et al., 1986).

In Table 2 the results for adhesion to extracellular matrix proteins of three different human tumour cells are reported: WILD20 inhibited in a dose-dependent manner adhesion to both laminin and fibronectin of all the tumour cell lines tested.

Table 2
Adhesion of human tumour cells to extracellular matrix proteins in the presence of various WILD20 concentrations

Substrate (20 μg/ml)	WILD20 (μg/ml)	% Adhesion	% Variation
E/J (G3) human bladder carcinoma			
LM [a]	–	81	–
LM+	10	73	−9.88
LM+	20	52	−35.80
FN [b]	–	81	–
FN+	10	75	−7.41
FN+	20	48	−40.74
RT112 human transitional bladder tumour cells			
LM	–	66	–
LM+	2.5	69	+4.54
LM+	5	62	−6.06
LM+	10	44	−33.33
FN	–	67	–
FN+	2.5	69	+2.98
FN+	5	62	−7.46
FN+	10	49	−26.87
Melanoma B16 tumour cells			
LM	–	79	–
LM+	0.1	80	+1.26
LM+	1	79	0
LM+	10	9	−88.61
FN	–	68	–
FN+	0.1	70	+2.94
FN+	1	68	0
FN+	10	4	−94.12

All tumour cells ~ 50000/well. Incubation with WILD20 during 1 h at 37°C. All results in quadruplicate. [a] LM = laminin; [b] FN = fibronectin.

The inhibition of tumour cell proliferation and adhesion led us to believe that WILD20 altered the cell membrane. This agrees with the effects found with other inhibitors of the arachidonic acid cascade (Rose and Connolly, 1990).

Perturbation of tumour cell membrane was evaluated by incubating two different tumour cells with various concentrations of WILD20 and inoculating the cells s.c. or i.p. into mice once the cell viability was verified. Mean survival time and survivor numbers were evaluated over a 50-day time span. Results reported in

Table 3
In vitro incubation of tumour cells with various concentrations of WILD20 and subsequent inoculation: effect on survival rate and survival time

Tumour	Mice (strain)	Group	μM	Cell conc.	Admin. route	M.S.T. (days)	P	Survivors	P
Lewis lung carcinoma	C57BL6	Controls	–	6.4×10^4	s.c.	27.75	–	0/12	–
		WILD20	120			50.00	0.0001	12/12	0.0001
		WILD20	80			38.91	0.003	5/12	0.02
		WILD20	40			35.83	0.019	3/12	0.11
Lymphoma L5178Y	CDF1	Controls	–	6.0×10^5	i.p.	9.25	–	0/12	–
		WILD20	100			50.00	0.0001	12/12	0.0001
		WILD20	50			48.75	0.0001	11/12	0.0001

Tumour cells were incubated with WILD20 in RPMI for 2 h at 37°C, 5% CO_2, counted and verified for viability by trypan blue exclusion test; cells suspended in 0.1 ml were then injected in a mouse. Experiments were stopped at day 50 after inoculation. Statistical evaluation: Mann-Whitney *U*-test (M.S.T.) and Fisher's exact test (survivors).

Table 3 demonstrate a statistically evident, dose-dependent influence of WILD20 on tumour growth.

The efficacy of WILD20 on experimental tumours in vivo was investigated: results were negative when tumour cells and drug were administered at separate sites; the results were, however, positive when tumour cells and drug were inoculated in the same site (i.p.). The results obtained under the latter conditions with S180 ascitis tumour are reported in Table 4 and with L1210 and P388 in Table 5. In Table 5 synergy with Cy is also reported. The administration times were chosen according to the life expectancy of the animals affected by each individual tumour. These results led the authors to presume a possible use of WILD20 in local tumour chemotherapy, via perilesional injection around a solid tumour. This technique has been used with biological response modifiers such as bacillus Calmette-Guérin (BCG), glucan and OK432 (Huang et al., 1990; Shirai et al., 1990; Nishiyama, 1991; Rutten et al., 1991) and with various cytokines, such as interferons (Silagi et al., 1988; Dunham et al., 1990; Mozzanica et al., 1990), interleukins (Silagi and Schaefer, 1986; Belardelli et al., 1989; Iigo et al., 1990; Ciolli et al., 1991), tumour necrosis factor (TNF) (Musiani et al., 1989; Alecu et al., 1990; Bosco et al., 1990; Healsmith et al., 1991; Nishizawa et al., 1991; Epstein, 1992).

Table 4
Effect of WILD20 i.p. on mean survival time of mice injected with S-180 ascitis tumour

Groups	M.S.T.	% Variation	P
Controls (saline)	17.9	–	–
WILD20 (0.01 mg/kg)	27.5	+53.6	0.001
WILD20 (0.001 mg/kg)	19.0	+6.1	n.s.

CD1 mice, male, 10 animals/group. WILD20 at days 3 through 7 from inoculation. WILD20 was injected at the same site as the tumour cells. S-180 cells: 1.5×10^5/mouse/0.1 ml i.p. Evaluation of deaths: within 60 days from tumour inoculation. Statistical evaluation: Mann-Whitney *U*-test.

Table 5
Synergy between cyclophosphamide and WILD20 administered in mice i.p. injected with tumour cells

Groups	Tumour	Dose (mg/kg)	Admin. times (days)	M.S.T.	P	% Variation	Survivors (day 60)	P
Controls	L1210	–	–	15.18	–	–	0/11	–
Cy [a]		50	1, 3, 7	29.81	0.0058	+96	0/11	–
WILD20		1	0, 2, 4, 7	15.90	n.s.	+4.7	0/11	–
Cy + WILD20				34.30	0.0003	+126.3	3/11	0.11
M.S.T.: Cy vs. Cy + WILD20 $P = 0.11$								
Controls	P388	–	–	22.17	–	–	0/10	–
Cy		50	1	45.80	0.009	+106.6	3/10	0.11
WILD20		1	7–14	28.71	0.047	+29.4	1/10	0.50
Cy + WILD20				55.65	0.00013	+151.0	9/10	0.0001
M.S.T.: Cy vs. Cy + WILD20 $P = 0.01$								

CDF1 mice (male) inoculated with 7.5×10^4 L1210 cells/mouse on day 0. C57BL6 mice (male) inoculated with 7.5×10^4 P388 cells/mouse on day 0. Statistical evaluation: Mann-Whitney *U*-test (M.S.T.) and Fisher's exact test (survivors). [a] Cy = cyclophosphamide.

Table 6
Perilesional injection of WILD20 and recombinant interleukin-2 (rIL-2) in mice injected with Lewis lung carcinoma (3LLC)

(a) Effect on mean survival time and survival percentage established at day 60 after tumour inoculation

Group	Dose (mouse)	M.S.T (days)	P	Survivors (day 60)	P
Controls (saline)	–	32.10	–	0/20	–
WILD20	2 μg	29.20	0.837	0/20	–
rIL-2	1×10^4 U	36.10	0.038	6/20	0.01
WILD20 + rIL2	2 μg + 1×10^4 U	44.40	0.0001	14/20	0.0001
M.S.T.: WILD20 vs. rIL2 + WILD20 $P = 0.0001$; rIL2 vs. rIL2 + WILD20 $P = 0.008$					

(b) Effect on tumour weight established at day 20 after tumour inoculation

Group	Dose (mouse)	No. of tumour-free mice/group	P	Tumour weight (g) mean ± S.E. (day 20)
Controls (saline)	–	1/8	-	0.988 ± 0.23
WILD20	2 μg	4/8	0.13	0.405 ± 0.20
rIL2	1×10^4 U	6/8	0.02	0.152 ± 0.11
WILD20 + rIL2	2 μg + 1×10^4 U	8/8	0.0006	0.00

C57BL6 mice, male, were intradermally injected with 9.25×10^4 3LLC cells in 0.05 ml/mouse at day 0. WILD20 2 μg/mouse and 1×10^4 rIL-2 U/mouse were peritumorally injected in different sites at days 1, 2, 3, then daily from day 6 to day 17 after tumour inoculation. Statistical evaluation: Mann-Whitney *U*-test (M.S.T.), Fisher's exact test (survivors and tumour-free mice).

Synergy between WILD20 and recombinant interleukin-2, in analogy with the well-known synergy between recombinant interleukin-2 and interferon (Silagi et al., 1988; Ghyka et al., 1991) or Cy (Silagi and Schaefer, 1986), was also studied. The results reported in Table 6 were obtained using 3LLC and were evaluated as mean survival time and tumour weight. The synergy between WILD20 and recombinant interleukin-2 administered peritumorally was statistically significant.

4. Discussion

WILD20 showed no direct effect on phosphokinase C as demonstrated by the lack of inhibition of phosphorylation in platelets (Tubaro et al., 1993a). This is an unexpected effect in the light of the inhibition found by Hannun et al. (1991) with lysogangliosides and sphingosine, and would represent a marked biochemical difference between molecular families.

However, WILD20 showed a strong inhibitory effect on phosphokinase C translocation from cytoplasm to membrane (Tubaro et al., 1993a). In this case, the results were in full agreement with those reported by Hannun et al. (1991) for glycosphingolipids and sphingosine.

As a consequence of this effect WILD20 was found to inhibit cellular phospholipase A_2 expression, which is commonly known to be phosphokinase C-mediated (Chakraborti et al., 1991).

Activation of phosphokinase C is one of the mechanisms by which metastases propagate (Korczak et al., 1989) through up-regulation of cell adhesion (Herbert and Maffrand, 1991). Thus staurosporine, which inhibits phosphokinase C, inhibits carcinoma cell invasion (Schwartz et al., 1990). Also, sphingosine, which is a part of the WILD20 molecule, inhibits phosphokinase C and is known to inhibit tumour cell attachment to laminin and collagen (Merrill et al., 1986; Inokuchi et al., 1991).

Glycosphingolipids are also considered responsible for some aspects of metastasis. In tumoral cells they modulate the cell surface interaction with basement membrane components, modulating the interaction of laminin and its receptors (Inokuchi et al., 1991). Gangliosides, in particular those on the cell surface, are able to mediate the adhesion of some tumour cells, such as neuroblastoma, to a suitable ganglioside-binding substratum (Mugnai and Culp, 1987).

The results reported here on cell proliferation are demonstrative of WILD20's similarity with other blocking agents of lipoxygenase of the cycloxygenase pathways of the arachidonic acid cascade (Rose and Connolly, 1990), and arachidonic acid is known to produce prostaglandins involved in the regulation of tumour cell proliferation, differentiation and metastasis (Conde et al., 1991; Fulton et al., 1991; Tzanakakis et al., 1991).

As opposed to gangliosides, which are known to bind fibronectin (Neale et al., 1991), WILD20 inhibits the adhesion of tumoral cells to laminin and fibronectin, and this effect could explain the effects of WILD20 on carcinogenesis of tumour cells incubated with WILD20.

In agreement with these results is the WILD20-dependent prolongation of animal survival when both the tumour cell challenge and the drug were injected simultaneously in the peritoneal cavity. WILD20 appeared to induce a strong dose-related membrane alteration that impaired the oncogenicity of tumour cells, probably via adhesion inhibition. The authors are aware of the apparent unlikelihood of a drug simultaneously inhibiting both proliferation and differentiation, but the results obtained appear to foster this hypothesis.

Especially interesting are the results of peritumoral WILD20 administration, considering the interest this form of cancer treatment has aroused in recent years. Peritumoral recombinant interleukin-2 treatment is a good example, and the most studied. Intratumorally, peritumorally and perilymphatically recombinant interleukin-2 shows inhibiting effects in the experimental animal (Vaage, 1987; Maas et al., 1991; Balemans et al., 1993) as well as in human models (Pizza et al., 1984; Musiani et al., 1989; Shirai et al., 1990; Squadrelli-Saraceno et al., 1990; Vaage, 1991). This effect appears to be bound to up-regulation of activated T-lymphocyte subsets infiltrating neoplastic tissues; infiltration induces a systemic T-cell-mediated response (Balemans et al., 1993). Similarly, Maas et al. (1991) reported the reduction of distant solid tumours obtained with small intratumoral doses of recombinant interleukin-2. This systemic response appears to be at least partly attributable to interferon-γ induction (Chakraborti et al., 1991).

In conclusion, the results reported here demonstrate a direct effect of WILD20 on various tumour cells and a synergistic effect of WILD20 with doxorubicin and cisplatin. WILD20 appears to down-regulate the oncogenicity of tumour cells by perturbing the cell membrane, to down-regulate tumour cell adhesion to extracellular matrix proteins and to impair solid tumour growth when peritumorally administered, especially together with recombinant interleukin-2. These findings merit exploration of the therapeutical possibilities of this agent in human cancer patients.

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