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Epidermal Growth Factor Receptor Expression and Suramin Cytotoxicity *in vitro*

S. Olivier, P. Formento, J.L. Fischel, M.C. Etienne and G. Milano

Twenty-five cell lines derived from nine different human cancers were tested for the cytotoxic activity of suramin. Two different initial cellular concentrations were used: C1 (800–2000 cells per well) and C2 (3000–7000 cells per well). Suramin concentrations ranged from 50 to 2500 μg/ml. Cytotoxicity was assessed by the MTT test. Epidermal growth factor receptors (EGFR) were assayed by competition analysis and Scatchard plots. In sixteen cell lines suramin had an unexpected growth stimulation effect at low concentration (50–125 μg/ml). IC_{50} varied from 21 μg/ml (osteosarcoma, OS2) to 1408 μg/ml (melanoma, CAL 24) and, within melanoma cell lines, it varied from 120 μg/ml (CAL 41) to 1408 μg/ml (CAL 24). The individual IC_{50} values were positively and significantly linked with the initial cellular density. Eighteen cell lines had measurable EGFR (six with two families of sites, twelve with one): K_d varied between 0.004 nmol/l for the highest affinity site (melanoma, CAL 7) to 1.852 nmol/l for the lowest affinity site (lung, CAL 12). There was no relation between presence or absence of EGF binding sites and distribution of IC_{50}, but for cells with measurable EGFR there was a weak but significant correlation between the number of EGF binding sites per cell and the corresponding IC_{50} ($r = -0.53$, $P = 0.021$).
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INTRODUCTION

SURAMIN may reduce tumour growth. At a molecular level, suramin inhibits the activity of protein kinase C [1] and the cellular binding of growth factors such as transforming growth factor β and epidermal growth factor (EGF) [2]. Suramin inhibits the cellular proliferation of several human tumour cells *in vitro*, including glioma [3], prostate carcinoma [4], colon cancer [5] and non-small cell lung cancer cells [6]. Bergh [6] found a relation between the cytotoxic activity of suramin and cellular expression in EGF receptors (EGFR). Such experimental data led to tests of the activity of suramin as an anticancer drug [7]; partial responses were recorded in patients with malignancies refractory to conventional cytotoxic chemotherapy. Since *in vitro* data about the activity of suramin on tumour cells were obtained in a variety of experimental conditions, we have evaluated the cytotoxic effect of suramin on a large panel of human tumour cell lines and investigated the relation between drug efficacy and cellular EGFR content.

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MATERIALS AND METHODS

Chemicals

Suramin (Bayer, batch number 4240152) was donated by R. Bellon Laboratories (Neuilly sur Seine). A working solution was prepared before use at 100 μg/ml in Dulbecco's modification of Eagle's medium (DMEM). Human recombinant ^{125}I-EGF (ref. IM 196, specific activity 4514×10^{10} Bq/mmol, 92.5×10^4 Bq per 250 μl) and unlabelled human recombinant EGF (ref. ARN 5100) were from Amersham. DMEM, RPMI 1640, glutamine and fetal bovine serum (FBS) were from Gibco. Penicillin and streptomycin were from Meyrieux. Transferrin and insulin were from Flow. Bovine serum albumin (BSA), 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethylsulphoxide (DMSO) were from Sigma.

Cell cultures

Twenty-five cell lines derived from nine different human cancers were tested (Table 1). Cells were routinely cultured in a humidified incubator at 37°C in 8% CO_2 in air. Cells were grown

Table 1. Cell lines

Tumour type	Usual name*	Origin	Doubling time (h)
Breast	MCF 7	ATCC, HTB 22	41
	T 47 D	ATCC, HBT 133	93
	ZR 75	ATCC, CRL 1500	100
Melanoma	CAL 1	CAL	60
	CAL 4	CAL	130
	CAL 7	CAL	160
	CAL 23	CAL	53
	CAL 24	CAL	61
	CAL 32	CAL	124
	CAL 41	CAL	96
	CAL 48 A	CAL	58
Head and neck	CAL 27	CAL (21)	45
	CAL 33	CAL (21)	78
Bladder	647 V	G. Julliard	40
Ovary	CAL 2	CAL	48
	CAL 9	CAL	54
Lung (NSC)	CAL 12	CAL	35
Osteosarcoma	OS 1	ATCC, HTB 85	88
	OS 2	ATCC, CRL 1543	57
	OS 3	ATCC, CRM 1547	30
Glioblastoma	GL 1	ATCC, CRL 1620	60
	GL 2	ATCC, HTB 14	118
	GL 3	ATCC, HTB 16	96
	GL 4	ATCC, HTB 17	216
Colon	WIDR	ATCC, CCL 218	43

*Official names where different: ZR 75 = ZR 75-1; OS 1, 2 and 3 = SAOS 2, HOS-TE 85/5 and TE 85mut, respectively; and GL 1, 2, 3 and 4 = A172, U87 MG, U138 MG and U378 MG, respectively.
ATCC = American Type Culture Collection, Rockville. CAL = Center A. Lacassagne, Nice. G. Julliard, University of California at Los Angeles. NSC = Non-small cell.

in DMEM containing 10% FBS and supplemented with L-glutamine (2 mmol/l), transferrin (0.64 μmol/l), insulin (0.1 μmol/l), penicillin (25 000 IU per 500 ml), streptomycin (86 μmol/l) and non-essential amino acids.

Experimental conditions are summarized in Table 1. Two different initial cellular concentrations were studied for each cell line (C1 was 800–2000 cells per well and C2 was 3000–7000 cells per well) to test the effect of initial cellular density on suramin cytotoxicity. Growth kinetics at C1 and C2 were almost the same. Incubations were stopped when controls without drugs reached 90% confluence. The final suramin concentrations (μg/ml) tested were 50, 70, 90, 125, 175, 250, 300, 400, 550, 750, 1000, 1400, 1850 and 2500.

Cytotoxicity

The cytotoxic effects of suramin were assessed by the MTT semi-automated test [8] in 96-well plates. The MTT test was done in fresh medium without suramin. Results were expressed as the percentage of absorbance compared with controls without drugs. Absorbance was set at 540 nm and measured on a 'Titertek Twin' reader. Each experiment was done six times. Sigmoid curves were generated from the dose-response graphs ('Graph Pad', ISI Software). The concentration giving 50% growth inhibition (IC_{50}) was computed by the program as the x value at the middle of the curve.

EGFR assay

Cells were grown in 24-well plates (10^5 cells per well) in 10% FBS-DMEM at 37°C. At 80–90% confluence, cells were rinsed three times with 500 μl RPMI 1640 containing 0.1% BSA at 2–4°C (plates placed on a tray with ice). Plates were then incubated for 30 min with the same medium (500 μl per well) at 4°C.

Cells were first screened for their capacity to bind EGF specifically. Total binding was measured after incubation with 0.2 nmol/l ^{125}I-EGF (3 h, 4°C, 0.1% BSA-RPMI); non-specific binding was measured in the presence of an excess of unlabelled EGF (20 nmol/l). Cells were considered as binding a detectable level of EGF when the total binding equalled or exceeded three times the non-specific binding. Cell lines exhibiting detectable EGF fixation were further investigated for their EGFR content. The cells were incubated in the RPMI medium for 3 h at 4°C in the presence of various concentrations of ^{125}I-EGF (0.01, 0.02, 0.04, 0.08, 0.12, 0.18, 0.2 nmol/l); for higher EGF concentrations, cells were incubated with 0.2 nmol/l of ^{125}I-EGF with increasing concentrations of unlabelled EGF (0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 20, 200 nmol/l). Plates were placed on a tray with ice to stop the reaction, and the supernatant was removed from each well. Cells were washed two times with phosphate-buffered saline (PBS) containing 0.1% BSA (4°C, 500 μl per well). After removal of the supernatant, cells were solubilized by 1 mol/l NaOH at 37°C (500 μl per well for 30 min). The radioactivity of each well was determined by gamma counting. Results were expressed in fmol per well. Scatchard analysis was used to calculate the number of receptor sites per cell (N) and the dissociation constant (K_d). Each point of every Scatchard plot was obtained in quadruplicate. Cells were counted in four wells run in parallel, resuspended in 200 μl PBS at room temperature and counted with a haemocytometer.

Statistics

The regression lines for the Scatchard plots and for the relation between IC_{50} values and log (N1 + N2) were computed with 'Stagraphics' (Uniware, Paris). (N1 and N2 represent receptors with one and two families of sites.) The Wilcoxon matched-pairs signed-rank test was used to analyse the IC_{50} values obtained for low and high initial cellular concentrations. Kruskall–Wallis analysis of variance was used to analyse the relation between IC_{50} values and the cellular content in EGFR, with 0 = no detectable binding sites, 1 = one family of binding sites and 2 = two families.

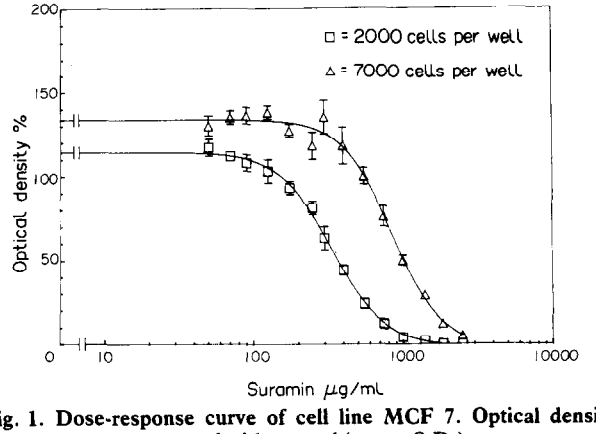

Fig. 1. Dose-response curve of cell line MCF 7. Optical density compared with control (mean, S.D.).

Table 2. Suramin effect

Cell line	H1, H2	$r^2$1, $r^2$2	IC_{50} (C1) (μg/ml)	IC_{50} (C2) (μg/ml)
MCF 7	−2.79, −3.01	0.99, 0.96	345 (2)	869 (7)
T 47 D	−1.86, −2.71	0.98, 0.98	621 (3) *	1163 (3)
ZR 75	−2.34, −2.21	0.99, 0.99	258 (1.5)*	381 (5)
CAL 1	−1.61, −1.25	0.97, 0.99	165 (1.5)*	177 (5)
CAL 4	−1.43, −1.63	0.97, 0.99	466 (1.5)*	609 (5)
CAL 7	−2.14, −1.77	0.99, 0.98	521 (1.5)*	574 (5)
CAL 23	−2.22, −3.17	0.99, 0.97	348 (1) *	388 (4)
CAL 24	−1.16, −5.50	0.98, 0.98	1330 (2) *	1408 (5)
CAL 32	−1.84, −2.90	0.99, 0.99	300 (1.5)*	841 (5)
CAL 41	−2.09, −1.88	0.99, 0.99	115 (1.5)	120 (5)
CAL 48 A	−3.67, −7.44	0.99, 0.98	475 (1.5)	741 (4)
CAL 27	−3.40, −1.23	0.99, 0.99	165 (1.5)	221 (5)
CAL 33	−6.10, −4.40	0.99, 0.99	159 (1.5)*	149 (5)
647 V	−4.61, −2.93	0.99, 0.99	262 (1.5)*	565 (5)
CAL 2	−2.36, −2.86	0.99, 0.97	165 (1.5)*	241 (5)
CAL 9	−1.35, −0.97	0.98, 0.95	259 (1) *	230 (4)
CAL 12	−6.59, −4.79	0.98, 0.99	161 (0.8)	238 (4)
OS 1	−1.43, −2.72	0.96, 0.97	382 (1) *	795 (3)
OS 2	−1.84, −1.03	0.90, 0.97	21 (1) *	63 (3)
OS 3	NC	NC	NA*	140 (3)
GL 1	−4.15, −2.81	0.99, 0.99	356 (1.5)	559 (4)
GL 2	−2.22, −3.29	0.98, 0.98	385 (1.5)	577 (5)
GL 3	−2.43, −6.07	0.97, 0.90	223 (1.5)	474 (4)
GL 4	−2.91, −2.84	0.95, 0.97	146 (1.5)*	322 (5)
WIDR	−1.57, −2.46	0.99, 0.99	135 (2)	649 (6)

*Increased cell growth at low suramin concentrations (50–125 μg/ml).
H(1), H(2) = Hill slopes of respective sigmoid curves for initial cellular densities C1 and C2.
$r^2$1, $r^2$2 = Goodness-of-fit between the experimental and fitted dose-response points for C1 and C2.
IC_{50} (C1), IC_{50} (C2) = suramin concentrations inhibiting 50% of cell growth compared with controls. C_1 and C_2 are initial cellular densities expressed as cells per well × 10^3.
NA = Not available. NC = Not calculated, IC_{50} determined graphically.

RESULTS

Figure 1 shows typical suramin dose-effect curves for cell line MCF-7 at the two initial cell densities tested. IC_{50} values for all cell lines and experimental conditions tested are given in Table 2. In sixteen cases, suramin had an unexpected growth stimulation effect at low concentrations (50–125 μg/ml); in these cases, cellular growth averaged 150% of control. The distribution of IC_{50} varied both between and within tumour cell categories: from 21 μg/ml (osteosarcoma, OS2) to 1408 μg/ml (melanoma, CAL 24), and, within melanoma cell lines, from 120 μg/ml (CAL 41) to 1408 μg/ml (CAL 24). Individual IC_{50} values increased significantly between the low initial cellular density and the high density ($P < 0.0001$).

Figure 2 illustrates EGF binding for head and neck cancer cell lines CAL 27 and CAL 33. These cell lines had two distinct families of sites. Eighteen of the twenty-five cell lines tested had measurable EGFR: six cell lines with two families of sites and twelve with one (Table 3). K_d values varied between 0.004 nmol/l

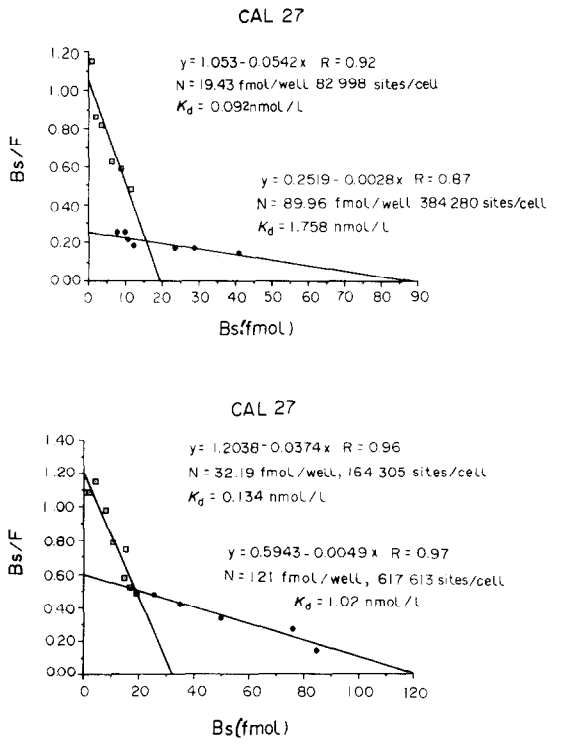

Fig. 2. EGF binding (Scatchard plot) for head and neck cancer cell lines CAL 27 and CAL 33.

for the highest affinity sites (melanoma, CAL 71) and 1.852 mmol/l for the lowest (lung, CAL 12). There was no relation between the presence or absence of EGF binding sites and the distribution of IC_{50} ($P = 0.981$). However, cells with measurable EGFR levels exhibited a weak but significant correlation between the number of EGF binding sites per cell line and the corresponding IC_{50} (Fig. 3). The cell lines with the highest EGFR levels were most sensitive to the effects of suramin.

DISCUSSION

The attention that has been paid to cellular oncogenes is now being turned toward growth factors, not only as concerns their control of cell growth but also the involvement of growth factor initiated pathways in the aetiology of cancer [9]. Although practical benefits in terms of the development of new anti-cancer tools, based on interactions with growth factors themselves or their products, are still awaited, some EGFR kinase inhibiters seem promising [10]. Suramin competes with the fixation of several potent growth factors, including transforming growth factor beta, EGF, platelet-derived growth factor and heparin-binding growth factor type 2 [2, 11, 12]. The potential activity of suramin as an anti-proliferative agent has been shown experimentally [3–6] and clinically [7].

Our data provide new insights into the mechanism of action of suramin. Suramin was cytotoxic against all cell lines investigated, but the IC_{50} values were highly variable. The least sensitive tumour cells were of breast cancer origin and, in most cases, from melanoma. The efficacy of suramin against glioma [3], colon cancer [5] and lung cancer cells [6] was confirmed. The lowest IC_{50} values were recorded for an osteosarcoma cell line. The two head and neck carcinoma cell lines tested also had a low IC_{50}. These results may be useful for objectively guiding further clinical trials on suramin-sensitive tumours. One other

Table 3. EGFR

Cell line	$K_d(1)$ (nmol/l)	N(1) (sites per cell)	$K_d(2)$ (nmol/l)	N(2) (sites per cell)
MCF 7	0.014	4563	0.561	14 845
T 47 D	—	—	0.082	4155
ZR 75	ND	ND	ND	ND
CAL 1	ND	ND	ND	ND
CAL 4	—	—	0.299	53 549
CAL 7	0.004	10 747	0.617	239 038
CAL 23	—	—	0.347	182 000
CAL 24	ND	ND	ND	ND
CAL 32	ND	ND	ND	ND
CAL 41	ND	ND	ND	ND
CAL 48 A	ND	ND	ND	ND
CAL 27	0.092	82 998	1.758	384 280
CAL 33	0.134	164 305	1.02	617 613
647 V	—	—	0.259	60 965
CAL 2	ND	ND	ND	ND
CAL 9	—	—	0.289	30 689
CAL 12	0.051	70 962	1.852	175 215
OS 1	0.011	6725	0.071	9687
OS 2	—	—	1.068	29 423
OS 3	—	—	0.246	12 997
GL 1	—	—	0.281	33 610
GL 2	—	—	0.240	67 608
GL 3	—	—	0.139	14 115
GL 4	—	—	0.322	133 402
WIDR	—	—	0.289	11 032

$K_d(1)$, N(1), $K_d(2)$ and N(2) = Dissociation constant and number of sites for EGF binding with one or two families of sites. ND = not detectable.

important observation was the fact that, in sixteen of the twenty-five cell lines, low suramin concentrations (50–125 μg/ml) stimulated growth.

IC_{50} values increased significantly when the initial cellular density rose; this can be explained by competition between cell-secreted growth factors and suramin. This competition hypothesis was supported by the fact that cellular binding of suramin-competing growth factors is reduced when cell density increases [13]. Cellular binding of EGF measured by Scatchard analysis was similar to data reported by others. For colon carcinoma cell lines, Wan *et al.* [14] reported K_d values ranging between 0.08 and 0.19 nmol/l; we measured 0.29 nmol/l in a cell line of the same tumour origin. Two families of EGF binding sites per lineage were characterized for six of our cell lines; for twelve others, only a single family was detectable. This concurs with Veale *et al.* [15] who reported two families of sites in most primary human non-small cell lung cancers while Carlin *et al.* [16] described a single family of sites in human hepatocellular carcinoma cell lines.

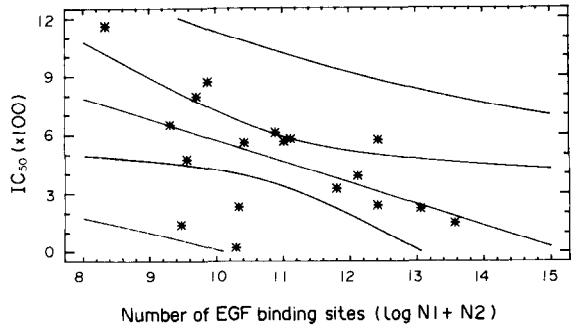

Fig. 3. Distribution of suramin IC_{50} values as function of total number of EGF binding sites (N1 + N2). Straight line represents line of best fit (r = 0.53, P = 0.021). Two envelopes represent 95% confidence and prediction limits.

EGFR assays in primary tumours appear useful, especially as a possible prognostic indicator [17–20]. Although suramin interacts with the cellular binding of several growth factors, we investigated the relation between the number of cellular EGF binding sites and suramin activity in our panel of cell lines. Cell lines with measurable levels of EGF binding sites showed a weak but significant correlation between the number of EGFR per cell line and the corresponding suramin IC_{50} values. Cells with the highest EGFR levels were the most sensitive to the cytotoxic effects of suramin. These data obtained for cell lines of different tumour origin confirmed previous data from human lung cancer cells [6]. However, and not surprisingly, the presence of EGFR in our cell lines was not a necessary condition for the manifestation of suramin cytotoxicity, because cells without detectable levels of this growth factor receptor were also sensitive to this drug's effects. This observation contradicts the hypothesis that knowledge of the tumoral EGFR content would allow selection of good candidates for treatment by suramin.

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Elective Regional Lymph Node Dissection in Malignant Melanoma

Michael Binder, Hubert Pehamberger, Andreas Steiner and Klaus Wolff

In patients with malignant melanoma elective regional lymph node dissection (ELND) is controversial. This retrospective study evaluated differences in prognosis in patients who were treated with or without ELND. 168 patients were included who initially presented with stage I melanoma (without palpable regional lymph nodes), and who had a minimum observation period of at least 5 years, or had died of malignant melanoma within 5 years of diagnosis. In 66 patients a wide local excision (WLE) followed by ELND was done. In 102 cases only a WLE was done. Assignment of patients to the two groups was non-random but there was no significant difference in age, sex, type and location of primary tumour and depth of invasion. No significant difference was found in survival of the two groups. The 5 year survival of the WLE group was 85.7% and that of the WLE plus ELND group was 89.1%. The 10 year survival rate was 77.9% and 73.1%, respectively. Neither in the whole series nor after sub-division of the patients into three classes according to Breslow depth of invasion (up to 1.5, 1.51–2.5, and greater than 2.5 mm) was a significant influence of ELND on the survival of patients apparent. Cox's regression model did not show ELND as a prognostic factor. ELND cannot be recommended as a routine treatment in patients with stage I melanoma.
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INTRODUCTION

ELECTIVE REGIONAL lymph node dissection (ELND) in primary malignant melanoma (stage I) is controversial [1]. Prospective randomized studies have failed to demonstrate improved disease-free interval and/or survival in melanoma patients by ELND [2–4]. But these studies have been criticised [5] and several investigators have claimed that at least some groups of melanoma patients—i.e. intermediate risk patients presenting with a melanoma of 1.5–4.0 mm invasion according to Breslow—might benefit from ELND [6–9]. Our retrospective study was done to evaluate differences in prognosis in patients who were treated with or without ELND.

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PATIENTS AND METHODS

The pigmented lesion group at the Department of Dermatology I, University of Vienna maintains a registry of melanoma patients that includes over 1200 patients. 168 patients with primary stage I melanoma qualified for this retrospective study. All had presented with stage I melanoma between 1970 and 1983, and were available for a minimum observation period of 5 years or had died before this period had elapsed. All patients received only surgical treatment; adjuvant immunotherapy and/or chemotherapy were not given. Surgical procedures consisted of a wide local excision (WLE) alone or WLE followed by ELND either immediately or within 2 weeks after WLE, according to standards at the time. 66 patients were treated with WLE followed by ELND and 102 with WLE alone.

The retrospective nature of this study precluded a randomization of the therapeutic procedures. In the 1970s it had been our strategy to recommend ELND to high-risk patients as