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ORIGINAL ARTICLE

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Inhibition of intracellular Ca^{2+} signalling, cytotoxicity and antitumor activity of the herbicide oryzalin and its analogues

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Abstract *Purpose*: Studies were conducted on oryzalin (3,5-dinitro-*N*,*N*-di(*n*-propyl)sulfanilamide), a widely used dinitroaniline sulfonamide herbicide, which was identified from plant extracts as an inhibitor of mitogen- and growth factor-mediated intracellular free Ca^{2+} ($[Ca^{2+}]_i$) signalling in mammalian cells. *Methods and Results*: Oryzalin inhibited vasopressin, bradykinin and platelet-derived growth factor $[Ca^{2+}]_i$ signalling in Swiss 3T3 fibroblasts with IC_{50} values of 14, 16 and 18 μ*M*, respectively. $^{45}Ca^{2+}$ uptake into nonmitochondrial stores of saponin-permeabilized Swiss 3T3 cells was inhibited by oryzalin with an IC_{50} of 34 μ*M*. Oryzalin inhibited colony formation of HT-29 colon carcinoma cells with an IC_{50} of 8 μ*M* and inhibited the growth of a number of other cancer cell lines and primary human tumors in vitro with IC_{50} values in the range 3 to 22 μ*M*. A number of oryzalin analogues were studied and an association was found between the ability to inhibit $[Ca^{2+}]_i$ signalling and inhibition of the growth of HT-29 human colon cancer cells ($P = 0.001$) and of CCRF-CEM human leukemia cells ($P = 0.016$). Oryzalin at doses up to 600 mg/kg administered orally or subcutaneously daily to mice for 3 to 10 days beginning a day after tumor inoculation inhibited the growth of murine B16 melanoma by 63% but showed no appreciable activity when administered subcutaneously or intraperitoneally to mice beginning a number of days after tumor inoculation against a variety of human tumor xenografts. The peak plasma concentration of oryzalin following repeated subcutaneous administration of oryzalin at 600 mg/kg per day to mice was 37 μ*M* and of its major metabolite *N*-depropyl oryzalin was 53 μ*M*. *Conclusion*: It is unlikely that the absence of significant antitumor activity of oryzalin is a result of the inability to achieve adequate plasma concentrations.

Key words Intracellular Ca^{2+} · Cell proliferation
Oryzalin

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Introduction

The cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) is tightly regulated in eukaryotic cells with resting levels around 0.1 μ*M* [8]. Transient increases in $[Ca^{2+}]_i$ to micromolar levels are used by cells as important intracellular signalling events that mediate the effects of a variety of external stimuli including growth factors, hormones, and neurotransmitters [5]. The most extensively studied pathway of $[Ca^{2+}]_i$ signalling involves the phospholipase C-mediated hydrolysis of phosphatidylinositol(4,5)bisphosphate (PtdIns(4,5)P_2). There are three major classes of PtdIns-specific phospholipase Cs (PtdInsPLC-β, γ and δ) [26]. PtdInsPLC can be activated by growth factors and mitogens by at least two mechanisms, one involving guanine nucleotide binding (G) proteins that activates membrane bound PtdInsPLC-β [33, 35, 39], the other involving tyrosine phosphorylation and binding through *src*-homology-2 (SH2) on cytoplasmic PtdInsPLC-γ to ligand-activated protein tyrosine kinase receptors such as the platelet-derived growth factor (PDGF) receptor [37].

PtdInsPLCs hydrolyse PtdIns(4,5)P_2 to give the water soluble inositol(1,4,5)trisphosphate (Ins(1,4,5)P_3) and a lipophilic diacylglycerol (DAG) [6]. Ins(1,4,5)P_3 releases Ca^{2+} from nonmitochondrial stores, producing a

transient increase in $[Ca^{2+}]$, while DAG is an activator of a Ca^{2+}- and phospholipid-dependent protein serine/threonine kinase, protein kinase C (PKC) [18]. Proteins phosphorylated by PKC include growth factor receptors, ion channels and transcription factors [17, 22]. Together, the increase in $[Ca^{2+}]$ and the increased activity of PKC leads to a sequence of events that culminates in expression of certain genes [20]. There are other ways by which $[Ca^{2+}]_i$ can be increased, including the influx of extracellular Ca^{2+} through voltage-operated or receptor-operated membrane Ca^{2+} channels [29], or by the release of Ca^{2+} from nonmitochondrial stores by polyunsaturated fatty acids [32].

There is considerable evidence that $[Ca^{2+}]_i$ signalling is an important regulator of cell growth. Lymphocytes and oocytes can be induced to undergo proliferation by the Ca^{2+} ionophore A23187, which produces increases in $[Ca^{2+}]_i$, together with a nonmetabolizable DAG analogue that activates PKC [19, 34]. Lowering external Ca^{2+} [7] or the use of a Ca^{2+} channel blocker [38] to decrease Ca^{2+} influx during mitogenic stimulation inhibits cell growth. Intracellular Ca^{2+} chelators that buffer the increases in $[Ca^{2+}]_i$ caused by growth factors and mitogens prevent cell growth [9, 36]. Cancer cells are less susceptible than normal cells to growth inhibition by a low extracellular Ca^{2+} concentration [7].

During studies to identify antiproliferative drugs that modulate growth factor-induced $[Ca^{2+}]_i$ signalling we found an activity that inhibits growth factor-induced $[Ca^{2+}]_i$ responses in extracts of *Geranium carolinium* (wild geranium), a plant commonly found in the southern USA. This activity was found to be due to the presence of a widely used pre-emergent herbicide oryzalin (3,5-dinitro-*N*,*N*-di(*n*-propyl)sulfanilamide). This was an intriguing observation given the widespread use of oryzalin as a herbicide and the potential for human exposure, as well as oryzalin's lack of appreciable animal or human toxicity [11]. We now report the ability of oryzalin and its analogues to inhibit $[Ca^{2+}]_i$ signalling, their toxicity to cancer cells in culture, and their antitumor activity and pharmacokinetics.

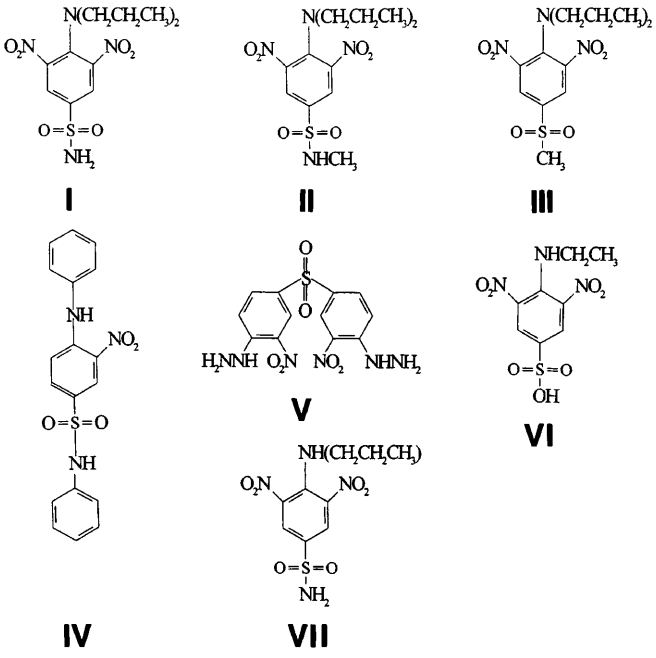

Fig. 1 Structures of oryzalin and some of its analogues: **I** oryzalin, **II** LY-67061, **III** LY-66750, **IV** LY-47173, **V** LY-802822, **VI** LY-108560, **VII** *N*-depropyl oryzalin

Materials and methods

Cells

Swiss mouse 3T3 fibroblasts were obtained from Dr. H.R. Herschmann, University of California, Los Angeles, Calif., CCRF-CEM human leukemia from St. Jude Children's Research Hospital (Memphis, Tenn.), HT-29 and SW480 human colon carcinosarcoma cells, HL-60 human promyelocytic leukemia cells, A375 human melanoma and A549 human lung cancer cells from the American Tissue Type Collection (Rockville, Md.). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and harvested with 0.05% trypsin and 0.5 m*M* EDTA before becoming confluent.

Fractionation of *Geranium carolinium*

Fresh parts of *Geranium carolinium* were chopped and extracted with ethanol. The ethanol extract was partitioned between water and chloroform, and the chloroform extract was further partitioned between hexane and aqueous methanol. The aqueous methanol was triturated in acetone and the acetone solution dried to give 46.6 g of material. This material was dissolved in chloroform and mixed with 750 silica gel and dried to give a slurry, which was applied to the top of a 6.0 × 120 cm column of silica gel and eluted with a gradient of methanol in chloroform. One of the peaks that eluted with 0 to 2% methanol in chloroform was shown to be active as an inhibitor of $[Ca^{2+}]_i$ signalling and, when crystallized from benzene, yielded 4.5 g of cubic orange crystals. [^{1}H NMR ($CDCl_3$) σ[ppm]: 8.30 (s,2H), 5.05 (s,2H), 2.29 (t,4H,J = 7.5 Hz), 1.60 (multiolet = 6, 4H, J = 7.5 Hz), 0.90 (t, 6H, J = 7.5 Hz). ^{13}C NMR ($CDCl_3$) σ[ppm]: 10.38, 20.63, 53.93, 127.71, 135.96, 141.84. EIMS: m/z 317 (100%), 346, 275. Exact mass: found 346.0793, calculated 346.0947. Melting point: 146–147 °C. Elemental analysis: found C 42.06%, H 5.25%, N 16.6%; theory C 41.6%, H 5.24%, N 16.18%]. Its formula was deduced as $C_{12}H_{18}N_4O_6S$ and its structure was identified as 3,5-dinitro-*N*,*N*-di(*n*-propyl)sulfanilamide (oryzalin; Fig. 1).

Chemicals

Oryzalin and its analogues were provided by Dr. Homer Pearce, Lilly Research Laboratories, Indianapolis, Ind. For animal studies oryzalin and its analogues were formulated for oral (p.o.) administration as a 120 mg/ml suspension in 4% dimethylsulfoxide, 5% polysorbate 20 (Tween 20) in 0.9% NaCl, or for intraperitoneal (i.p.) and subcutaneous (s.c.) administration as a 30 mg/ml solution in 32% soybean oil, 32% ethanol, 23% 0.9% NaCl, 8% Tween 80 and 5% cetyl alcohol, or in 2% polyethyleneglycol, 8% Cremophor and 90% 0.9% NaCl. The Ca^{2+}-sensitive photoprotein aequorin was purchased from Dr. John Blinks, Friday Harbor Laboratories (Seattle, Wash.), Ins(1,4,5)P_3 from Calbiochem (San Diego, Calif.), human PDGF, B chain homodimer, from Bachem (Torrence, Calif.), [Arg^8]vasopressin and bradykinin from Sigma Chemical Co. (St Louis, Mo.), and $^{45}Ca^{2+}$ (25 mCi/mg) from Amersham (Arlington Heights, Ill.).

Measurement of $[Ca^{2+}]_i$

Measurements of $[Ca^{2+}]_i$ were made in Swiss 3T3 fibroblasts loaded with aequorin by a low-Ca^{2+} centrifugation technique as previously described [27]. The aequorin-loaded cells were plated at a concentration of 10^6 cells in a 35-mm plastic culture dish containing 2 ml DMEM with 10% fetal calf serum. After 18 h in an incubator with 5% CO_2 in air, the medium was replaced with 2 ml DMEM for 3 h. $[Ca^{2+}]_i$ was estimated by light emission from the aequorin-loaded cells by placing the cells over a sensitive photomultiplier tube in a light-tight apparatus, with the culture dish maintained at 37 °C and continually flushed with 5% CO_2 in air. The mitogens vasopressin (10^{-7} *M*), bradykinin (2×10^{-7} *M*), and PDGF (3.3×10^{-9} *M*), dissolved in 0.2 ml warmed DMEM, were added sequentially to the dish at 10-min intervals through separate, light-tight, temperature-controlled inlet lines. We have previously reported that this order of addition of mitogens does not interfere with the $[Ca^{2+}]_i$ responses produced by the individual mitogens [28]. At the end of the experiment the cells were lysed with 1% Triton X-100 solution containing 5 m*M* $CaCl_2$ and the total light signal was integrated. $[Ca^{2+}]_i$ was calculated by the method of Allen and Blinks [1] employing the ratio of the light signal obtained by exposure to the mitogen to the total light signal following cell lysis. Oryzalin and its analogues were added to the cells 3 h before addition of the mitogens and growth factor. In a survey of 31 oryzalin analogues the analogues were tested at 5, 10 and 25 μg/ml to obtain an estimate of the concentration of analogue required to inhibit the $[Ca^{2+}]_i$ response by 50% (IC_{50}). Oryzalin and four active analogues were chosen for further study and IC_{50} values for inhibition of $[Ca^{2+}]_i$ responses obtained by a four-point assay.

Ca^{2+} uptake and release

Ca^{2+} uptake and release by saponin-permeabilized HT-29 colon carcinoma cells was measured by a modification of a method previously described [32]. Briefly, the cells in suspension at 2×10^6 cells/ml were permeabilized with medium containing 0.005% saponin for 25 min at 37 °C with gentle stirring. Cells were washed three times to remove saponin and incubated at 37 °C at 4×10^6 cells/ml in medium containing 1 m*M* ATP, 3% polyethylene glycol, 50 μ*M* $^{45}Ca^{2+}$ (160 μCi/μmol) and sufficient EGTA to buffer the free Ca^{2+} concentration to 10^{-7} *M* [13]. Mitochondrial function was inhibited by including in the incubation medium 0.5 m*M* dinitrophenol, 16 μ*M* antimycin A and 2 μg/ml oligomycin. Aliquots (0.1 ml) of the cell suspension were taken at various times and cells collected on glass microfiber filters (GF/A, Whatman International, Maidstone, UK) and washed with medium containing 1 m*M* $LaCl_3$. The cells were then digested in 0.5 ml 1 *N* KOH at 60 °C for 1 h prior to liquid scintillation counting. In some studies, 10 μ*M* $Ins(1,4,5)P_3$, a supramaximal concentration for Ca^{2+} release [32], was added to the cells after 8 min incubation and $^{45}Ca^{2+}$ release was measured as the difference in the cellular $^{45}Ca^{2+}$ content 1 min later, compared to nontreated cells.

Cell proliferation assays

Inhibition of HT-29 colon carcinoma cell growth by oryzalin and its analogues was measured in the soft agarose colony-forming assay with continuous drug exposure over 7 days as previously described [2]. Inhibition of cell viability over 4 days drug exposure was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay [24]. Inhibition of colony formation by human primary tumors in the human tumor colony-forming assay was measured as previously described [30].

Antitumor activity

The in vivo antitumor activity of oryzalin and its analogues was studied using either mouse B16 melanoma cells inoculated s.c. into C57Bl/6 mice with drug treatment initiated 1 day after tumor inoculation and tumor volume measured 12 days later [15], or human VRC5, CX-1 or HT-29 colon cancer cells inoculated into CD1 Nu/Nu mice with treatment initiated 7 to 14 days after tumor inoculation and tumor weights measured 10 to 12 days later [31].

Pharmacokinetic studies

Female C57Bl/6 mice weighing 20 to 25 g were given oryzalin at 300, 600 or 1200 mg/kg s.c. twice daily for up to 5 days. Preliminary studies had shown that the peak oryzalin concentration occurred 1 h after s.c. administration. Groups of three mice at each time-point, either before or 1 h after the administration of oryzalin, were anesthetized with diethylether and exsanguinated by retroorbital bleeding. Blood was collected into heparinized tubes, plasma immediately separated and stored frozen at −80 °C until assay. High-performance liquid chromatography (HPLC) was used to measure oryzalin and its metabolites in plasma [12]. Briefly, 0.5-ml plasma samples were extracted on a C18 solid-phase extraction column (BondElut; Varian Sample Preparation Products, Harbor City, Calif.), the columns were washed with 3 ml water, vacuum dried and eluted with 3×200 μl acetonitrile. Aliquots of the eluate, 10 to 50 μl, were taken for HPLC on a 150-mm, 5-μm particle size, C-18 reverse-phase column (Alltech Associates, (Deerfield, Ill.) and eluted with 47% water and 53% acetonitrile at a flow rate of 2 ml/min. The retention time of oryzalin was 4.7 min and of *N*-depropyl oryzalin (Fig. 1, **VII**), the major metabolite present in plasma, was 2.2 min. Calibration curves for oryzalin and *N*-depropyl oryzalin were prepared over the concentration range 0.1 to 50 μg/ml.

Results

Inhibition of $[Ca^{2+}]_i$ signalling by extracts of Geranium carolinium

Aqueous methanol extracts of *Geranium carolinium* contained an activity that inhibited growth factor $[Ca^{2+}]_i$ signalling in Swiss 3T3 fibroblasts. Subfractionation of the extract gave a yellow crystalline compound that was identified as oryzalin (Fig. 1, **I**). Oryzalin was found to be a potent inhibitor of growth factor-induced $[Ca^{2+}]_i$ signalling (Fig. 2) with IC_{50} values for the inhibition of vasopressin-, bradykinin- and PDGF-mediated $[Ca^{2+}]_i$ signalling of 14 μ*M*, 16 μ*M* and 18 μ*M*, respectively. Because oryzalin has been reported to inhibit microtubule polymerization in cells of higher plants [14, 23], the effects of taxol and vinblastine, both microtubule-active drugs in mammalian cells [4] were examined. Neither agent affected $[Ca^{2+}]_i$ signalling. Oryzalin did not inhibit other intracellular signalling enzymes studied; these included rat brain PKC, c-*src* protein tyrosine kinase, PtdInsPLC and phosphatidylinositol-3-kinase (results not shown).

Inhibition of proliferation

Oryzalin inhibited colony formation by HT-29 colon carcinosarcoma cells with an IC_{50} of 13 μ*M*. Oryzalin inhibited the growth of several other human cancer cell lines measured by the MTT assay with IC_{50} values of: 2.9 μ*M* for HL-60 promyelocytic leukemia, 7.2 μ*M* for A375 melanoma, 8.7 μ*M* for LnCAP prostate, 16.8 μ*M*

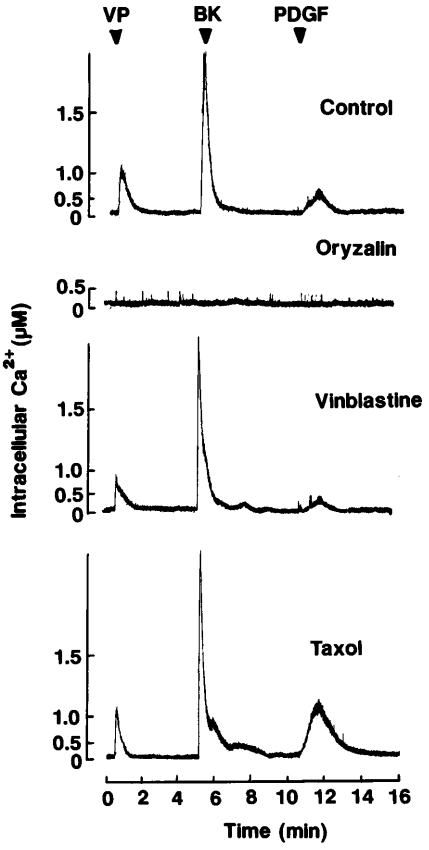

Fig. 2 Effects of oryzalin and other microtubule-active drugs on growth factor- and mitogen-mediated $[Ca^{2+}]_i$ signalling in Swiss 3T3 fibroblasts. Cells were loaded with the Ca^{2+}-sensitive photoprotein aequorin, grown for 20 h in DMEM with 10% fetal calf serum and then exposed to DMEM without serum and the drugs oryzalin (72 μM), vinblastine (62 μM) and taxol (59 μM) for 3 h. Vasopressin (10^{-7} M), (*VP*), bradykinin (2×10^{-7} M) (BK), and PDGF (3.3×10^{-9} M) were added at the arrows

for SW 480 colon, and 22.5 μM for A549 lung. The ability of oryzalin to inhibit the colony formation by human primary tumors was also measured. The mean IC_{50} values (number of tumors in parentheses) were breast (two) 11.8 μM, kidney (one) 1.1 μM, sarcoma (two) 4.9 μM, melanoma (two) 13.2 μM. Melanoma cell lines (four), and ovary (three), liver (one) and lung (one) cancer cell lines showed IC_{50} values above 15 μM.

Inhibition of $[Ca^{2+}]_i$ signalling by oryzalin analogues

A series of 31 oryzalin analogues were tested for their ability to inhibit $[Ca^{2+}]_i$ signalling and to inhibit the growth of HT-29 colon carcinosarcoma cells and CCRF-CEM leukemia cells. There was a significant association (two-tailed Fisher's exact test) between the mean value for inhibition of $[Ca^{2+}]_i$ signalling mediated by all three mitogens and inhibition of the growth of HT-29 cells ($P = 0.001$) and CCRF-CEM cells ($P = 0.016$). Secondary analysis not adjusted for multiple comparisons revealed that the associations remained

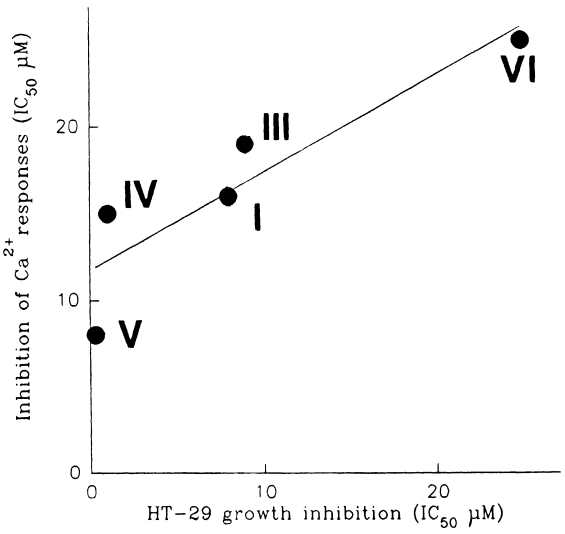

Fig. 3 Inhibition of HT-29 colon cancer cell colony formation and mean inhibition of growth factor- and mitogen-mediated $[Ca^{2+}]_i$ signalling in Swiss 3T3 fibroblasts by oryzalin and some of its analogues. Values are IC_{50} values in both cases. The line is a computer generated regression of the data. Roman numerals refer to compounds shown in Fig. 1

when inhibition of $[Ca^{2+}]_i$ responses to individual mitogens was compared with cell growth inhibition for both cell types ($P < 0.05$ in all cases except for PDGF versus HT-29 colon carcinoma for which $P = 0.051$). The correlations between HT-29 colon cancer cell growth inhibition and mean inhibition of $[Ca^{2+}]_i$

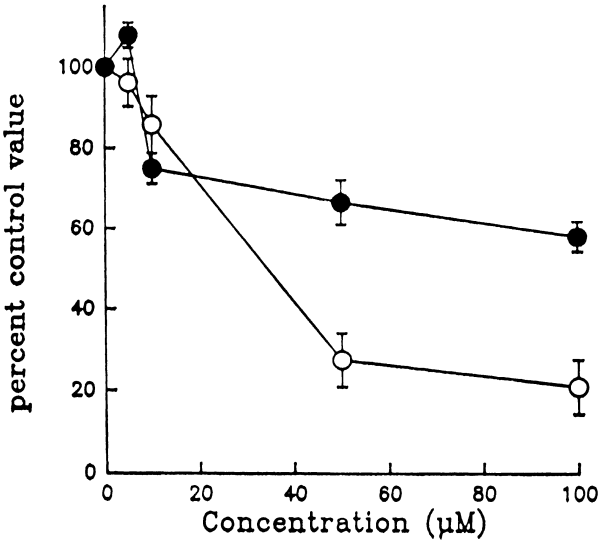

Fig. 4 Effects of oryzalin on (○) $^{45}Ca^{2+}$ uptake and (●) Ins(1,4,5)P_3-mediated $^{45}Ca^{2+}$ release by nonmitochondrial stores of saponin-permeabilized Swiss 3T3 fibroblasts. $^{45}Ca^{2+}$ uptake was measured in the presence of an ATP-generating system at 37 °C over 8 min as described in the text. Ins(1,4,5)P_3 (10 μM), was added at 8 min and $^{45}Ca^{2+}$ released measured at 9 min as the difference between the $^{45}Ca^{2+}$ that remained in the cells and $^{45}Ca^{2+}$ in cells without Ins(1,4,5)P_3. Oryzalin was added at the concentrations shown. Each point is the mean of five determinations and *bars* are SE

responses by oryzalin and four analogues chosen for further study are shown in Fig. 3.

Ca^{2+} uptake and release

The effects of oryzalin on Ca^{2+} uptake and Ins(1,4,5)P_3-mediated Ca^{2+} release in saponin-permeabilized Swiss 3T3 fibroblasts are shown in Fig. 4. Oryzalin was a potent inhibitor of Ca^{2+} uptake (IC_{50} 34 μM), but a weaker inhibitor of Ins(1,4,5)P_3-mediated Ca^{2+} release (IC_{50} > 100 μM).

Antitumor activity

Oryzalin produced up to 65% inhibition of the growth of B16 melanoma in mice when administered p.o. or s.c. beginning 1 day after tumor inoculation, daily for up to 10 days at doses up to 2000 mg/kg (Table 1). Oryzalin was, however, without activity against established human VRC5, CX-1 and HT-29 colon cell tumors when administered either i.p. or s.c. daily for 10 days at up to 600 mg/kg per day. There was some lethality seen with p.o. doses of oryzalin of 1200 mg/kg per day or greater and precipitated drug was found in the i.p. cavity of these animals. The oryzalin analogues **II**, **III** and **IV** were tested for antitumor activity against established murine X5563 plasma cell myeloma, C3H murine mammary carcinoma and 6C3HED murine lymphosarcoma. The compounds were administered i.p. at doses up to 300 mg/kg daily for 10 days. There was no significant inhibition of tumor growth or toxicity (more than 20% weight loss) seen for any of the compounds (results not shown).

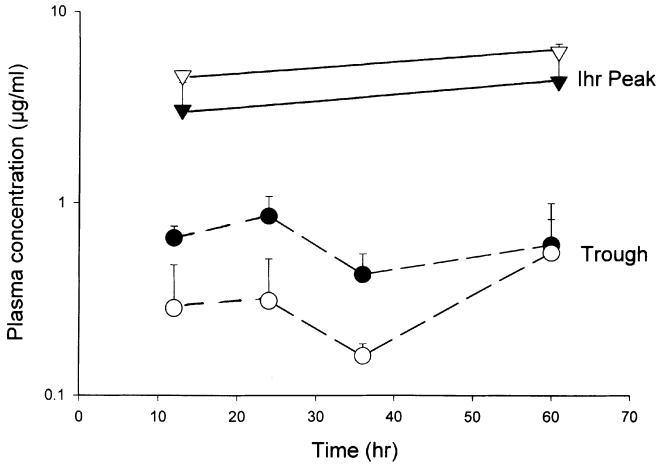

Fig. 5 Plasma concentrations of oryzalin (●, ▼) and its metabolite *N*-depropyl oryzalin (○, ▽) inthe plasma of mice given oryzalin 300 mg/kg/day s.c every 12 h. Trough values (*dotted lines*) immediately preced and peak values (*continuous lines*) 1 h followed a dose of oryzalin. Each point is the mean of three mice and *bars* are SE

Pharmacokinetics

Concentrations of oryzalin in the plasma of mice following twice daily s.c. administration of 300 mg/kg over 5 days were measured (Fig. 5). Trough concentrations of oryzalin were around 0.8 µg/ml and peak concentrations 1 h after administration of oryzalin around 4 µg/ml. A

Table 1 Antitumor activity of oryzalin

Tumor	Dose (mg/kg)	Schedule	Route	Vehicle[a]	% tumor inhibition	Surviving[b] animals
Mouse						
B16 melanoma	300	Days 1, 5, 9	s.c.	a	63	8/8
	600	Days 1, 2, 3	s.c.	a	63	8/8
	600	Days 1–10	p.o.	b	65	7/7
	1200	Days 1–10	p.o.	b	63	5/8
	1000	Days 1–10	p.o.	b	16	7/10
	2000	Days 1–10	p.o.	b	57	4/10
	3000	Days 1–10	p.o.	b	30	4/10
Human						
VRC5 colon	150	Days 1–10	s.c.	c	0	8/8
	300	Days 1–10	s.c.	c	0	8/8
	600	Days 1–10	s.c.	c	28	6/6
	150	Days 1–10	i.p.	c	0	9/9
	300	Days 1–10	i.p.	c	22	9/9
CX-1 colon	150	Days 1–10	s.c.	c	12	10/10
	300	Days 1–10	s.c.	c	0	10/10
	150	Days 1–10	i.p.	c	0	10/10
	300	Days 1–10	i.p.	c	17	10/10
HT-29 colon	150	Days 1–10	i.p.	c	7	10/10
	300	Days 1–10	i.p.	c	15	9/10

[a]Vehicles: *a* 32% soybean oil, 8% Tween 80, 32% ethanol, 23% 0.9% NaCl, 5% cetyl alcohol; *b* 4% dimethylsulfoxide, 5% Tween 20, 91% H_2O; *c* 2% polyethylene glycol, 8% Cremophor, 90% 0.9% NaCl

[b]Animals surviving/animals injected

Table 2 Peak plasma concentrations for oryzalin and *N*-depropyl oryzalin. Oryzalin was administered s.c. every 12 h at the daily doses shown. The plasma values were measured following 3 days administration of oryzalin 1 h after the last dose. Each value is the mean of three mice and *bars* are SE

Oryzalin dose (mg/kg)	Oryzalin (μg/ml)	*N*-depropyl oryzalin (μg/ml)
250	5.8 ± 2.8	6.5 ± 3.9
600	14.8 ± 9.7	15.3 ± 10.2
1200	9.6 ± 2.9	16.5 ± 4.7

major metabolite peak was seen in the plasma of mice which in other studies we have identified as *N*-depropyl oryzalin [12]. The peak and trough concentrations of *N*-depropyl oryzalin were 5.4 and 0.3 μg/ml. The maximum peak concentrations of both oryzalin and *N*-depropyl oryzalin were achieved at a dose of 600 mg/kg per day and did not increase further at 1200 mg/kg per day (Table 2).

Discussion

Oryzalin is a dinitroaniline sulfonamide pre-emergent herbicide that binds to tubulin and inhibits microtubule disassembly in algae and higher plants [14, 23]. Oryzalin does not affect microtubule function in animal cells [21, 23]. We identified oryzalin present in extracts of *Geranium carolinium* which had, presumably, been treated with the herbicide, as an inhibitor of growth factor-mediated Ca^{2+} signalling. Dinitroanilines such as oryzalin have previously been reported to inhibit the uptake of Ca^{2+} in vitro by plant mitochondria [16]. This, however, is the first report of an effect of oryzalin on Ca^{2+} intracellular signalling in animal cells. The inhibition of Ca^{2+} signalling by oryzalin was seen with a variety of mitogens and growth factors that act by different signalling pathways. They included vasopressin, which releases Ca^{2+} primarily by a mechanism involving the G protein-mediated activation of PtdInsPLC-β and formation of $Ins(1,4,5)P_3$ [25], bradykinin, which in addition releases Ca^{2+} by the formation of arachidonic acid mediated by the activation of phospholipase A2 [3], and PDGF which activates PtdInsPLC-γ by tyrosine phosphorylation and *src*-homology domain binding, thus forming $Ins(1,4,5)P_3$ [10]. This widespread action suggests that oryzalin is acting on an early point in Ca^{2+} signalling shared by all the pathways. This is likely to be the depletion of Ca^{2+} in nonmitochondrial $Ins(1,4,5)P_3$ and arachidonic acid-sensitive stores. We found that oryzalin is an inhibitor of Ca^{2+} uptake into nonmitochondrial stores in saponin-permeabilized cells with an IC_{50} of 34 μ*M*.

Oryzalin and its analogues were found to inhibit the growth of a variety of cancer cell lines in culture. This occurred at micromolar levels of the compounds and was significantly correlated with inhibition of Ca^{2+} signalling by the compounds. The in vitro potency of oryzalin as an inhibitor of cell proliferation is surprising in light of the reported lack of toxicity of oryzalin to animals and humans. The LD_{50} of oryzalin administered i.v. to adult male or female rats or gerbils is greater than 10 g/kg [11]. When administered at doses up to 600 mg/kg to mice inoculated 1 day previously with B16 melanoma tumor cells, oryzalin produced up to 65% tumor growth inhibition. However, oryzalin did not exhibit activity against a panel of human tumor xenografts when administration was begun several days after tumor inoculation. The analogues **II**, **III**, **IV** and **V** were also tested against established murine tumors, and at nontoxic doses they exhibited no antitumor activity.

Pharmacokinetic studies showed that the maximum concentration of oryzalin that could be achieved in the plasma of mice given 600 mg/kg per day was around 13 μg/ml (37 μ*M*) with trough concentrations at 300 mg/kg, around 0.8 μg/ml (2.3 μ*M*). Thus, the achievable plasma concentrations of oryzalin in mice are in the same range as the in vitro IC_{50} concentrations for inhibition of cancer cell growth of 3 to 22 μ*M*. A major metabolite of oryzalin is *N*-depropyl oryzalin which has an in vitro cytotoxicity similar to that of oryzalin [12]. Concentrations of *N*-depropyl oryzalin achieved in mouse plasma were 16 μg/ml (53 μ*M*). Thus, it appears that the lack of antitumor activity of oryzalin against established tumors is not a consequence of inadequate plasma concentrations of oryzalin or its major active metabolite *N*-depropyl oryzalin.

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