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Furoxan derivatives as cytotoxic agents: preliminary *in vivo* antitumoral activity studies

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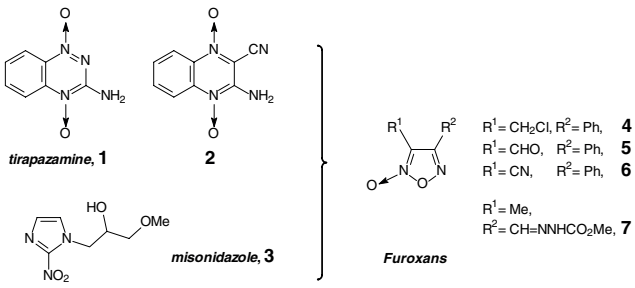
Furoxan derivatives with *in vitro* cytotoxic activity were investigated as antitumoral agents *in vivo*. The compounds were tested in murine models of both CCRFS-180 II sarcoma and mammary adenocarcinoma. Two of the furoxan derivatives considered here, 3-formyl-4-phenyl-1,2,5-oxadiazole N^2-oxide and 3-carbonitrile-4-phenyl-1,2,5-oxadiazole N^2-oxide, present *in vivo* antitumoral activity. They were able to produce more than 90% of tumoral necrosis under the experimental protocol of administration and posology employed. NO-releasing capacity of furoxans may explain the anti-neoplastic activity of these compounds.

1. Introduction

It is well known that the imperfect neovascularisation seen in growing solid tumours results in limited and inefficient blood vessel networks and in restricted and often chaotic blood flow. This and the high and variable interstitial pressures caused by the growing tumour lead to the presence of a variable but significant proportion of hypoxic cells. One strategy aiming to provide substantial increases in the clinical efficacy of antitumoral drugs, especially against the more slowly growing solid tumours, is the development of relatively non-toxic prodrug forms of those cytotoxins that can be selectively activated in tumour tissue. Hypoxia appears to be a common and unique property of cells in solid tumours and is an important mechanism for the tumour-specific activation of prodrugs (Denny 2001). The major classes of compounds used as hypoxia-selective cytotoxins are aromatic and aliphatic *N*-oxides (Cerecetto and González 2001), quinones, nitro aromatic compounds, and cobalt complexes. All of them represent groups which are bioreduced in the solid tumour due to the high levels of reductase enzymes present.

In this sense, we have investigated the bioreductive capacity of some characteristic *N*-oxide derivatives (Monge et al. 1998a, 1998b; Cerecetto et al. 2000; Boiani et al. 2001; Cerecetto et al. 2001; Boiani et al. 2004; Cerecetto et al. 2004a, 2004b). In general, these derivatives showed poor selectivity *in vitro* against V79 cells under oxic and hypoxic conditions. However, derivatives of 1,2,5-oxadiazole *N*-oxide (furoxans) showed a wide spectrum of cytotoxic activities. These compounds were designed attempting to combine a bioreductive moiety, *N*-oxide, like compounds **1** and **2**, and an heterocycle structurally related to imidazole, like compound **3**. Particularly, furoxans **4**–**6** showed high *in vitro* cytotoxic activity whereas compound **7** was moderately cytotoxic (Table 1) (Monge et al. 1998b; Boiani et al. 2001). To our knowledge, the effect of furoxans on the anti-neoplastic properties *in vivo* has not been studied.



The excellent bio-reductive quinoxaline dioxides, e.g. **2**, present very low solubility in physiological media (Monge et al. 1995a; Zamalloa et al. 1997). Therefore, they are non-convenient pharmaceutical products. The good solubility in physiological solutions shown by the furoxan derivatives together with their excellent *in vitro* cytotoxicity, make them good candidates for pre-clinical trials. Besides, they adhere to Lipinski's 'rule of 5'. Lipinski has described desired ranges for certain properties thought to be important for pharmacokinetics and drugs development. According to 'rule of 5' a good drug shows CLopP < 5, number of hydrogen bond donors ≤ 5, number of hydrogen bond acceptors ≤ 10 and molecular weight < 500 (Lipinski et al. 1997). A compound that fulfils at least three out of the four criteria is said to adhere to Lipinski's 'rule of 5'. Table 1 lists the values of these properties for furox-

Table 1: *In vitro* activity, physicochemical properties and Lipinski's 'rule of 5' for the studied furoxan derivatives

Compd.	*In vitro* cytotoxicity[a] SFoxia/SFhypox [b] (at 10 μM)	CLogP [c]	H-bond donors	H-bond acceptors	Molecular weight	'Rule of 5' criteria met
4	0.2/1.6 (1.5/1.2)	3.30	0	4	210	All
5	0.0/0.0 (0.0/0.0)	2.31	0	6	190	All
6	0.0/0.0 (37.0/100.0)	3.07	0	5	187	All
7	68.0/48.0 (100.0/100.0)	0.47	1	7	200	All

[a] Using V79 cells, at 20 μM, [b] SF: survival fraction. Percentage, respect to non-treated, of living V79 cells treated with the corresponding drugs, in both conditions (oxia and hypoxia) (Boiani 2001; Monge 1998b), [c] LogP calculated (CLogP) by Villar algorithm implemented in PC SPARTAN Pro package (PC SPARTAN Pro, Wavefunction, Inc. 18401 Von Karman Avenue, Suite 370. Irvine, California 92612 USA., PC SPARTAN Pro User's Guide, Wavefunction Inc., California, 1999)

Table 2: Determined maximum tolerated dose (MTD) and pharmacological dose (PD) for drugs 2, 4, 5, 6, and 7

Compd.	**2**	**4**	**5**	**6**	**7**
MTD[a,b]	100[c]	100[d]	20[e]	250[f]	600[g]
MTD[h]	0.5	0.5	0.1	1.3	3.0
PD[a]	16.7[i]	12.5[j]	3.5[i]	PD_6 A: 25.0[k] PD_6 B: 20.8[l]	100.0[i]
PD[h]	0.08	0.06	0.02	PD_6 A: 0.13 PD_6 B: 0.11	0.5

For details of each determination see Experimental section, [a] MTD and PD are reported as mg of drug/kg of animal weight, [b] At higher dose than determined MTD death or decreasing weight more than 10% were observed, [c] Studied dose: 200, 150, 120; 100, and 70 mg/kg, [d] Studied dose: 200, 150, 120, 100, 90, 70, 50 and 20 mg/kg, [e] Studied dose: 100, 70, 50, 25, 20, 15, and 10 mg/kg, [f] Studied dose: 300, 250, 200, 100, 50, and 20 mg/kg, [g] Studied dose: 600, 400, 300, 200, 100, 70, 50, and 20 mg/kg, [h] MTD and PD are reported as mmol of drug/kg of animal weight, [i] 1/6 MTD, [j] 1/8 MTD, [k] 1/10 MTD, [l] 1/12 MTD

ans **4**–**7** and suggests that these compounds constitute reasonable starting points for pharmacological *in vivo* studies.

In this paper, we address this aspect studying the antineoplastic properties *in vivo* for furoxans **4**–**7** as well as for the quinoxaline dioxide **2**. Data on the ability of some derivatives to promote necrosis on rat sarcoma and mammary adenocarcinoma without others systemic effects are also presented.

2. Investigations and results

Firstly, in order to determine the pharmacological doses (PD), the maximum tolerated doses (MTD) *in vivo* for compounds **2**, **4**–**7** was established. The MTD, defined as a single intraperitoneal (i.p.) dose of drug that produces a 10% weight decrease at the third day post-administration as compared to the non-treated animal (Monge et al. 1995b, 1995c), was determined in healthy tumour free animals (see Experimental) (Table 2). Compounds **6** and **7** present the highest MDT in agreement with the *in vitro* cytotoxicity against V79 cells (see Table 1).

From MDT pharmacological doses (PD) were determined. The PD's were defined as those which any side effect was evidenced during the experiments. This study attempted to determine the toxic effects of PD on tumour-free animals during the chronic treatment. The initial PD was established as 1/6 of the MTD (Lee et al. 1992). This dose was administered following the schedule shown in Figure 1a, including animals treated with vehicle solution (V.I., vehicle-injected) as negative control.

During the first and second week of the PD determination experiment the mice treated with drugs **5** and **6**, at PD initially established (see above), presented moderate fell-erection that disappeared in the last weeks of the treatment. This phenomenon was the only one observed related to the vasodilatory properties of furoxans (Feelisch et al. 1992; Ferioli et al. 1993), blood pressure or another vasodilator parameter were not accounted during the pharmacological trials. Macroscopic observations at autopsy and the results of the histological studies lead us to redefine the individual PD for compounds **4** and **6**. The histological studies showed that the liver, in some animals treated with drug **4**, presented a fibrous retraction with thickening of the Glisson capsule, mononuclear inflammatory process and some necrotic foci. So, in order to diminish these toxic effects the final PD for drug **4** was reduced

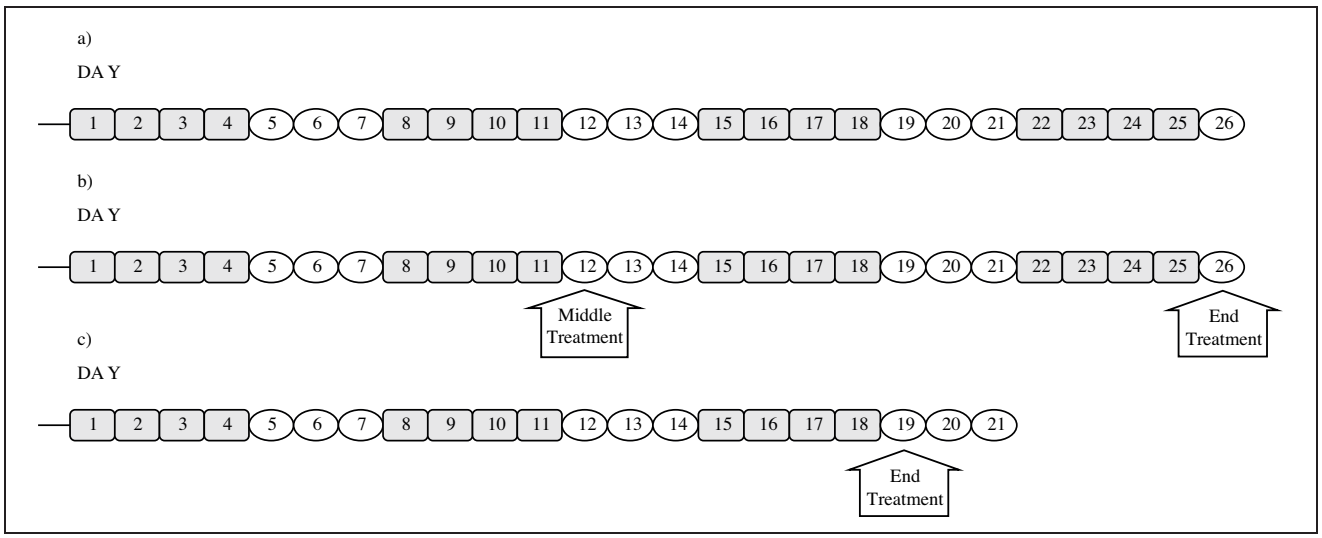


Fig. 1: Schedules of trials. a) Posology establishment, study effects of initial PD on tumour-free animals. b) Treatment with PD on sarcoma-carrier animals. c) Treatment with PD on carcinoma-carrier animals. Codes: ▭ treatment's days, ◯ rest's days

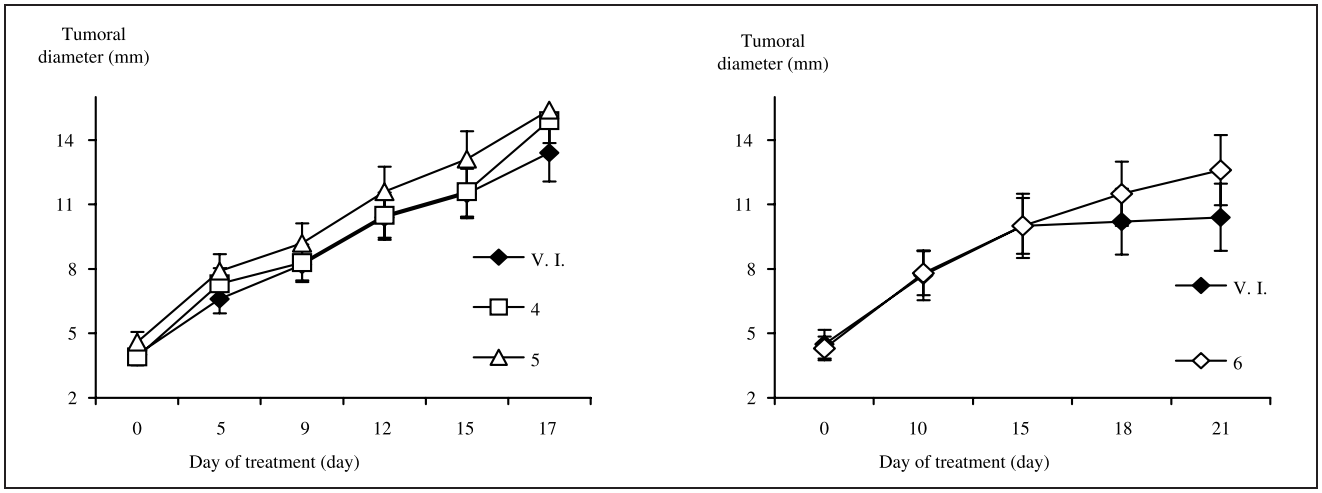


Fig. 2: *In vivo* treatment of the murine mammary adenocarcinoma. For details see section 4.2.7. a) Tumoral diameter vs day of treatment with drugs **4**, **5**, and V.I. b) Tumoral diameter vs day of treatment with drug **6**, and V.I.

to 1/8 of the MTD. The macroscopic observations in the dissection process for the animals treated with **6** showed adherence in liver, spleen, and intestine that prompted us to correct the PD. The final PDs for compound **6**, affording none of these effects, are reported in Table 2.

In vivo antitumoral trials were developed in murine models of CCRFS-180 II sarcoma and mammary adenocarcinoma (Basombrío and Prehn 1972). The compounds were tested with the same schedule of administration as for the establishment of PD using two groups of mice (Fig. 1b and 1c), each carrying implants of either sarcomas or mammary carcinomas. The trial lasted 26 days for sarcoma experiments and 19 to 21 days for adenocarcinoma experiments. Suitable controls of non-treated (N.T.) and V.I. animals were also included. For sarcoma model trial all drugs (**2**, **4**–**7**) were assayed, whereas for the adenocarcinoma model were tested the most active furoxans in the sarcoma model (drugs **4**–**6**) only. The effect of each drug against each tumour type was daily evaluated by measuring the tumour diameter. On days 12 and 26 for the sarcoma model (Fig. 1b) and on day 21 for the carcinoma model (Fig. 1c) the animals were sacrificed and the drug effects were analysed. The parameters used to evaluate the effects were weight and size of the dissected tumours, presence of metastasis and organ and tumour histology. The percentage of necrotic tumoral tissues was determined by observing the complete tumoral nodule and expressed as the average for each group of animals. Clinical biochemistry and haematological studies post-treatment were used to determine health of the animals and the participation of the immune response in the defence against the tumour.

The results of the trials show that no statistical differences between treated and non-treated animals were observed in the weight, form, evolution of size of tumours and presence of metastasis. Always, in the one-month trial the tumours grew continuously without significant differences between animals treated with drugs and V.I. animals (see i.e. Fig. 2). Never the existence of metastasis was observed.

These macroscopic evaluations of the animals treated with PD indicate that compounds **2**, **4**–**7** did not display any remarkable anti-tumoral activity in both models. However, histological studies revealed interesting differences. In the sarcoma model, treatment with compounds **5**, **6**, and to a less degree **2** lead to a significant increase in the percentage of the necrotic tumoral zones (Fig. 3a and 3b). Also this phenomenon was observed when the administration of drugs **5** and **6** was interrupted at day 11 (Fig. 3a) (Fig. 4).

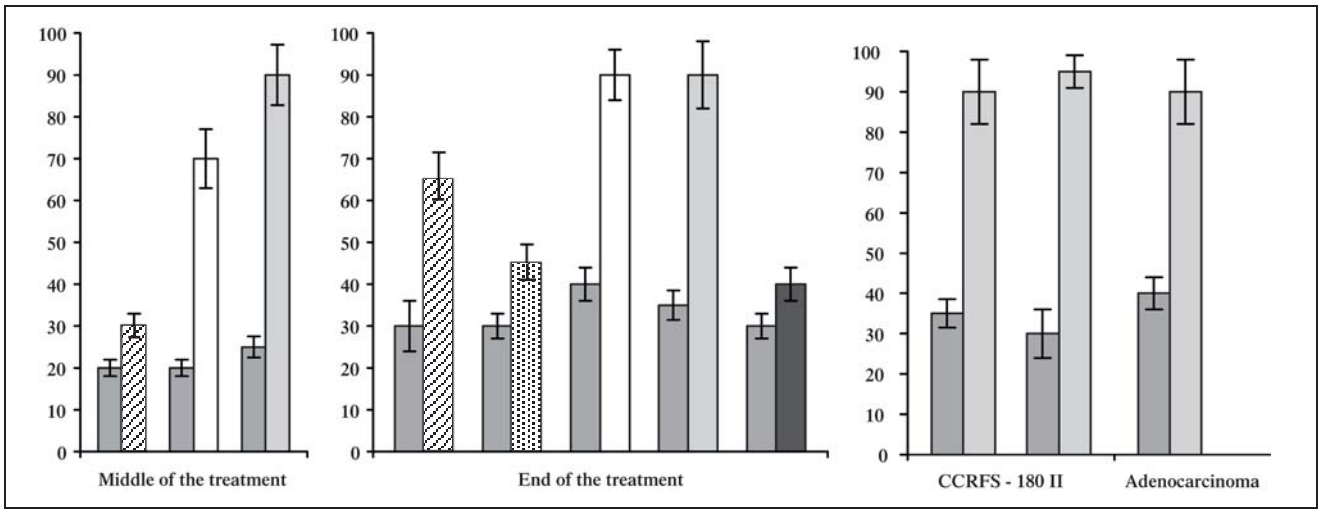


Fig. 3: Percentage of tumoral necrosis after the treatment with vehicle (■) and with drugs **2** (▨), **4** (⬚), **5** (□), **6** (▩) (PD_6 B), and **7** (■). a) Model of sarcoma, middle of the treatment. **b)** Model of sarcoma, end of the treatment. c) Trials with drug **6** (▩) at the end of the treatment, left to right: sarcoma, dose = PD_6 B; sarcoma, dose = PD_6 A; adenocarcinoma, dose = PD_6 B

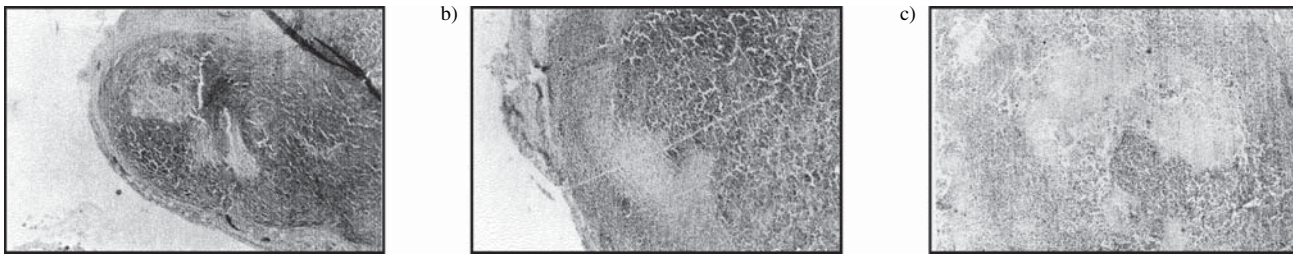


Fig. 4: Examples of the histological studies of sarcoma nodules in the middle of the treatment. For details see section 4.2.7. a) Tumoral nodule from V.I.-treated animals. Necrosis focus, occupying ca. 20% of the tumoral mass, without special distribution. b) Tumoral nodule from drug **2**-treated animals. Necrosis focus such as V.I.-treated animals with diffuse necrosis distribution but occupying ca. 30% of the tumoral mass. c) Tumoral nodule from drug **6**-treated animals. Necrosis in the core of the nodule occupying ca. 90% of the tumoral mass

Table 3: Histological results for selected organs in animals carrying CCRFS-180 II tumours

Animal treated with	Lungs[a]	Kidneys	Liver	Spleen	Heart	Intestine
V.I.	(II)	(I)	(II)	(I)–(II)	(I)	(I)
2	(II)	(I)	(II)	(I)–(II)	ND [b]	(I)
4	(II)	ND	(II)–(III)	(II)	(I)–(II)	(I)
5	(II)	(I)	(II)	(I)	(I)	(I)
6 PD_6 A	(II)	(I)–(II)	(II)	(I)	(I)	ND
PD_6 B	(II)	(I)	(II)	(I)	(I)	(I)
7	(II)	(I)	(II)	(I)–(II)	ND	ND

[a] The histological results were summarized as: (I) when the organ no present relevant changes respect to normal tissue; (II) when the organ present moderate kind of change; (III) when the organ present a great number of changes, [b] ND: not determined

Different patterns of necrosis were found for compounds **5** and **6** and **2**. For the first ones necroses were observed in the tumoral core whereas diffuse necroses were found for the last one. Moreover, animals treated with compounds **4**, **5**, and **6** presented an important degree of vascularization in the periphery of the tumoral nodules with respect to the V.I.- or drug **2**-treated animals. Adjacent to arterioles and capillaries viable cells in the mitotic phase were found. For animals treated with drug **7** histological observation revealed that the necrotic focus level and the appearance of tumoral nodules did not differ to that of V.I.-treated animals.

In the mammary tumour model, tumoral histological studies were performed only for drug **6**, the furoxan with the highest MTD and activity on the sarcoma model. Again, the relevant data in these studies was the high number of necrotic cells in the core of tumoral nodules. The percentage of necrotic areas observed was close to 100% (Fig. 3c). At the periphery of tumoral nodules 2–3 layers of viable cells were seen. These cells presented a high mitotic index displaying typical mitoses and "back to back" growing.

In addition, clinical biochemistry, haematological and histological studies of organs of tumour carrying animals, at the end of the trial, showed no statistically relevant differences for any of the analysed parameters between treated-, V.I.- and N.T.-animals (Table 3 and Table 4). These results, together with the behaviour of the animals, imply that drug PDs used in these trials did not produce relevant toxicity. However, animals treated with compound **4** at PD presented moderate histological changes in liver and heart in respect to V.I.-animals. Specifically, the livers presented diffuse congestion and focal necrosis, whereas the myocardium, without necrosis, showed arteriolar invasion by lymphocytes. Besides, animals treated with drug **6** at the highest assayed dose (PD_6 A) displayed little histological changes in kidneys in respect to V.I.-animals. The interstitial vessels of kidneys showed diffuse congestion when the animals were treated with PD_6 A, but these alterations were not observed when PD_6 B was used. Otherwise, animals treated with quinoxaline **2** displayed higher GPT and GOT values in respect to V.I.-animals but they were in agreement with the normal ones (Ofert et al. 1993).

3. Discussion

The promotion of necrosis by furoxans **5** and **6** could be the result of the particular biochemical behaviour of this kind of compounds. Furoxans represent a well known class of thiol-dependent nitric oxide (NO) donors (Kontogiorgis and Hadjipavlou-Litina 2002; Cerecetto and Porcal 2005). The bioactivation of furoxans *in vivo* is likely to be mediated mainly by non-enzymatic pathways involving the reaction with critical sulfhydryl groups. Otherwise, certain nuclear protein thiol groups appear to be intimately involved in gene control and neoplastic proliferation (Knock 1967; Fukuyama et al. 1996). Thus, compounds exerting an influence on the activity of such thiols may have potential anticancer effects. Furoxans which generate NO by interaction with sulfhydryl groups, may mimic the

Table 4: Mean values of the biochemical and the haematological findings in animals carrying CCRFS-180 II tumours

Animal treated with	Leukocyte (/μL)[a,b]	Haemoglobin (g/L)[b,c]	Hematocrite (%)[b,d]	GPT (UI/L)[b,e]	GOT (UI/L)[b,f]	Glucose (g/L)[b,g]
Healthy	6,300	16.3	53.6	ND[h]	ND	ND
N.T.	5,600	13.8	44.0	ND	ND	ND
V.I.	7,200	14.4	46.3	16.6	75.8	0.97
2	18,600	16.1	45.4	145.0	196.0	0.79
4	9,800	13.8	41.0	13.6	49.8	0.65
5	10,800	12.8	44.7	19.8	31.2	1.11
6 PD_6 A	ND	ND	ND	ND	ND	ND
PD_6 B	5,000	12.3	42.1	16.2	204.5	0.74
7	15,200	14.6	48.4	19.3	112.0	0.46

[a] Normal value: 5,000–13,700/μL, [b] From reference (Ofert 1993), [c] Normal value: 11.0–14.5 g/L, [d] Normal value: 35.0–45.0%, [e] Normal value: 28–184, [f] Normal value: 55–251, [g] Normal value: 0.97–1.86, [h] ND: not determined

action of activated macrophages which are thought to exert their cytotoxic effect against tumour cells by an enhanced release of NO (Hibbs et al. 1988; Moharram et al. 2004). Moreover, it was suggested that high concentrations of NO may lead to partial deamination of DNA bases and DNA strand breaks (Wink et al. 1991). Besides, considering the vasodilating properties and the vascular permeability capacity of NO in solid tumours (Medana et al. 1994; Gasco et al. 1998; Maeda et al. 1994), NO-releasing compounds could increase the tumour blood uptake and thus enhance uptake of drugs.

Furoxan **6** is a recognized NO-releasing product in the presence of thiol-containing compounds (Kontogiorgis and Hadjipavlou-Litina 2002; Cerecetto and Porcal 2005). Furoxans **4**, **5** and **7** and quinoxaline **2** did not show remarkable NO-releasing capacity in the presence of cysteine, although **5** has demonstrated moderate NO-releasing capacity in absence of thiols (data not shown). So, the NO-releasing property of furoxans may explain, at least in part, the anti-neoplastic activity of the studied products.

Effect of furoxans on *in vivo* anti-neoplastic properties were studied. For the first time furoxans are reported to promote tumoral necrosis at a dose where no side effects are detected. Furoxans **5** and **6** behave as antitumoral agents *in vivo* on CCRFS-180 II sarcoma and mammary adenocarcinoma. Pharmacologically active doses were deduced from the maximum tolerated dose. With the schedule of administration proposed in the present paper these drugs demonstrated that they are able to produce a necrotizing effect on tumours. Further studies with other tumoral models, doses and posology with these drugs are currently in progress.

4. Experimental

4.1. Synthesis of the compounds

Quinoxaline **2** and furoxans **4**–**7** were synthesized according to the methods reported by Monge et al. (1998b), Boiani et al. (2001) and Monge et al. (1995a). The purity of the compounds was established by microanalysis and thin layer chromatography.

4.2. Pharmacology

4.2.1. Formulation of drugs for in vivo trials.

Compounds **2** and **4**–**7** were suspended in sterile physiological saline: Tween 80 (4 : 1) solution (vehicle solution) immediately prior to injection. These preparations were made under aseptic conditions and in all cases an homogeneous suspension were obtained by shaking under ultrasound conditions.

4.2.2. Animals

The experiments were carried out on two month-old BALB-*c* female mice (20–22 g) bred under specific pathogen-free (SPF) conditions. Animals were housed in wire mesh cages at $20 \pm 2\,^{\circ}C$ with natural light-dark cycles. The animals were fed *ad libitum* with a standard pellet diet and water, and were used after a minimum of 3 days acclimation to the housing conditions (Institute of Laboratory Animal Resources, National Research Council, US 1996). Control and experimental group consisted of 10–20 animals. The experimental protocols with animals were evaluated and supervised by the local Ethics Committee and the research adhered to the Principles of Laboratory Animal Care (Morton and Griffiths 1985). Animals were evaluated applying international protocols and they were dealt with in a humane way in accordance with recognized guidelines on experimentation (American Veterinary Medical Association 1993). At the end of experiments they were anaesthetised with ethyl ether and sacrificed by cervical dislocation.

4.2.3. Biological samples

For the *in vivo* studies two kinds of biological samples were obtained: 1) Blood for biochemical and haematological studies was drawn by sectioning the subclavian artery, and maintained in EDTA or heparin anticoagulant at 0 °C. The determinations were carried out no more than 24 h post extraction. 2) Organs (lung, kidney, liver, spleen, heart, and intestine) and tumours were obtained by autopsy. The tumours were measured and weighed together with the organs and were maintained in aqueous formalin solution (10%) for further histological studies.

4.2.4. In vivo generation of carcinomas

Sarcomas was obtained by intradermal inoculation of $10–150 \times 10^6$ tumour cells (CCRFS-180 II). The tumour cells, obtained from multiparous females, were taken from the 2^{nd}–8^{th} *in vitro* passage. Pharmacological studies were initiated on day 15 after inoculation, when the tumours had 1 cm of diameter. The mammary tumour was grown intradermally in the flank, from an inoculation of $10–150 \times 10^6$ tumour cells. The tumour cells, obtained from multiparous females, were taken from the 2^{nd}–3^{rd} *in vivo* passage (Basombrío and Prehn 1972).

4.2.5. Determination of MTD

The MTD was defined as a single i.p. dose of drug that produce in the third day post administration a 10% weight decrease respect to the non-treated animal (Monge et al. 1995b, 1995c). MTD was determined in healthy tumour free animals, for quinoxaline **2** (reference drug) and furoxans **4**–**7** with 1.0 mL i.p. applications. Five to eight doses of each drug were studied, administered as a solution prepared as indicated above (see 4.2.1.). As a negative control, animals treated i.p. with 1.0 mL of vehicle solution (V.I., vehicle-injected), were included. Treated animals were weighed and observed daily for alterations in skin, physical aspect, activity and faeces aspect. On day 3 the animals were sacrificed, dissected, and the organs and blood were submitted for further studies.

4.2.6. Treatment of healthy animals with pharmacological doses at the established posology

The initially established PD, 1/6 of the MTD, was injected i.p. (0.5 mL) daily during 4 days with 3-days rest interval on tumour-free animals. This schedule was repeated 4 times. The experiments lasted 25 days of which 16 were devoted to treatment (Fig. 1a). Also, a negative control, V.I. animals, was included. During the 25-days experiment the animals were daily weighted and observed, and the microenvironment was examined. At the end of the experiments (day 26^{th}) the animals were sacrificed and dissected and the organs and blood were submitted for further studies.

4.2.7. In vivo antitumoral trials

Drugs were tested with the same schedule of administration described above for the establishment of PD using two groups of mice, each carrying implants of either sarcomas or mammary carcinomas. Suitable controls of N.T. and V.I. animals were also included. The effect of each drug against each tumour type was daily evaluated by measuring the tumour diameter. On days 12 and 26 for the sarcoma model and on day 21 for the carcinoma model the animals were sacrificed. The dissected tumours were weighed and measured, the presence of metastases was determined and histological studies on organs and tumours were performed. These studies were carried out on hematoxilin-eosin-stained sections. Percentage of necrotic tumoral tissues was estimated in each tumour by observing the complete tumoral nodule and expressed as the average for each group of animals.

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