Eur J Med Chem (1996) 31, 735–740

Short communication

Synthesis of 1-(isoxazol-3-yl)triazene derivatives. Antimetastatic activity of 3,3-dimethyl-1-(5-methylisoxazol-3-yl)triazene

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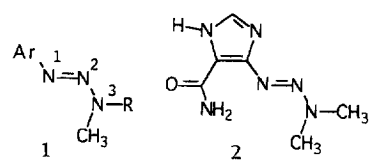
(Received 4 January 1996; accepted 11 March 1996)

Summary — The synthesis of some 3,3-dialkyl-1-(isoxazol-3-yl)triazenes is reported, together with preliminary biological tests on 3,3-dimethyl-1-(5-methylisoxazol-3-yl)triazene **3a**. This compound exerts a selective antimetastatic action in mice bearing Lewis lung carcinoma, reducing the weight but not the number of metastases. Some unfavorable properties in the toxicological profile of **3a** have discouraged further investigation of this class of compounds.

triazene / 1-(isoxazol-3-yl)triazene derivative / 3,3-dimethyl-1-(5-methylisoxazol-3-yl)triazene / antimetastatic activity

Introduction

Triazenes **1** [Ar = (hetero)aryl, R=alkyl] have been thoroughly investigated for their antitumor activity [1]. 5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide **2** (Dacarbazine) [2] is currently used in the treatment of malignant melanoma [3].

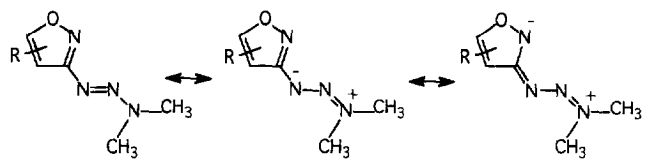


Due to the failure to separate the toxophoric from the pharmacophoric determinants in compounds **1** [4–6], interest has shifted to structurally different triazenes, such as 1,3-dialkyl-3-acyltriazenes [7], bistriazenes [8] and imidazotetrazinones [9].

3,3-Dimethyl-1-(hetero)aryltriazenes were found to possess selective antimetastatic action, apparently due to a mechanism other than that of cytotoxicity [10]. A quantative structure–activity relationship (QSAR) study on a series of *p*-substituted phenyldimethyltriazenes indicated dissociation between antimetastatic and antitumor effects in an experimental Lewis lung carcinoma model [11]. It is accepted that: (i) electron-donating substituents on the aryl group of **1** increase the toxicity of the compounds, due to the rapid generation of the diazonium ion [12]; and (ii) the mutagenicity (Ames test) of a series of heterocyclic 3,3-dimethyltriazenes correlates positively with the electron-donating and hydrophobic properties of the heteroaryl system [13].

We decided, in connection with other projects [14–16], to synthesize a series of 3,3-dimethyl-1-(isoxazol-3-yl)triazenes **3** and to evaluate their antimetastatic effects in mice bearing Lewis lung carcinoma. 3,3-Dimethyl-1-(3,5-dimethyl–isoxazol-4-yl)-triazene [17], a few 1,3-di(isoxazol-3-yl)triazenes [18–21] and 3-aryl-1-(isoxazol-5-yl)triazenes [22, 23] are known; 3,3-dialkyl-1-(isoxazol-3-yl)triazenes **3** are new. The isosteric replacement of the aryl group of **1** with the 'hydrophilic' isoxazol-3-yl moiety might result, via a mesomeric effect (scheme 1), in an overall electron-withdrawal from the triazene chain, thus reducing the toxicity of compounds **3** (points (i) and (ii) above). The bond order and length of the N_2–N_3 bond of **3a**, 3,3-dimethyl-1-phenyltriazene and 3,3-dimethyl-1-(4-nitro-phenyl)triazene were calculated by semiempirical quantum-mechanical methods (AM1, PM3 and MNDO): the values obtained with

these methods indicate, at a qualitative level, a close similarity between the N2–N3 bond of **3a** and 3,3-dimethyl-1-(4-nitrophenyl)triazene; for example the MNDO method gave for **3a**, 3,3-dimethyl-1-(4-nitrophenyl)triazene, and 3,3-dimethyl-1-phenyltriazene, bond orders of 1.15, 1.13, and 1.09 respectively and values of bond lengths of 1.324, 1.327 and 1.337 Å.



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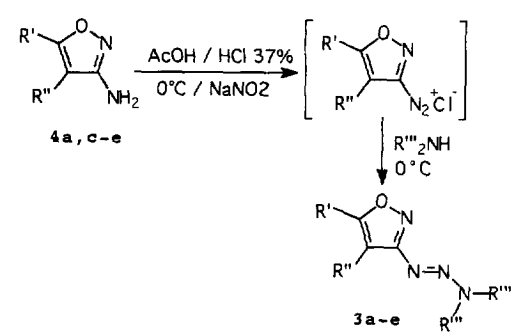
Scheme 1.

The assumption of the presence of a mesomeric effect in **3a** has subsequently found support in spectroscopic observations. The ^{1}H-NMR spectra of 3,3-dialkyl-1-aryltriazenes show one or two signals for the alkyl groups in N_3 as a function of the rotational freedom around the N_2–N_3 bond and of the temperature [24–26]. Whereas in 3,3-dialkyl-1-(substituted-phenyl)triazenes the N_3-alkyl signals have a coalescence point at low temperature [24, 26], in 3,3-dimethyl-1-(4-nitrophenyl)triazene [24], Dacarbazine **2** [25] and **3** (see the *Experimental protocols*) the N_3-methyl signals are distinct even at room temperature, because of the pronounced electron-withdrawing effect exerted by the (hetero)aryl group on the triazene chain.

Lastly, the higher thermodynamic stability of compounds **3** with respect to **2** was demonstrated by means of mass spectrometry techniques [27].

Because of the preliminary character of the present communication we report here the pharmacological tests of the simplest term of our series, ie, compound **3a**.



Compounds	R'	R"	R'"
a	Me	H	Me
b	Me	H	Et
c	Me	COOEt	Me
d	Ph	COOEt	Me
e	H	Ph	Et
f	H	COOEt	Me
g	Me	NO2	Me
h	Me	NO2	Et

Scheme 2.

Chemistry

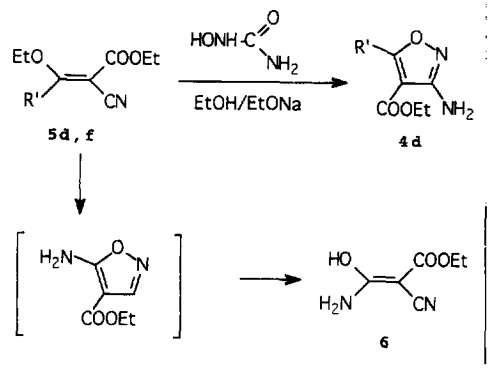
1-Aryl-triazenes are synthesized by *N*-coupling of aryldiazonium salts with the appropriate amine; in our case diazotization of 3-isoxazolamine **4a,c–f** and subsequent coupling with dimethylamine or diethylamine gave the desired triazenes **3a–e** in yields of 28–76% (scheme 2). We used strongly acidic conditions to avoid coupling between the isoxazol-3-yldiazonium ion and the unreacted 3-isoxazolamine which occurs in dilute HCl [18–21].

Compounds **3g,h** were obtained by nitration of the corresponding triazenes **3a,b** with nitric acid in sulfuric acid. The structure of **3a–e,g,h** was confirmed by ^{1}H-NMR and MS data; the latter show molecular ions and significant fragments corresponding to the isoxazolyldiazonium fragment ($M^+ - NMe_2$). We failed to obtain compound **3f**; diazo-coupling of **4f** with aqueous dimethylamine under the conditions reported above gave only unidentified decomposition products.

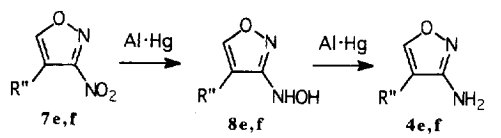
Compound **4a** is commercially available; **4c,d** were prepared by the procedure reported for **4c** [28] (scheme 3). This method cannot be applied to the synthesis of **4f**, because treatment of ethyl 2-cyano-3-ethoxyacrylate **5f** with *N*-hydroxyurea in MeONa/MeOH yields **6** (70%), from the hydrolysis of the unstable 4-carbethoxy-5-aminoisoxazole (scheme 3) [28]. We prepared **4f** by reduction of **7f** [16] (scheme 4); similarly reduction of **7e** [16] gave compound **4e**.

Results and discussion

The effects of **3a** on mice bearing Lewis lung carcinoma were evaluated with regard to the growth of the primary tumor and to its metastatic dissemination.



Scheme 3.



Scheme 4.

Starting 24 h after tumor implantation, **3a** was administered ip at the maximum tolerated dose (120 mg/kg/day) for 14 days. The animals were sacrificed 21 days after the implantation of the tumor, and the weight of the primary tumors and the number and weight of the metastases were evaluated. The maximum tolerated dose of compound **3a** ($LD_{0.05}$) in a 14-day treatment schedule is 120 mg/kg/day (60 mg/kg/day for Dacarbazine [29]).

The cytotoxic and antimetastatic effects of **3a** are summarized in table I. Compound **3a** did not inhibit the growth of the primary tumor; however, it reduced the weight of the metastases by 93% (*%T/C* = 7±3; table I) with disappearance of visible metastases in some of the animals. This antimetastatic effect is greater than that of 3,3-dimethyl-1-phenyltriazene (*%T/C* = 15) and similar to that of the most active terms of the aryl analogues such as 3,3-dimethyl-1-(4-sulfonamidophenyl)triazene (*%T/C* = 3) or 3,3-dimethyl-1-(4-methylthiophenyl)triazene (*%T/C* = 4) [11]. In contrast, the reduction in the number of metastases was poor (*%T/C* = 84±18); dissociation between the two effects, ie, the weight and the number of the metastases has been reported neither for Dacarbazine [30] nor for the phenyl series [11].

This dissociation effect needs further study. An explanation may be sought in the different pharmacokinetics of Dacarbazine, 4-(3,3-dimethyl-1-triazeno) benzoic acid potassium salt and **3a**; whereas the antimetastatic effects of the former two compounds persist after discontinuation of treatment [10], the prevention metastatic migration by **3a** seems to be limited to the time of the treatment. This, and the lack of cytotoxic effects of **3a**, would explain the number of micrometastases observed, probably originating upon discontinuation of the treatment. To verify this hypothesis we used a protocol in which **3a** was administered over the 21 days of the experiment (see the *Experimental protocols*).

The administration of **3a** at 100 or 80 mg/kg/day for 21 days was well tolerated in non-tumored animals (all these animals survived the experiment and the deaths started 19 days after the last treatment), but in tumor-bearing mice, the same protocol resulted in a great number of deaths (11/14), mostly on the 18th day. This made statistical analysis of the data impossible .

The control tumored animals, treated with the vehicle alone, survived the experiment and it may be inferred that the number of deaths observed during the treatment of the tumored animals with **3a** may be due to a negative interaction of this compound with the

Table I. Effects of the treatment with **3a** on primary tumor growth and metastatisis formation in mice bearing Lewis lung carcinoma[a].

	Weight of primary tumor (mg)			*Effect on metastases*					*Thymus*[f]	*Spleen*[f]
	14 days[b]	*21 days*[c]	*% T/C*[c,d]	*Number*	*% T/C*[d]	*Weight (mg)*	*% T/C*[d]	*% free*[e]	*(mg)*	*(mg)*
Control	2649 ± 298	4642 ± 578		50 ± 6		277 ± 44		0	19.5 ± 2.7	294 ± 27
Treated	2012 ± 438	3200 ± 804	69 ± 17	42 ± 9	84 ± 18	20 ± 8	7 ± 3	30	6.7 ± 1.7[g]	97 ± 24[g]

[a]Each value is the mean (± SE) obtained using groups of ten BD2F1 mice treated ip with **3a** at a dose of 120 mg/kg/day daily on days 1–14 after tumor inoculation. [b]Primary tumor weight evaluated 14 days after tumor implantation. [c]Primary tumor weight evaluated 21 days after tumor implantation. [d]*% T/C* is the percent ratio between the average values (± SE) of the treated group and controls. [e]Percentage of animals without visible metastases. [f]Weight of thymus or spleen at sacrifice. [g]Means significantly different from untreated controls, Mann–Whitney U test $P < 0.05$.

immunoresponse of the host. The remarkable reduction of the weight of the thymus and spleen (see table I) indicates that compound **3a** is immunotoxic; however, Dacarbazine shares this property [31] and therefore the explanation of these data must be more complex.

Thus while further study is necessary to understand the action of **3a**, the unfavorable properties in the toxicological profile of this molecule have discouraged in vivo testing of congeners **3b–e,g–h**.

Experimental protocols

Chemistry

Solvents and reagents were of the highest commercial grade and were used without additional purification. Melting points were determined on a Büchi SMP-510 capillary melting point apparatus, and are uncorrected. Infrared spectra were obtained on a Perkin-Elmer 257 or a Bruker FT-48 spectrometer; absorbances are reported in ν (cm^{-1}). NMR spectra were recorded on a Bruker AC 200 spectrometer unless stated differently; chemical shifts are reported in ppm and given in δ units relatively to TMS as an internal standard. EI-MS spectra (70 eV) were taken on a Fisons Trio 1000 and the FAB spectra on a Fisons VG Autospec. Elemental analyses were performed on a Carlo Erba 1106 analyzer and were within ±0.4% of calculated values.

Quantum-mechanical semiempirical calculations.
Structures were built using Sybyl (Molecular Modeling System, version 6.1, Tripos Ass, St Louis, USA) and minimized with Mopac in order to fully optimize the geometry; calculations of the physicochemical properties of the N_2–N_3 bond were performed by Mopac using AM1, PM3 and MNDO methods.

*Ethyl 3-amino-5-phenyl-4-isoxazolcarboxylate **4d**.*
This compound was prepared by partial modification of the method reported for **4c** [28]. Thus, *N*-hydroxyurea (2.5 mmol) in dry ethanol (4 mL) and ethyl 2-cyano-3-ethoxy-3-phenylacrylate [32] (2.4 mmol) were added in that order to a solution of Na (2.8 mmol) in dry ethanol (1 mL). The mixture was stirred overnight, water was added carefully, the mixture was extracted with EtOAc and the organic phase was dried and evaporated to give a crude product which was purified by flash chromatography (cyclohexane/ethyl acetate 7:3) to give 56% of pure **4d** as a white solid. Crystallization (diethyl ether/hexane) gave white crystals, mp 105–106 °C; IR (Nujol): ν 3420, 3300 (NH_2), 1700 (C=O), 1620 (C=N) cm^{-1}; ^{1}H-NMR: δ 1.31 (t, 3H, CH_2CH_3); 4.32 (q, 2H, CH_2CH_3); 5.05 (br s, 2H, NH_2); 7.43–7.93 (m, 5H arom); MS (EI): *m/z* (%) 232 (M^+, 89) 186 (M^+ – CH_3CH_2OH, 100) 105 (PhCO, 22) 77 (Ph, 13); the fragmentation pattern is closely related to that of 5-phenyl-3-isoxazolamine and is inconsistent with that of 3-phenyl-5-isoxazolamine [33]. Anal $C_{12}H_{12}N_2O_3$ (C, H, N).

*Reduction of 3-nitroisoxazoles **7e,f** to 3-isoxazolamines **4e,f**. General procedure*
The appropriate nitroisoxazole **7e,f** (1 mmol) [16] was dissolved in a mixture of diethyl ether (4.5 mL) and water (0.45 mL) and refluxed with an excess of amalgamated aluminium. The starting material disappeared (TLC) within 1–3 h, giving the hydroxylamine **8e,f**, which proceeded to form the desired **4e,f** (5–6 h). The mixture was cooled, filtered and the filtrate evaporated to give a crude material which upon flash chromatography (cyclohexane/ethyl acetate, 7:3) gave pure **4e,f**. When the reaction was interrupted after 1 h and worked-up as described, flash chromatography afforded mainly *N*-(4-phenylisoxazol-3-yl)hydroxylamine **8e** [MS (EI): *m/z* (%) 176 (M^+, 100)], or ethyl 3-hydroxylamino-4-isoxazolcarboxylate **8f** [mp 119–124 °C (ethyl acetate); ^{1}H-NMR: δ 1.36 (t, 3H, CH_2CH_3), 4.33 (q, 2H, CH_2CH_3), 6.73 (br s, 1H), 7.80 (br s, 1H), 8.66 (s, 1H, isoxazole C*H*); MS (EI): *m/z* (%) 172 (M^+, 89), 144 (M^+ – NHOH, 100)].

*4-Phenyl-3-isoxazolamine (**4e**).* This compound was obtained as a white solid, yield 82%. Crystallization (diisopropyl ether) gave white crystals, mp 113–115 °C; IR (Nujol): ν 3440, 3280, 3180 (NH_2), 1630 (C=N) cm^{-1}; ^{1}H-NMR (60 MHz): δ 4.1 (br s, 2H, NH_2), 7.3–7.5 (m, 5H arom), 8.1 (s, 1H, isoxazole C*H*); MS (EI): *m/z* (%) 160 (M^+, 68), 131 (18), 104 (100), 89 (32), 77 (18). Anal $C_9H_8N_2O$ (C, H, N).

*Ethyl 3-amino-4-isoxazolcarboxylate **4f**.* This compound was obtained as a white solid, yield 36%. Crystallization (diethyl ether/petroleum ether) gave white crystals, mp 91–92 °C; IR (Nujol): ν 3300, 3200 (NH_2), 1680 (C=O), 1600 (C=N) cm^{-1}; ^{1}H-NMR: δ 1.38 (t, 3H, CH_2CH_3), 4.33 (q, 2H, CH_2CH_3), 4.95 (br s, 2H, NH_2), 8.59 (s, 1H, isoxazole C*H*); MS (EI): *m/z* (%) 156 (M^+, 87), 128 (M^+ – CH_2=CH_2, 37), 110 (M^+ – CH_3CH_2OH, 100), 84 (15), 68 (8). Anal $C_6H_8N_2O_3$ (C, H, N).

*Preparation of 3,3-dialkyl-1-(isoxazol-3-yl)triazenes **3a–c,e**. General procedure*
The 3-isoxazolamine **4a,c,e** (2 mmol) was dissolved in a 1:1 mixture of glacial acetic acid and 37% hydrochloric acid (2 mL). The solution was cooled to 0 °C and treated portionwise, over 45 min, with ground sodium nitrite (2 mmol); the mixture was stirred at 0 °C for 15 min, then carefully poured into 6 mL of a cooled (0 °C), 40 % (ω) water solution of dimethylamine **3a,c** or diethylamine **3b**; cold (0 °C), pure diethylamine (8 mL) was used in the preparation of **3e**. The mixture was stirred at 0 °C for 30 min, then warmed to rt. After addition of water (10 mL), it was extracted with ethyl acetate: the organic phase was washed (water), dried and evaporated in vacuo to give a product **3a–c,e** which was purified by flash chromatography (cyclohexane/ethyl acetate 7:3).

*3,3-Dimethyl-1-(5-methylisoxazol-3-yl)triazene **3a**.* Pale yellow solid, yield 76%. Crystallization (isopropyl ether) gave pale yellow crystals, mp 71–72 °C ; IR (deuterochloroform): ν 1610 (C=N) cm^{-1}; ^{1}H-NMR: δ 2.38 (s, 3H, isoxazole CH_3), 3.24 (s, 3H, NCH_3'), 3.53 (s, 3H, N-CH_3"), 6.10 (s, 1H, isoxazole C*H*); MS (EI): *m/z* (%) 154 (M^+, 37), 110 (100). Anal $C_6H_{10}N_4O$ (C, H, N).

*3,3-Diethyl-1-(5-methylisoxazol-3-yl)triazene **3b**.* Yellow oil, yield 59%; IR (deuterochloroform): ν 1610 (C=N) cm^{-1}; ^{1}H-NMR: δ 1.23 (dt, 6H, $NCH_2'CH_3'$ and $NCH_2'CH_3$"), 2.33 (s, 3H, isoxazole CH_3), 3.74 (dq, 4H, $NCH_2'CH_3'$ and NCH_2"CH_3"), 6.07 (s, 1H, isoxazole C*H*); MS (EI): *m/z* (%) 182 (M^+, 38), 110 (100). Anal $C_8H_{14}N_4O$ (C, H, N).

*Ethyl 3-(1,1-dimethyltriazenyl)-5-methyl-4-isoxazolcarboxylate **3c**.* Yellow oil, yield 40%; IR (chloroform): ν 1700 (C=O), 1600 (C=N) cm^{-1}; ^{1}H-NMR: δ 1.34 (t, 3H, CH_2CH_3), 2.65 (s,

3H, isoxazole CH_3), 3.30 (s, 3H, NCH_3'), 3.59 (s, 3H, NCH_3''), 4.30 (q, 2H, CH_2CH_3); MS (FAB): *m/z* (%) 227 (MH^+, 100), 182 (56). Anal $C_9H_{14}N_4O_3$ (C, H, N).

3,3-Diethyl-1-(4-phenylisoxazol-3-yl)triazene ***3e***. Amorphous solid, yield 28%; IR (chloroform): ν 1610 (C=N) cm^{-1}; ^{1}H-NMR (60 MHz): δ 1.2 (dt, 6H, NCH_2CH_3, and $NCH_2'CH_3''$), 3.8 (dq, 4H, $NCH_2'CH_3'$ and $NCH_2''CH_3''$), 7.0–7.8 (m, 5H arom), 8.4 (s, 1H, isoxazole C*H*); MS (EI): *m/z* (%) 244 (M^+, 10), 172 (59), 116 (100), 89 (51), 57 (48). Anal $C_{13}H_{16}N_4O$ (C, H, N).

Ethyl 3-(1,1-dimethyltriazenyl)-5-phenyl-4-isoxazolcarboxylate ***3d***.
This compound was prepared by partial modification of the method for **3a–c,e**: **4d** (2 mmol) in a 1:1 mixture of glacial acetic acid and 37% hydrochloric acid (4 mL) was cooled to 0 °C and treated portionwise (over 45 min) with sodium nitrite (2 mmol). The mixture was stirred at 0 °C for 15 min, then the resulting creamy mass was treated carefully at 0 °C with a 40% (w) water solution of dimethylamine (6 mL), stirred at 0 °C for 30 min, and warmed to rt. The standard work-up gave a material which was purified by flash chromatography (cyclohexane/ethyl acetate 7:3) to give 30% of **3d**, mp 90–91 °C (Et_2O/petroleum ether); IR (chloroform): ν 1720 (C=O), 1600 (C=N) cm^{-1}; ^{1}H-NMR: δ 1.30 (t, 3H, CH_2CH_3), 3.32 (s, 3H, NCH_3'), 3.58 (s, 3H, NCH_3''), 4.31 (q, 2H, CH_2CH_3), 7.90–7.47 (m, 5H arom); MS (FAB): *m/z* (%) 289 (MH^+, 100), 244 (12), 147 (66), 105 (79). Anal $C_{14}H_{16}N_4O_3$ (C, H, N).

Nitration of compounds ***3a,b*** *to* ***3g,h***. *General procedure*
Compound **3a** (or **3b**) (2 mmol) was dissolved in cooled concentrated sulfuric acid (1.2 mL) (the process is exothermic), then nitric acid 90% (0.3 mL) was added carefully and the mixture stirred at rt for 1 h. The mixture was cautiously poured into water (20 mL) and extracted with CH_2Cl_2. The organic phase was dried and evaporated to give crude **3g,h,** which were purified by flash chromatography (cyclohexane/ethyl acetate 1:1).

3,3-Dimethyl-1-(5-methyl-4-nitroisoxazol-3-yl)triazene ***3g***. Yield 35%. Colorless needles, mp 66 °C (methylene chloride/petroleum ether); IR (Nujol): ν 1606 (C=N), 1520 (C–NO_2) cm^{-1}; ^{1}H-NMR: δ 2.80 (s, 3H, isoxazole CH_3), 3.36 (s, 3H, NCH_3'), 3.64 (s, 3H, NCH_3''); MS (EI): *m/z* (%) 199 (M^+, 17), 155 (100). Anal $C_6H_9N_5O_3$ (C, H, N).

3,3-Diethyl-1-(5-methyl-4-nitroisoxazol-3-yl)triazene ***3h***. Yellow oil, yield 70%; IR (film): ν 1610 (C=N), 1517, 1356 (C–NO_2) cm^{-1}; ^{1}H-NMR (60 MHz): d 1.3 (dt, 6H, $NCH_2'CH_3'$ and $NCH_2''CH_3''$), 2.7 (s, 3H, isoxazole CH_3), 3.8 (dq, 4H, $NCH_2'CH_3'$ and $NCH_2''CH_3''$); MS (EI): *m/z* (%) 227 (M^+, 7), 155 (100). Anal $C_8H_{13}N_5O_3$ (C, H, N).

Pharmacology

Drug toxicity was evaluated in non-tumored female BD2F1 mice weighing 18–20 g (Charles River, Calco, Como, Italy.). The animals were treated daily ip for 14 consecutive days, using the same treatment schedule employed in the antitumor and antimetastatic studies. Compound **3a** was administered ip in volumes of 0.05 mL per 10 g of body weight as a freshly prepared sonicated suspension in 0.9% NaCl aqueous solution containing 1% carboxymethylcellulose. Toxicity was determined using groups of five mice for each drug level. The $LD_{0.05}$ (dosage causing a lethality of 0.05%) was derived graphically from the % effect (lethality) vs log of dose plots, following the method of Litchfield and Wilcoxon [34].

The evaluation of **3a** on tumor growth and metastatic dissemination was performed in female BD2F1 mice inoculated im with a single cell suspension of 10^6 Lewis lung carcinoma cells prepared as already reported [35]. Daily treatment was started 24 h after tumor implantation and was continued for 14 days.

The primary tumor volume was determined at 14 and 21 days after tumor inoculation by caliper measurements of short (*a*) and long (*b*) axes (cm) using the following equation:

$$\text{Tumor weight} = \pi/6 \times a^2 \times b \qquad [1]$$

The number of metastases was determined at sacrifice on day 22 after tumor inoculation, by examining the surface of the lungs by a low-power microscope. The weight of metastases was determined as the sum of their individual weights calculated according to equation [1] after determination of their dimension by an ocular micrometer. At the sacrifice, thymus and spleen weights were also recorded.

Acknowledgments

Thanks are due to the Ministero dell'Università e della Ricerca Scientifica e Tecnologica, Roma for financial support and to G Costantino (University of Perugia, Italy) for the semiempirical quantum-mechanical calculations.

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