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NEW DERIVATIVES OF 5-NITROINDAZOLE WITH POTENTIAL ANTITUMOR ACTIVITY

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New 5-nitroindazole derivatives substituted in 1st position with radicals that contains the di-(β-chloroethyl)-amine group have been synthesized in order to obtain substances with enhanced antitumor activity. The structure of the new compounds has been confirmed by elemental and spectral analyses (^{1}H-NMR and FT-IR). The potential cytostatic action of the compounds studied on Guérin experimental tumors proves that new alkylating agents significantly suppress the proliferation of the neoplastic cells.

INTRODUCTION

The remarkable pharmacodynamics properties of many chemical compounds widely used in medicine are the result of interactions between certain groups of atoms within molecule and biological environment. The indazolic ring and di-(β-chloroethyl)-amine group are structures often considered as potential candidate for synthesis of substances for medical applications due to their notable biological activity. The substances containing indazolic ring have antiinflammatory, fungicidal, bactericidal, hepatoprotective, analgesic-antipyretic, antibacterial, antiangiogenic, antiprotozoal and cytostatic properties.[1-9] Di-(β-chloroethyl)-amine group is known as one of the most active alkylating agents, with antitumoral activity that can be explained by the capacity of alkylating a number of cellular components (amines, alcohols etc) through nucleophilic substitution. In order to eliminate the high toxicity of substances containing di-(β-chloroethyl)-amine group, methods of grafting di-(β-chloroethyl)-amine group on various organic substrates (both biologically active and with no toxicity) were proposed.[10-17]

Synthesis of organic compounds containing indazole ring and di-(β-chloroethyl)-amine group is very useful both for chemical and biological studies on the mechanisms of mutual influence within the molecule or of the global influence over the biological activity as well for the potential applications in medical treatments as new substances with high antitumor activity.

The present study continues the previous work related to the synthesis of the compounds containing di-(β-chloroethyl)-amine group and proposes new different ways of preparation of chemical complexes with di-(β-chloroethyl)-amine group grafted on the pyrazol ring of the substrate.[10-15]

RESULTS AND DISCUSSION

The procedure for the synthesis of the bioactive compounds studied in this work involves different stages, schematically shown in Figs.1-3. Sodium

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salt (I) was obtained by dissolving of 5-nitroindazole in hot alcoholic solution containing the sodium etoxid. The reaction between (I) and sodium monochloroacetate produces a sodium salt of acid 5-nitroindazole-1-yl-acetic (II) (see Fig. 1).

The reactions between sodium salts (I) or (II) and tris β-chloroethyl amine create new organic compounds with alkylating group grafted on indazolic substrate through an ethylene bridge: 1-[N-di-(β-chloroethyl)-aminoethyl]-5-nitroindazole (III) and ester form 1-[N-di-(β-chloroethyl)-aminoethyl)-carboxy-methyl]-5-nitroindazole (IV), respectively (see Fig. 2).

Quantitative chemical analysis and the spectral investigations using FT-IR and ^{1}H-NMR methods, as well as good yield obtained for pure products prove that the reactions occur at the nucleophile center in the molecule of sodium salt, which is negatively charged nitrogen-bearing for the compound (I) and oxygen derived from carboxyl group for the compound (II).

Attaching the group alkylated by ethylene and ester bridge is important because they ensure the split of the molecule *in vivo*, thus releasing the alkylating fragment.

The reaction of sodium salt of the 5-nitroindazole (I) with monochloroacetic acid chloride creates N-chloroacethyl-5-nitroindazole (V) which in reaction with di-(β-chloroethyl)-amine produces N-[di-(β-chloroethyl)-aminoacethyl]-5-nitroindazole (VI) (see Fig. 3).

The substituted acetyl group is also present in the biological substrate (proteins, etc.) so the compound (VI) having this common group with the substrate will be probably more easily accepted by this.

The compounds (I)-(VI) are new and unreported in the literature. The preparation steps are presented for each particular compound in the Experimental Part. The specific characteristics and chemical structures were established by chemical and spectral analysis (FT-IR and ^{1}H-NMR).

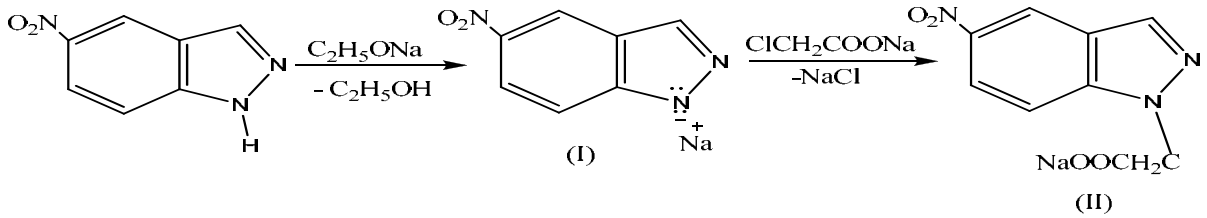

Fig. 1 – Synthesis steps for the compounds (I) and (II).

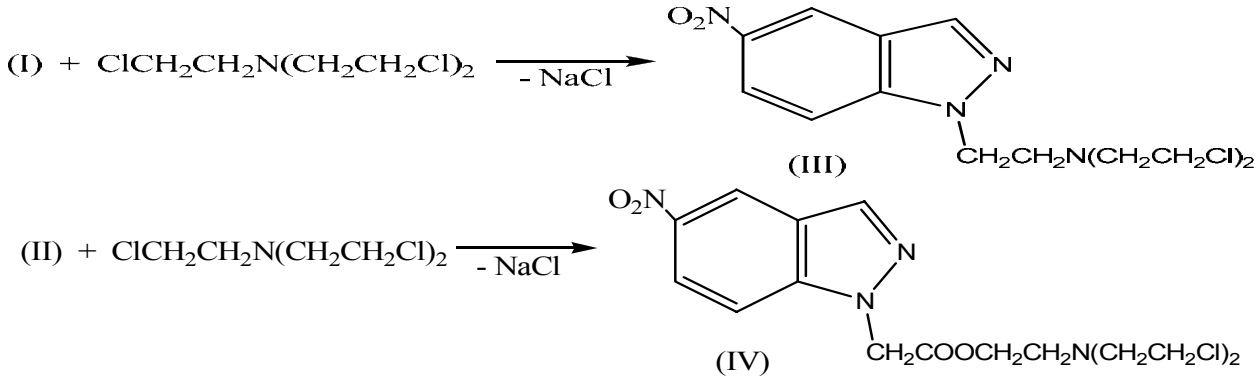

Fig. 2 – Synthesis steps for the compounds (III) and (IV).

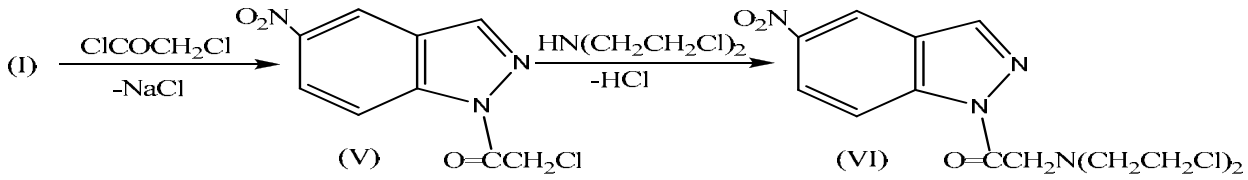

Fig. 3 – Synthesis steps for the compound V and VI.

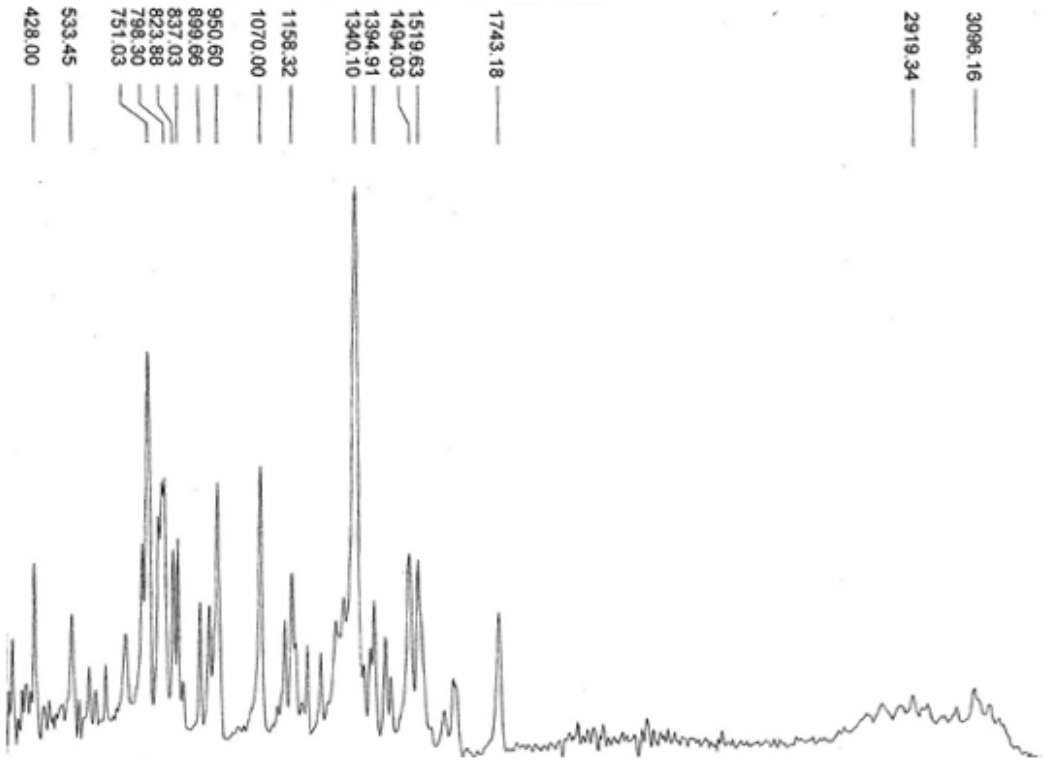

Fig. 4 – FT-IR spectrum for the compound (IV).

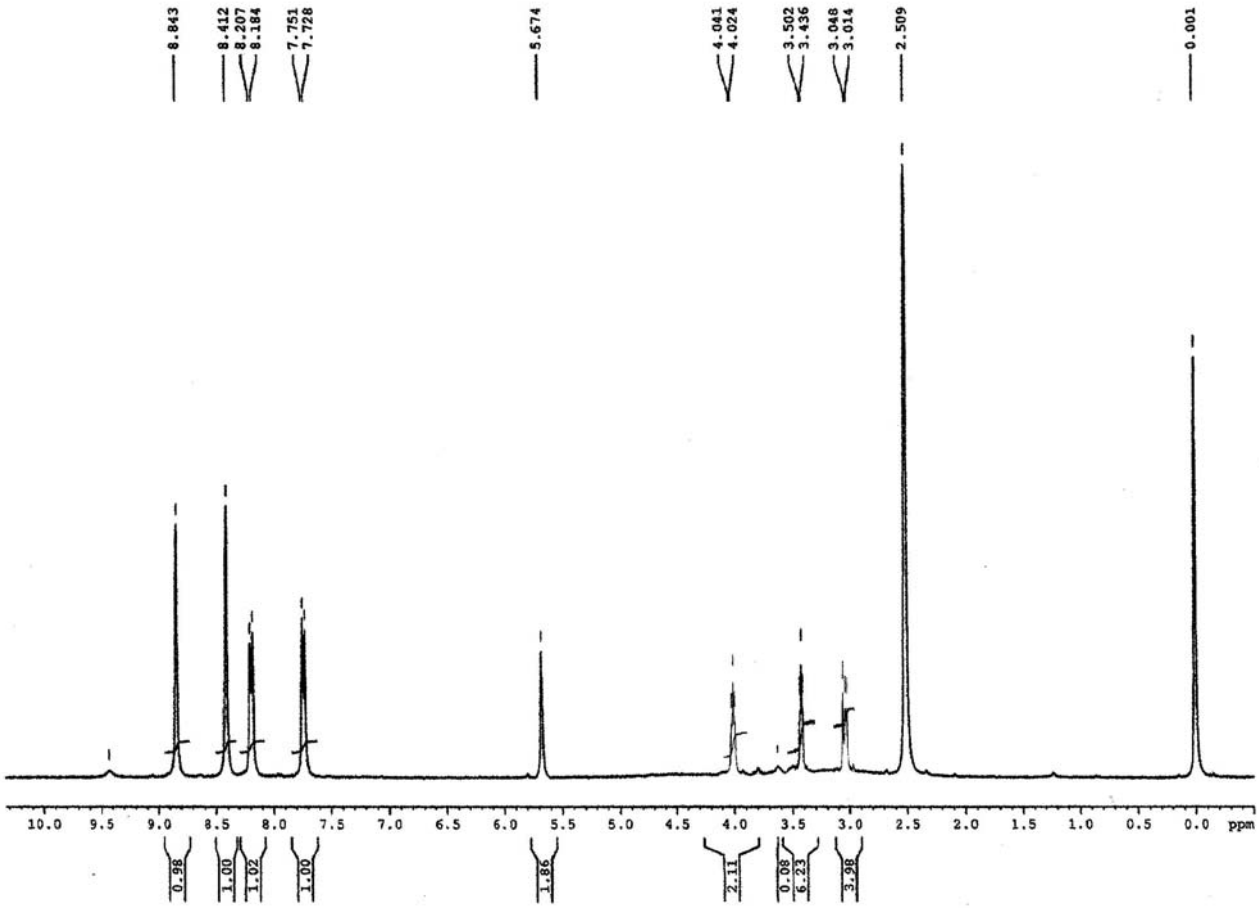

Fig. 5 – ^{1}H-NMR spectrum for the compound (IV).

As general remarks, in the FT-IR analysis, the frequencies for NO_2 group symmetric and asymmetric ranged between 1334-1340 cm^{-1} and 1519-1595 cm^{-1}. The carboxylate ion in compound (II) is identified by an absorption band at 1610 cm^{-1} and C-Cl bond from compound (V) is identified by a weak intensity band at 749 cm^{-1}. From the absorption spectra for N-mustards (III), (IV) and (VI), C-N stretching frequency was identified at 1305-1307 cm^{-1} while the C-Cl stretching frequency ranged between 747-789 cm^{-1}.

Fig. 4 illustrates a typical FT-IR spectrum, particularly for the compound (IV), showing the identification of the frequencies characteristic to the groups of interest (expressed in cm^{-1} and written upper to the spectrum).

The analysis of ^{1}H-NMR spectra adds new information on the properties of the chemical structures (I)-(VI). Thus, N-mustard protons present signals at δ=3.02-3.43 ppm and at 4.02-4.74 ppm. Fig. 5 shows a typical ^{1}H-NMR spectrum of the compound (IV).

Toxicity and potential antitumoral activity

The biomedical activity of the compounds (I)-(VI) was tested on mice lots.

a) Toxicity

The compounds (I)-(VI) may have antitumor activity functioning as antimetabolites (I), (II) and (V) or as alkylating agents (III), (IV) and (VI). The toxicity activity was evaluated using the lethal dose DL_{50}.

The results are presented in Table 1.

Grafting di-β-chloroethyl-amine through ethylene bridges, ester group or acetyl on the nucleus of indazole derivatives (I), (II), (V) led to mustards (III), (IV), (VI), which present a lower toxicity than free di-(β-chloroethyl)-amine and slightly up from the intermediaries support. This result is acceptable if taking into account data from literature, according to which the use of heterocyclic structures as transport agents have a positive influence on reducing the cytotoxic grouping. [2, 6, 9, 19-21]

b) Antitumoral activity

The antitumoral activity of N-mustards (III), (IV) and (VI) was established by the tumor evolution in the Guérin experimen.

We have found that for the untreated rats, tumors have greatly expanded, reaching 50-60 cm^3 and even more of a surveillance during 10-15 days, with clinical dissemination, macroscopic nodal in packages and multiple visceral dissemination. In contrast, for the group of treated animals, the tumor growth was slower. After a few days of treatment, ulceration occured only at a rate of 65-70% of tumors, dissemination was slower and at a lower percentage (30-35%). The administration of products to animals with induced tumors resulted in a prolongation of survival up to 40-45 days compared to untreated animals.

Table 2 present the results of the antitumor inhibition for the new N-mustards.

Table1

LD_{50} (mg/kg body) for indazole derivatives (I)-(VI)

Compound	LD_{50} (mg/kg body)			
	24 hours	48 hours	7 days	Average value
I	1675	1675	1640	1663
II	1775	1775	1730	1760
III	1580	1580	1550	1570
IV	1620	1620	1600	1613
V	1590	1590	1565	1581
VI	1555	1555	1535	1548
Di-β-chloroethyl-amine	378			

Table 2

Antitumor activity of N-mustards (III), (IV), (VI)

Compound	Route of administration	Experimental animals	Inhibition%, Guérin carcinom
III	p.o.	Rats	60
IV	p.o.	Rats	70
VI	p.o.	Rats	56
Endoxan			84

Experimental data show that the tested N-mustards present a remarkable antitumor activity. More selective is N-mustard (IV) which produces an inhibition of Guérin carcinoma close to that of endoxan (considered as a reference cytostatic). The probable explanation is that besides the alkylating effect, the compounds may have a specific antimetabolite effect.

EXPERIMENTAL

A. The experimental procedure and the main characteristics of the synthetized compounds (I)-(IV).

I) Sodium salt of the 5-nitroindazole (I). In a flask provided with ascendant condenser, ethyl alcohol (100 mL), sodium (0.1 mol) and 5-nitroindazole (0.1 mol) are added. The mixture of reaction, after 15-20 minutes of stirring, is heated under reflux on water bath, for 90 minutes. The ethanolic excess was removed by distillation under reduced pressure. The sodium salt of 5-nitroindazole was filtered under vacuum and then dried. The compound is slightly soluble in water.

Characteristics: Yellow solid, yield 82.5% (15.26 g) m.p.=250-253°C. Anal. calc. for $C_7H_4N_3O_2Na$: 45.40%C; 2.16%H; 22.70%N. Found: 45.77%C; 2.35%H; 23.11%N. IR(ν cm^{-1}): 3100 (CHAr); 1480 (C=N); 1335 (NO_2 sym); 1555 (NO_2 asym); ^{1}H-NMR (DMSO-d6, 400 MHz), δ (ppm): 7.70-7.74 (d, 1H, Ar); 8.17- 8.19 (d, 1H, Ar); 8.40 (s, 1H, Ar); 8.82 (s, 1H, Ar).

II) Sodium salt of the 5-nitroindazole-1-yl-acetic acid (II). In a flask the sodium salt (I) (0.02 mol) is dissolved in 100 mL anhydrous ethanol and then, under stirring 0.02 mol sodium salt of monochloroacetic acid in 40 mL anhydrous ethyl alcohol are added. The mixture of reaction is heated under reflux by stirring for 2 hours and then was filtered. The excess of ethyl alcohol was removed by distillation under reduced pressure to 40-45 mL. The crude solid (II) is filtered and dried under vacuum and then it is washed with anhydrous ethyl alcohol. The sodium salt (II) is slightly soluble in water.

Characteristics: White yellow solid; yield 87% (4.22 g); m.p.= 203-205°C. Anal. calc. for $C_9H_6N_3O_4Na$: 44.44%C; 2.47%H; 17.28%N. Found: 44.82%C; 2.76%H; 17.59%N. IR(ν cm^{-1}): 3096 (CHAr); 1491 (C=N); 1336 (NO_2 sym); 1595 (NO_2 asym); 1416 (CH_2). ^{1}H-NMR (DMSO-d6, 400 MHz), δ(ppm): 3.78 (s, 2H, CH_2); 7.72-7.75 (d, 1H, Ar); 8.18- 8.20 (d, 1H, Ar); 8.41 (s, 1H, Ar); 8.84 (s, 1H, Ar).

III) N-[di-(β-chloroethyl)-aminoethyl]-5-nitroindazole (III). The suspension of 0.01 mol, sodium salt (I) in 25 mL anhydrous dioxane is treated with 0.02 mol tris-(β-chloroethyl)-amine hydrochloride. The mixture of reaction is heated at 50-55ºC, for 45 minutes. The compound (III) is precipitated with anhydrous ethyl ether after filtering sodium chloride from dioxin solution and then is purified from anhydrous ethyl alcohol.

Characteristics: Cream-colored solid, yield 72% (2.38 g); m.p.=194-196°C. Anal. calc. for $C_{13}H_{16}Cl_2N_4O_2$: 47.12%C; 4.83%H; 21.45%Cl; 16.91%N. Found: 47.41%C; 5.24%H; 21.87%Cl; 17.27%N. IR(ν cm^{-1}): 3095 (CHAr); 1491 (C=N); 1305(C-N); 1336 (NO_2 sym); 1534 (NO_2 asym); 748, 789 (C-Cl). ^{1}H-NMR (DMSO-d6, 400 MHz), δ(ppm): 3.09-3.11 (m, 4H, $2CH_2$); 3.37-3.41(m, 6H, 3 CH_2) 4.62-4.74 (m, 2H, CH_2); 7.73-7.75 (d, 1H, Ar);); 8.18- 8.20 (d, 1H, Ar); 8.41 (s, 1H, Ar); 8.85 (s, 1H, Ar).

IV) N-[di-(β-chloroethyl)-aminoethyl-carboxy-methyl]-5-nitroindazole (IV) The same procedure for compound (III) was used, starting from 0.01 mol sodium salt (II), 30 mL anhydrous dioxane and 0.01 mol tris-(β-chloroethyl)-amine.

Characteristics: Yellow solid, yield 74% (2.87 g); m.p.=146-148°C. Anal. calc. for $C_{15}H_{18}Cl_2N_4O_4$: 46.27%C; 4.62%H; 18.25%Cl; 14.39%N. Found: 46.49%C; 4.96%H; 18.52%Cl; 14.67%N. IR(ν cm^{-1}): 3096 (CHAr); 1494 (C=N); 1743(C=O ester); 1158 (C-O-C); 1340 (NO_2 sym); 1519 (NO_2 asym); 751, 793 (C-Cl). ^{1}H-NMR (DMSO-d6, 400 MHz), δ(ppm): 3.01-3.04 (m, 4H, $2CH_2$); 3.43-3.50 (m, 6H, 3 CH_2) 4.02-4.04 (m, 2H, CH_2); 5.67 (s, 2H, CH_2) 7.72-7.75 (d, 1H, Ar); 8.18- 8.20 (d, 1H, Ar); 8.41 (s, 1H, Ar); 8.84 (s, 1H, Ar).

V) N-chloroacethyl-5-nitroindazole (V). The sodium salt (I) (0.01 mol) is dissolved in anhydrous dioxane (50 mL) and the monochloroacetic acid chloride (0.01 mol) is added. The mixture of reaction is heated under reflux on water bath for 120 minutes, by stirring under cooling. A sticky product is obtained and by repeated washing with anhydrous ethyl ether changes into a fine powder. By recristallization in acetone the pure product is obtained.

Characteristics: Yellowish white solid, yield 79% (1.88 g); m.p.=166-168°C. Anal. calc. for $C_9H_6ClN_3O_3$: 45.09%C; 2.50%H; 14.82%Cl; 17,53%N. Found: 45.51%C; 2.87%H; 15.04%Cl; 17.93%N. IR(ν cm^{-1}): 3097 (CHAr); 1493 (C=N); 1624(C=O amide); 1338 (NO_2 sym); 1535 (NO_2 asym); 749 (C-Cl). ^{1}H-NMR (DMSO-d6, 400 MHz), δ(ppm): 5.26 (s, 2H, CH_2), 8.45-8.47 (d, 1H, Ar); 8.50- 8.57 (d, 1H, Ar); 8.75 (s, 1H, Ar); 8.91 (s, 1H, Ar).

VI) N-[di-(β-chloroethyl)-aminoacethyl]-5-nitroindazole (VI). The suspension of 0.01 mol compound (V) in 100 mL anhydrous dioxane is treated with 0.012 mol di-(β-chloroethyl)-amine, free base in ether. The mixture of reaction is heated under reflux, for 4 hours. The excess of dioxane was removed by distillation under reduced pressure and the product is recrystallization from hot benzene.

Characteristics: Yellowish white solid, yield 65% (2.24 g); m.p.=181-183°C. Anal. calc. for $C_{13}H_{14}Cl_2N_4O_3$: 45.21%C; 4.05%H; 20.57%Cl; 16.23%N. Found: 45.37%C; 4.32%H; 20.92%Cl; 16.54%N. IR(ν cm^{-1}): 3094 (CHAr); 1490 (C=N); 1622 (C=O amide); 1334 (NO_2 sym); 1533 (NO_2 asym); 747, 788 (C-Cl). ^{1}H-NMR (DMSO-d6, 400 MHz), δ(ppm): 3.02-3.08 (m, 4H, $2CH_2$); 3.31-3.34 (m, 4H, $2CH_2$); 4.67 (s, 2H, CH_2); 7.74-7.76 (d, 1H, Ar); 8.17- 8.20 (d, 1H, Ar); 8.40 (s, 1H, Ar); 8.83 (s, 1H, Ar).

B. Evaluation of the toxicity and potential antitumoral activity

a) Toxicity

The lots were composed of 10 mice of either gender with a weight of 20±2 g and a safety limit of 90%. After intraperitoneal administration of drug suspensions in Tween80, the mortality was registered after 24 hrs, 48 hrs and 7 days.[18]

b) Potential antitumoral activity

We evaluated the cytostatic effect of the synthesized N-mustards (III), (IV), (VI), by transplanting subcutaneously the Guérin experimental tumors T8 to 15 males rats weighing 100-150 g (±15 g) each. The substance administration was started when tumor volume reached sizes of 5-6 cm^3 (product of three diameters larger tumors).

The inhibition of tumor growth was assessed by tumor weights from animals slaughtered at 24 hours after last administration and was calculated using the formula:

$$I(\%) = \frac{C - T}{C} \times 100 \quad (1)$$

where: C is the average weight of tumors in the control group and T the average weight of tumors in the treated group.

The compound was extemporaneously suspended in saline solution and esophageal probe was administered to 10 animals in daily dose of 20 mg/animal; 5 animals were treated as control group. The animals were monitored for 10-15 days, watching the effects on hematopoietic system and the variations in the peripheral blood cell count.

All experiments were organized in conformity with Ordinance for animal protection used for scientific or experimental purposes, no. 37/2002 from 30/01/2002 published in Monitorul Oficial, Part I, no. 95 from 02/02/2002.

CONCLUSIONS

Six new derivatives of 5-nitroindazole (I)-(VI) were synthesized, three of them (III), (IV), (VI) containing grafted di-(β-chloroethyl)-amine group. The structure of the new compounds was confirmed by elemental and spectral analysis (FT-IR and ^{1}H-NMR).

The toxicity activity evaluated using the lethal dose DL_{50} demonstrates that all compounds have low toxicity. The experimental study of the inhibition of the antitumor activity for N-mustards (III), (IV) and (VI) shows a significant regression on Guérin carcinoma, close to the reference cytostatic - endoxan.

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