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Halomethyl-1,2,3-Triazole Derivatives: A New Type of Alkylating Agent Active in Mouse Transplantable Tumors

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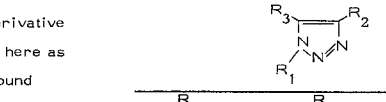
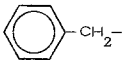
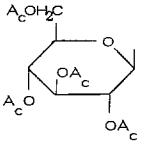
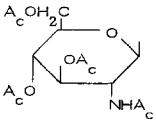
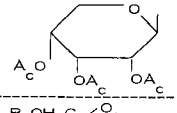
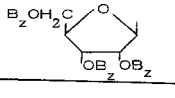
Summary. *A number of halomethyl-1,2,3-triazole derivatives have been tested for their effect on ICR Swiss female mice bearing the Ehrlich carcinoma ascites (ECA) tumor. Two of these compounds, namely 4-bromomethyl-1-(2,3,4,6-tetra-0-acetyl-β-D-glucopyranosyl)-1,2,3-triazole and 4-iodomethyl-1-(2,3,4,6-tetra-0-acetyl-β-D-glucopyranosyl)-1,2,3-triazole, referred to here as compounds H and K, respectively, promoted a significant increase in the median survival time (145% and 195%) when injected IP for 9 consecutive days at doses of 75 mg/kg/injection and 100 mg/kg/injection, respectively. DNA synthesis, as measured by (methyl-^{3}H)thymidine incorporation into trichloroacetic acid-precipitated material, was always inhibited by all the triazole derivatives to a greater extent than the incorporation of (5,6-^{3}H)uridine, (5-^{3}H)proline, and (6-^{3}H)-glucose. Moreover, inhibition of DNA synthesis was complete and irreversible following exposure to selected triazole derivatives. Compounds H and K inhibited the incorporation of di(^{3}H)methyl sulfate by ECA cells. In addition, compound K promoted the release of radioactivity associated with trichloroacetic acid-insoluble material coming from (8-^{3}H)guanosine-prelabeled cells. This release of radioactivity did not occur when cells were prelabeled with (methyl-^{3}H)thymidine. It is concluded from these results that these triazole derivatives act as alkylating agents.*

Introduction

Among the synthetic products showing antineoplastic activity, alkylating agents are distinguished both by the great number of chemical structures that have been synthesized [12] and by the clinical use of a number of them [6]. In this regard, a series of halomethyl-1,2,3-triazole derivatives have been synthesized in our Institute. They were designed as potential alkylating agents and, in fact, most of them have shown cytotoxic activity against HeLa cells [2]. Therefore, it was of interest to investigate the action of these compounds on tumor cells in more detail. For this purpose, only those triazole derivatives with an ED_{50} against HeLa cells of lower than 100 μg/ml [2] were considered in this study. Their chemical structures are shown in Table 1.

Table 1. Chemical structures of the triazole derivatives used in this work

Triazole derivative referred to here as compound		R_1	R_2	R_3
A			$-CH_2Cl$	H
B			$-CH_2Cl$	$-CH_2Cl$
C			$-CH_2Br$	H
D			$-CH_2I$	H
E			$-CH_2Cl$	H
F			H	$-CH_2Cl$
G			$-CH_2Cl$	$-CH_2Cl$
H			$-CH_2Br$	H
I			H	$-CH_2Br$
J			$-CH_2Br$	$-CH_2Br$
K			$-CH_2I$	H
L			$-CH_2Cl$	H
M			H	$-CH_2Cl$
N			$-CH_2Br$	H
O			$-CH_2Cl$	H
P			$-CH_2Br$	H
Q			$-CH_2I$	H
R			$-CH_2Cl$	$-CH_2Cl$
S			$-CH_2Br$	H

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Our findings have shown that compounds H and K increased the lifespan of tumor-bearing mice. They inhibited DNA synthesis by Ehrlich carcinoma ascites (ECA) cells, blocked di(^{3}H)methyl sulfate incorporation by ECA cells, and promoted the release of radioactivity from (8-^{3}H)guanosine-labeled nucleic acids but not from (methyl-^{3}H)thymidine-labeled DNA. It is concluded that these halomethyl-1,2,3-triazole derivatives act as alkylating agents.

Materials and Methods

Drugs. Triazole derivatives used throughout this work were synthesized in our Institute as described [2] and were generously supplied by the authors. Their chemical structures are shown in Table 1. Di(^{3}H)methyl sulfate (150 mCi/mmol), (methyl-^{3}H)thymidine (47 Ci/mmol), (5,6-^{3}H)uridine (47 Ci/mmol), (5-^{3}H)proline (23 Ci/mmol), (6-^{3}H)glucose (15 Ci/mmol), and (8-^{3}H)guanosine (7.1 Ci/mmol) were purchased from the Radiochemical Centre, Amersham (England) and were used throughout this work. 5-Fluorouracil was a gift from Roche SA.

Experimental Animals and Tumor Passage. ICR Swiss female mice weighing 19–22 g were bred in the animal house of our Institute and used throughout this work. Ehrlich carcinoma was maintained in ascitic form by IP injection to mice of about 5×10^5 cells obtained from a donor mouse bearing a 7-day-old tumor.

In vivo Cytostatic Activity. Cytostatic activity in mice bearing Ehrlich carcinoma ascites (ECA) tumor was estimated according to protocols 1100 and 1200 from the National Cancer Institute (USA) [5]. Test compounds were suspended in 0.9% (w/v) NaCl solution containing 0.05% (v/v) Tween 80 and injected IP at a fixed volume of 0.4 ml. The treatment and control groups contained 6 and 30 mice, respectively. The positive control compound was 5-fluorouracil (20 mg/kg/injection).

Incorporation of Radioactive Compounds into Trichloroacetic Acid-Precipitated Material. The incorporation of (methyl-^{3}H)thymidine (5,6-^{3}H)uridine, (5-^{3}H)proline, and (6-^{3}H)glucose by ECA cells was estimated as previously described [1]. When the incorporation of di(^{3}H)methyl sulfate was studied, the mixture (7.5 ml) containing 5×10^5 ECA cells/ml, 1.33 μM di(^{3}H)methyl sulfate (150 mCi/mmol), 0.67% dimethyl sulfoxide (because of the insolubility of triazole derivatives in water), and, where indicated, the corresponding test compound was incubated, with stirring, for 1.5 h at 37° C. At the end of the incubation period, 7 ml mixture was centrifuged for 5 min at 1000 rpm. The cell pellet was lysed in 3 ml ice-cold water, and 3 ml ice-cold 10% (w/v) trichloroacetic acid was added. The large amount of cells used prevented us from filtering through Whatman GF/C filters. Therefore, the cell lysate was centrifuged at 1000 rpm and the pellet was washed twice in 3 ml ice-cold 5% (w/v) trichloroacetic acid and resuspended in 0.3 ml water, and the resulting suspension (about 0.4 ml) was mixed with 2 ml PCS (Amersham, England) for estimation of the radioactivity in a liquid scintillation spectrometer.

Results

In vivo Cytostatic Activity. Some of the most active triazole derivatives against HeLa cells [2] were assayed for their cytostatic activity in mice bearing ECA tumor. Compounds B, C, D, I, J, P, and Q were devoid of activity in this system (results not shown). However, compounds H and K produced a significant increase in the lifespan of tumor-bearing mice, which merited further study (Table 2). Therefore, compound H was subse-

Table 2. Cytostatic activity of compounds H and K in tumor-bearing mice

Compound	Tumor	Daily dose[b] (mg/kg)	Toxicity day-5 survivors[c]	Animal weight difference[d]	Cures[e]	T/C × 100[f]
H	ECA	75	6/6	− 0.5	2	145
		50	6/6	− 0.6	−	131
		25	6/6	0.0	1	100
H	P 388[a]	50	6/6	− 2.0	−	163
		25	6/6	− 0.7	−	149
		12.5	6/6	− 1.0	−	131
		6.25	6/6	− 1.1	−	93
K	ECA	100	6/6	− 1.1	−	195
		50	6/6	− 0.3	−	160
		25	6/6	0.1	−	125

Conditions were as described under Materials and Methods

[a] Experiments with CDF_1 female mice bearing P 388 lymphocytic leukemia were carried out by the National Cancer Institute (USA)

[b] Nine IP doses were given at 24-h intervals, starting 24 h after IP injection of tumor cells

[c] Number of survivors on day 5/number of mice started on test

[d] The difference (g) between the weights of test and control animals

[e] Survivors on day of evaluation (day 30)

[f] T/C is the ratio (expressed as a percentage) of the median survival time of the treated group of mice divided by the median survival time of the control group. A value of T/C × 100 ≥ 125 is considered a statistically significant indication of the antitumor activity of the compound [5]

quently studied at the National Cancer Institute (USA) and was found also to produce a significant increase in the lifespan of mice bearing P388 lymphocytic leukemia (Table 2).

Mode of Action. Studies were carried out to elucidate the mode of action of the compounds that have the halomethyl-1,2,3-triazole structure. Most of the studies were performed on compounds H and K, as these showed the highest in vivo activities (Table 2). The incorporation of radioactive precursors by ECA cells in the presence of compound K (Fig. 1) or compound H (results not shown) indicates that DNA synthesis, as measured by (methyl-^{3}H)thymidine incorporation, was more extensively inhibited than RNA synthesis, as measured by (5,6-^{3}H)uridine incorporation, whereas (5-^{3}H)proline and (6-^{3}H)glucose incorporation was inhibited to a lesser extent. The remaining compounds showed a similar pattern of inhibition when they were assayed for their effect on the incorporation of radioactive precursors by ECA cells (Table 3). On the other hand, DNA synthesis was irreversibly inhibited by compounds B, C, D, H, J, P, and Q (Fig. 2).

The preferential [14, 18] and irreversible [13] inhibition of DNA synthesis might indicate that the inhibitors used are alkylating agents. To test this possibility, we studied the effect of compounds H and K on the incorporation of di(^{3}H)methyl sulfate by ECA cells. As

Table 3. Effect of the triazole derivatives A–S on the incorporation of radioactive precursors by ECA cells

Compound	ED_{50}			
	(methyl-^{3}H)-thymidine incorporation	(5,6-^{3}H)-uridine incorporation	(5-^{3}H)-proline incorporation	(6-^{3}H)-glucose incorporation
A	87	> 100	> 100	–
B	2	9	> 10	> 10
C	1	> 10	> 10	> 10
D	2	8	> 15	> 15
E	124	150	> 200	–
F	52	66	> 200	–
G	24	43	> 100	–
H	3	12	19	–
I	4	> 20	> 20	> 20
J	1	> 5	> 5	> 5
K	5	9	> 20	–
L	230	> 300	> 300	> 300
M	129	> 500	> 500	> 500
N	7	10	> 300	> 300
O	190	230	> 300	> 300
P	7	> 15	> 15	> 15
Q	2	> 10	> 10	> 10
R	40	66	88	–
S	9	9	16	–

The mixture (1.5 ml) containing cells, dimethyl sulfoxide, and radioactive precursors at the concentration indicated in the legend to Fig.1 and the test compound was incubated for 1.5 h at 37° C, after which 1 ml was taken and processed as described [1]. The values indicate the test compound concentration (μg/ml) that inhibits incorporation to 50% of the control incorporation

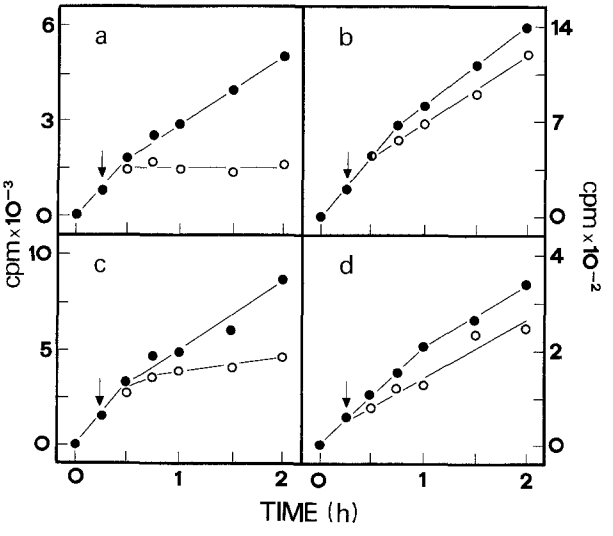

Fig. 1. Effect of compound K on the incorporation of radioactive precursors by ECA cells: 7.5 ml ECA cell suspension (5 × 10^5 cell/ml) containing 0.67% (v/v) dimethyl sulfoxide and 0.5 μCi (methyl-^{3}H)thymidine (**a**), 1.25 μCi (5-^{3}H)proline (**b**), 1.25 μCi (5,6-^{3}H)uridine (**c**) or 7.5 μCi (6-^{3}H)glucose (**d**) was incubated at 37° C in the absence (●—●) or in the presence (○—○) of 17 μg compound K/ml. When indicated, 1 ml incubation mixture was taken and processed as described [1]. The *arrows* indicate the addition of compound K

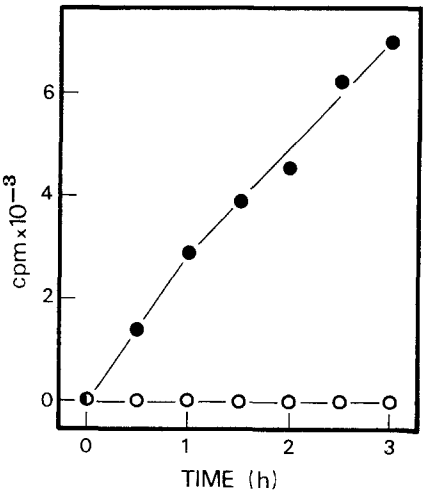

Fig. 2. Irreversible inhibition by triazole derivatives of DNA synthesis by ECA cells: 7.5 ml ECA cell suspension (5 × 10^5 cell/ml) containing 0.67% (v/v) dimethyl sulfoxide was incubated at 37° C for 90 min in the absence (●—●) or in the presence (○—○) of the inhibitors mentioned below. Cells were collected by centrifugation, resuspended in 7.5 ml fresh medium, and incubated at 37° C in the presence of 0.5 μCi (methyl-^{3}H)thymidine. When indicated, 1 ml of the incubation mixture was taken and processed as described [1]. The following inhibitors were tested at the concentrations indicated in parentheses: compounds J (5 μg/ml); B and C (10 μg/ml); D, P, and Q (15 μg/ml), and H (20 μg/ml)

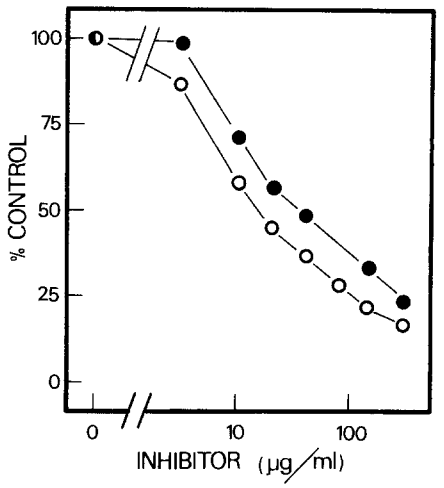

Fig. 3. Inhibitory effect of compounds H and K on the incorporation of di(^{3}H)methyl sulfate by ECA cells. Compounds H (●—●) and K (○—○) were tested at the concentrations indicated. Otherwise, the conditions were as indicated under Materials and Methods

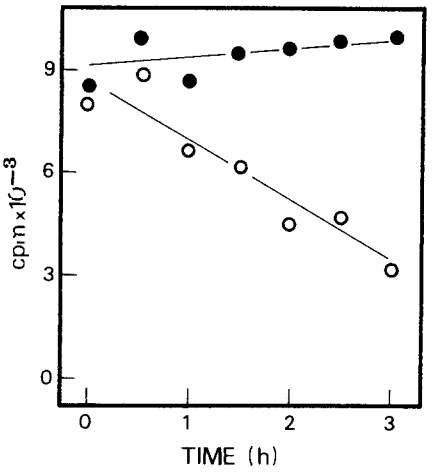

Fig. 5. Effect of compound K on the stability of nucleic acids. Cells (30 ml) were labeled in the presence of 10 μCi (8-^{3}H)guanosine and collected as described under the legend to Fig. 4. Then 7.5 ml this cell suspension containing 0.67% (v/v) dimethyl sulfoxide was incubated at 37° C in the absence (●—●) or in the presence (○—○) of 300 μg/ml of compound K. When indicated 1 ml incubation mixture was taken and processed as described [1]

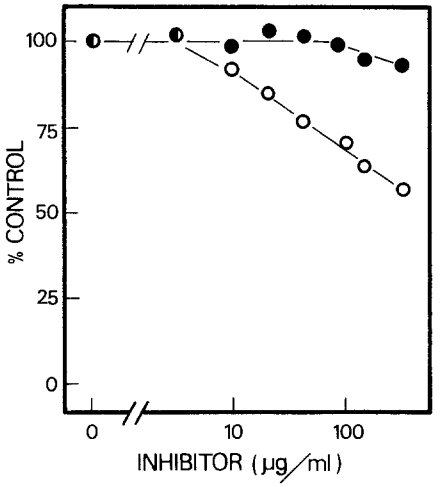

Fig. 4. Effect of compound K on the stability of nucleic acids: 100 ml ECA cell suspension (5 × 10^5 cell/ml) was incubated for 30 min at 37° C in the presence of 5 μCi either (methyl-^{3}H)thymidine (●—●) or (8-^{3}H)guanosine (○—○). Cells were collected by centrifugation and resuspended in 100 ml fresh medium. Then 7.5 ml each cell suspension was incubated for 90 min at 37° C in the presence of 0.67% (v/v) dimethyl sulfoxide and compound K at the indicated concentrations. At the end of the incubation period, 7 ml was taken and processed by the method followed in Fig. 3

shown in Fig. 3, they inhibited the incorporation of this radioactive alkylating agent, a result that also suggests that these tirazole derivatives act via an alkylating mechanism. On the other hand, Tomasz [16] has developed a method for detecting alkylation at the N-7 position of guanine by the concurrent labilization and release of ^{3}H attached to C-8. It has also been reported that a number of drugs induce DNA-strand breakage and promote base release [4, 8–11, 14, 15, 18]. In order to study this aspect of the mode of action of these triazole derivatives, ECA cells were prelabeled in the presence of (methyl-^{3}H)thymidine or (8-^{3}H)guanosine, collected by centrifugation and incubated in the presence of compound K. As shown in Fig. 4, the amount of radioactivity associated to trichloroacetic acid-precipitated material did not diminish when DNA was prelabeled with (methyl-^{3}H)thymidine but decreased when nucleic acids were prelabeled with (8-^{3}H)guanosine. This loss of radioactivity amounted to 70% after incubation of the cells for 3 h at 37° C in the presence of 300 μg compound K/ml (Fig. 5).

Discussion

The blockade of DNA synthesis in ECA cells (Table 3) correlates satisfactorily with the inhibition of HeLa cell growth [2]. However, this in vitro activity does not strictly correlate with their cytostatic activity in vivo, since several compounds, e.g., B, C, D, I, J, P, and Q were very active in tissue culture but showed little activity, if any, in mice bearing ECA tumor. Otherwise, structure – in vitro activity relationships for compounds A–S have been considered elsewhere [2].

The chemical structure of compounds A–S suggests that they could be alkylating agents. In fact, CH_3I is a

well known methylating agent and compounds with the general structure R-CH_2-halogen may act as alkylating agents, especially if R is an activator radical. Moreover, both cytostatic activity [2] and chemical alkylating ability increase in the same order (fluorinated compounds < chlorinated compounds < brominated compounds < iodinated compounds) for compounds A–S. On the other hand, these triazole derivatives inhibited DNA synthesis to a greater extent than RNA or protein synthesis, as alkylating agents do [14, 18], and this inhibition was irreversible. This finding, according to Painter [13], might imply that DNA is damaged (alkylated) by these triazole derivatives. In addition, compounds H and K inhibited the incorporation by ECA cells of di(^{3}H)methyl sulfate, a typical alkylating agent that binds preferentially to the N-7 position of guanine moieties [10, 18]. Compound K promoted the release of radioactivity from (8-^{3}H)guanosine-labeled nucleic acids but not from (methyl-^{3}H)thymidine-labeled DNA, a result which is in agreement with the well-known ability of alkylating agents of mainly binding to N-7 position of guanine moieties [9, 14, 18] promoting both the labilization of ^{3}H attached to C-8 of guanine [16] and the cleavage of the bond between the guanine moiety and the sugar moiety, with subsequent release of the alkylated base [9, 14, 18]. However, this decrease in the guanine content of nucleic acids cannot account for the inhibition of di(^{3}H)methyl sulfate incorporation by ECA cells, since this incorporation process (Fig. 3) was more drastically affected than the former (Fig. 4).

The results, especially those obtained with (8-^{3}H)guanosine-labeled nucleic acids, indicate that these triazole derivatives are alkylating agents that bind at least to the N-7 position of guanine moieties. This will provide the biochemical rationale [3] for selecting other drugs that could be used in combination with these in the treatment of experimental neoplasma. We want to emphasize that most of these triazole derivatives are nucleosides. It is now possible to design new alkylating nucleosides, using a sugar whose transport is enhanced in tumor cells [7, 17], to provide these compounds with a more selective toxicity towards cancerous cells.

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