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BIOLOGICAL EFFECTS OF UNSATURATED KETONUCLEOSIDES ON EUCARYOTIC CELLS IN CULTURE—THE ACTION OF 7-(3′,4′-UNSATURATED 2′-KETOHEXOSYL) THEOPHYLLINE

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Abstract—This study on the effects of 7-(3′,4′-unsaturated 2′-ketohexosyl) theophylline on KB cells in culture has confirmed the high cytotoxic potency of the compound. It also showed that at low doses, where no cytotoxic effect occurs, the ketonucleoside impaired DNA, RNA and protein syntheses and strongly inhibited cell multiplication. However, this action was transient, owing to the inactivation of the compound in the cell culture medium. The sensitivity of the cells to the 2′-ketonucleoside was found to depend upon the interval of time between the seeding and the addition of the compound to the culture medium—the shorter the interval of time, the more sensitive the cells. The biological effects induced by the compound were not exclusively restricted to cancer cells.

The 7- and 9-(2′-ketohexosyl) purines, which constitute a new class of active nucleosides, have been shown to inhibit KB cell growth, whereas the parent compounds, devoid the 2′-keto group, were completely inactive at the same dose levels [1]. More recent results have demonstrated that the 2′-ketonucleosides inhibit the growth of L 1210 leukemia cells injected intraperitoneally into mice; a significant increase of the survival was observed in treated animals as compared with that of controls [2].

The compound 7-(3′,4′-unsaturated 2′-ketohexosyl) theophylline [or more explicitly the 7-(3′-0-acetyl 4′,6′-dideoxy-β-L-glycero-hex-3′-enopyranosulosyl) theophylline] (Fig. 1) was chosen for this study because of its high inhibitory effect on cell growth [1].

The results confirm the high cytotoxic potency of the compound. They also show that at low doses, where no cytotoxic effect occurs, the ketonucleoside impaired DNA, RNA and protein syntheses and strongly blocked cell multiplication. However, this action was transient, owing to the inactivation of the compound in the cell culture medium. Cells appeared more sensitive to the ketonucleoside if exposed to the compound shortly after seeding. The biological effects induced by the compound were not exclusively restricted to cancer cells.

MATERIALS AND METHODS

Chemicals. The unsaturated ketonucleoside was synthesized from 7-(2′-keto-β-4-"fucosyl") theophylline according to the procedure described by Antonakis *et al.* [3, 4]; before use, it was dissolved in DMSO.

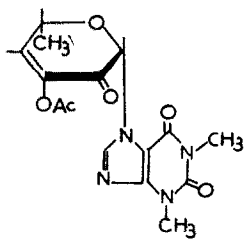

Fig. 1. The structure of 7-(3′-*O*-acetyl 4′,6′-dideoxy-β-L-glycero-hex-3′-enopyranosulosyl) theophylline.

[^{3}H]*methyl* thymidine (46 Ci/m-mole), 8[^{3}H]uridine (55 Ci/m-mole) and (U-^{14}C)L-amino acid mixture (40 mCi/m-mole) were purchased from CEA (Saclay, France).

Eagle's basal medium and Williams medium were obtained from Eurobio (Paris, France) and sera from Sorga (Paris, France).

Cell cultures. KB cells (Flow Laboratories Ltd, Irvine, Scotland) were grown as monolayer cultures in glass flasks and subcultured after trypsinization. The cells were grown in Eagle's basal medium supplemented with 10% calf serum.

Hepatic cell lines (LF, F_1, F_2, F_3) were established by one of us (E.C.M.). LF line derived from hepatoma cells grafted in the intraperitoneal (i.p.) cavity of 2-week-old male Wistar rats. The grafted hepatoma had issued from a primary hepatoma induced by 4-dimethyl-aminoazobenzene feeding and was kindly provided by C. and C. Frayssinet. LF cells were grown in Eagle's basal medium supplemented with 20% calf serum.

Abbreviations—DMSO, dimethylsulfoxide; TCA, trichloroacetic acid; PBS, phosphate buffer solution.

F_1, F_2 and F_3 lines were derived from a primary culture of 10-day-old rat liver hepatocytes according to the technique of Williams *et al.* [5]. The characteristics of the three cell lines will be detailed elsewhere [6]. They may be briefly summarized as follows: F_1 cells corresponded to spontaneously malignant transformed cells. Like LF cells, they were able to grow on soft agar and induce tumors after injection into syngeneic hosts. Up to now, F_2 and F_3 cells failed to grow on soft agar and did not give rise to tumors in syngeneic hosts. They may be considered as still untransformed hepatocyte cells. F_1, F_2 and F_3 cells were grown in Williams' medium supplemented with 15% fetal calf serum.

Antibiotics (neomycin, penicillin and streptomycin) and glutamine were routinely added to the various media.

Treatment of cells with ketonucleoside. Trypsinized cells were resuspended in fresh medium and distributed in a series of glass flasks. Each flask contained about 2×10^5 cells in 2 ml of medium at initial seeding. Cells were grown at 37°. After an interval of time as indicated in the tables (usually 24 hr after seeding, unless otherwise stated), 10 μl of DMSO containing the ketonucleoside or DMSO alone were added. The final concentration of DMSO was 0.5% for all experiments; it had no effect on cell growth. The ketonucleoside concentrations are indicated in the tables. The cultures were further incubated at 37° for a 24-hr period (unless otherwise stated). The cells were then dispersed with 0.02% EDTA made in PBS and counted in a Coulter counter.

Labelled precursor incorporation. Three days after seeding, the ketonucleoside was added to the cultures. Immediately after this treatment, one of the following labelled precursors was added for a 1-hr pulse: 0.5 μCi/ml of ([^{3}H]CH_3)thymidine, 0.5 μCi/ml of 8[^{3}H]uridine, or 1 μCi/ml of the (U-^{14}C)L-amino acid mixture. At the end of the pulse, the medium was drained and the cells were rinsed with PBS. The cells were then detached and resuspended in 0.02% EDTA. Aliquots were drawn for determination of the number of cells. Other aliquots (50 μl) were deposited on Schleicher and Schüll fiber glass filters for determination of the radioactivity. The filters were immersed in cold 10% TCA (w/v), washed three times with cold 5% TCA, rinsed with alcohol then with ether and air dried. The radioactivity was counted after addition of 5 ml of a phosphor solution (toluene containing 0.4% PPO and 0.01% dimethyl-POPOP) in a liquid scintillation spectrometer (Intertechnique, Paris, France). The measurements were made on duplicate samples. The results were expressed in cpm per 1000 cells.

RESULTS AND DISCUSSION

Among the various 7- and 9-(2'-ketohexosyl) purines previously tested, 7-(3',4'-unsaturated 2'-ketohexosyl) theophylline was one of the most active on KB cells (Fig. 1).

The data reported in Table 1 illustrate the dose-response effect of the compound on KB cells: with doses up to 2.5 μg per ml, there was no significant difference between treated and control cells. A dose of 10 μg per ml completely inhibited cell multiplication within the first 24 hr of treatment. At this concentration no cytotoxic effect was observed (there was no significant decrease in the number of cells after the addition of the compound). Later on, cell multiplication continued. At higher concentrations, a marked cytotoxicity was observed: after a 24-hr period of action the initial number of cells dropped considerably. After continued incubation cell multiplication resumed. These results might be explained by a recovery of the cultures owing to the total consumption of the ketonucleoside by cells or by its inactivation during cell culture. This point was further investigated and the results obtained argue in favor of the second hypothesis. It was shown that the compound not only lost its inhibiting capacity after a 24-hr incubation in cell cultures but also in the presence of the medium alone (Eagle's medium supplemented with 10% calf serum) (Table 2). The results reported in Table 3 show that the serum was not responsible for this fact since the inactivation of the ketonucleoside occurred in the *absence* of serum; it was even observed in salt solution (PBS). Identical results were obtained for either a 5-hr or a 1-hr period of preincubation, which demonstrated how rapid the inactivation process is in aqueous

Table 1. Dose-response effects of unsaturated 2'-ketonucleoside on growth of KB cells

Concentration of 2'-ketonucleoside in culture medium μg/ml	Number of cells per flask ($\times 10^{-6}$) Days after treatment				Multiplication rate for the following intervals of time		
	0	1	2	3	days 0–1	days 1–2	days 2–3
0	0.26	0.44	0.74	1.10	1.69	1.68	1.48
0.6	0.26	0.43	0.63	1.12	1.65	1.46	1.77
2.5	0.26	0.42	0.63	1.15	1.61	1.50	1.82
10	0.26	0.25	0.42	0.80	0.96	1.68	1.90
40	0.26	0.08	0.06	0.22	0.30	0.75	3.66
160	0.26	0.09	0.05	0.15	0.34	0.55	3.00

The cells were treated 24 hr after seeding. At this time ("day zero"), the number of cells per flask was determined (see Methods) just before the addition of the ketonucleoside dissolved in DMSO or DMSO alone (control). The same determination was made on days 1, 2, and 3. Each value reported in the table corresponds to the mean values obtained from two separate flasks.

The multiplication rate is expressed as the ratio between the number of cells per flask for 2 consecutive days.

Table 2. Demonstration of the inactivation of the unsaturated 2'-ketonucleoside by the culture medium at 37°

Experimental procedure applied to the ketonucleoside	Number of cells per flask 24 hr after the addition of the compound ($\times 10^{-6}$) Concentration used (μg per ml) 0	10	20
Freshly dissolved in DMSO	0.48	0.34	0.25
Kept in DMSO for 24 hr at 4°	0.48	0.37	0.26
Preincubated in cell cultures for 24 hr at 37°*	0.47	0.45	0.43
Preincubated in the culture medium alone for 24 hr at 37°†	0.47	0.48	0.49
Freshly dissolved in culture medium previously incubated for 24 hr at 37°	0.48	0.32	

One day after seeding, the cultures (containing 0.25×10^6 cells per flask) were treated with solutions of 2'-ketonucleoside according to different procedures.

* The compound was added to a KB cell culture; after a 24-hr incubation at 37°, the medium was recovered and added to a new 24-hr old KB cell culture whose own medium had previously been drawn off.

† The same procedure was used except that the compound was incubated in the culture medium without cells.

Each value corresponds to the mean of three flasks.

solution; the mechanism of this process is under investigation. Whatever this mechanism might be, the instability of the unsaturated ketonucleoside must be considered for its eventual use for biological purposes.

Additional results have shown that the degree of sensitivity of KB cells to the unsaturated ketonucleoside was modified by various experimental parameters such as the age and the concentration of the cells in culture. Thus, when the ketonucleoside was added after an interval of time varying from 0 to 3 days after seeding, the inhibition in the treated culture after a further 24-hr period dropped from 62 to 17 per cent with regard to the control value (Table 4); the younger the cells, the more sensitive they were to the effect of the compound added. The cells treated immediately after seeding were so sensitive that a cytotoxic effect was observed at a dose of 10 μg/ml (compare results in Tables 1 and 4). It is conceivable that the recent trypsinization of cells induced some changes in their properties (permeability, membrane viscosity...). In addition, when the cell number at the start of the treatment varied, owing to a different cell concentration at seeding, the extent of nucleoside action also varied: the higher the number of cells, the less marked was the inhibition of the multiplication rate (Table 5). In fact, it is wellknown that certain cell functions are cell density dependent (e.g. the uptake of precursors and their incorporation into macromolecules). Moreover, the percentage of cells in the different phases of the cycle varies according to the age and/or the density of the culture. These parameters might play an important role in the case where ketonucleoside

Table 3. Demonstration of the inactivation of the unsaturated 2'-ketonucleoside in aqueous solutions at 37°

Experimental procedure applied to the ketonucleoside	Number of cells per flask 24 hr after the addition of the compound ($\times 10^{-6}$) Duration of the preincubation of the compound at 37° (hr) 0	1	4
No addition (control)	0.24		
Freshly dissolved in DMSO	0.05		
Preincubated in PBS		0.25	0.25
Preincubated in Eagle's medium supplemented with serum		0.24	0.25
Preincubated in Eagle's medium without serum		0.24	0.23
Preincubated in Williams' medium supplemented with serum		0.25	0.26
Preincubated in Williams' medium without serum		0.24	0.24

Immediately after seeding, the cultures (containing 0.27×10^6 cells per flask) were treated with solutions of 2'-ketonucleoside preincubated according to different procedures. The concentration of the compound was 20 μg/ml. Each value reported corresponds to the mean of three flasks.

Table 4. Influence of the age of the cell cultures on the inhibiting action of unsaturated 2'-ketonucleoside on the cell multiplication rate

Age of the cultures at the start of treatment (days)	Number of cells per flask ($\times 10^{-6}$) At seeding	At the start of treatment	After a further 24-hr period at 37° Controls	Treated cells	% Decrease in the cell number with regard to control value
0	0.16	0.16	0.23	0.09	62
1	0.16	0.23	0.40	0.25	37
2	0.16	0.40	0.72	0.54	25
3	0.16	0.72	0.99	0.82	17

The age of the cultures corresponded to the interval of time between the seeding of the cells and the addition of the compound dissolved in DMSO or DMSO alone (controls). The concentration of the compound was 10 μg/ml. Each value reported corresponds to the mean of three flasks.

Table 5. Influence of the number of cells at seeding on the inhibiting action of the unsaturated 2'-ketonucleoside on the cell multiplication rate

Number of cells per flask ($\times 10^{-6}$) At seeding	At the start of treatment (24 hr after seeding)	24 hr after the start of the treatment Controls	Treated cells	% Decrease in cell number with regard to control values
0.05	0.09	0.11	0.06	47
0.10	0.13	0.19	0.12	37
0.20	0.25	0.46	0.28	39
0.40	0.53	0.85	0.64	24

The concentration of the ketonucleoside was 10 μg/ml. Each value reported corresponds to the mean of two flasks.

action would be phase cycle dependent. The phenomenon is complex and requires more experiments for complete clarification.

The following experiments were carried out to investigate the mode of action of the ketonucleoside on cell metabolism. As a first step, we studied three processes responsible for synthesis of cellular macromolecules: replication, transcription and translation. Because the 2'-ketonucleoside was rapidly inactivated in the culture medium, short-term experiments (1-hr period of action) were performed. The results show that DNA, RNA and protein syntheses were inhibited but to different degrees (Table 6). Replication was the first process to be impaired by the compound. At a dose which did not modify the cell growth rate for a 24-hr period (2.5 μg/ml) DNA synthesis was inhibited, whereas the transcription and translation processes were not significantly changed. This is in agreement with the presence of enlarged cells, which may be observed in the cultures.

At higher doses (5 and 10 μg/ml) replication inhibition increased, but both RNA and protein syntheses were also inhibited. At cytotoxic concentrations the ketonucleoside completely blocked cell metabolism. Thus, although replication appears as the process most sensitive to 2'-ketonucleoside action, it was by no means the only metabolic path-

Table 6. Action of the unsaturated 2'-ketonucleoside on replication, transcription and translation processes in KB cells

Concentration of 2'-ketonucleoside in culture medium μg/ml	% Inhibition of DNA synthesis	RNA synthesis	Protein synthesis
2.5	25 ± 2.5	13 ± 0.7	10 ± 5
5	39 ± 4.5	31 ± 4.3	26 ± 13.8
10	63 ± 11	48 ± 10.2	45 ± 9
20	75 ± 7.4	76 ± 8.5	75 ± 8.2
40	91 ± 2.5	90 ± 6.9	88 ± 7.4

Three days after seeding, the 2'-ketonucleoside dissolved in DMSO was added to KB cell cultures (containing about 0.8×10^6 cells per flask) at the concentrations indicated in the Table: DMSO alone was added to control cultures. Labelled precursors were immediately added to the cultures: 0.5 μCi/ml of ([^{3}H]methyl)thymidine (46 Ci/m-mole) or 0.5 μCi/ml of 8-[^{3}H]uridine (55 Ci/m-mole) or 1 μCi/ml of the (U-^{14}C) L-amino acid mixture (40 mCi/m-mole). At the end of the 1-hr pulse, the cultures were treated as described in Methods. The results are expressed as the mean ± S.E.M. of four experiments.

Table 7. Inhibiting action of the unsaturated 2'-ketonucleoside on cell multiplication in both untransformed and neoplastic cells

Intervals of time after seeding (hr)	% Inhibition of the multiplication rate Cell lines				
	KB	LF	F_1	F_2	F_3
6–22	36	15	11	40	17
29–45	23	10	17	13	29
54–70	24	3	13	15	5

The concentration of the ketonucleoside was 10 μg/ml. A constant 16-hr period of action of the compound was considered at different intervals of time after seeding.
The multiplication rate was defined in Table 1.

way which was impaired. The compound produced an overall inhibiting effect on cell metabolism. Experiments now in progress are aimed at providing information on the mechanism of 2'-ketonucleoside action. They attempt to determine which metabolic step(s) are perturbed: uptake of precursors, activity of enzyme system(s), or others.

The mechanism of ketonucleoside action may still be unknown but there is evidence that the carbonyl group plays a crucial role in the biological activity of the compound. Thus, the parent compounds, which are devoid of the keto group, were completely inactive as were compounds derived from the reduction of the keto group (isomers of the parent nucleosides or deoxyhexosyl purines [7, 8]). Experiments now under way will indicate to what extent the keto group is implicated in the inactivation of the compound in aqueous solution at 37°.

The last point concerns the specificity of the action of ketohexosyl purines for cancer cells. It has been shown that the unsaturated 2'-ketonucleoside inhibits multiplication of L 1210 leukemia cells intraperitoneally injected to mice [2]. It is also known that the KB cells used for experiments were derived from a human epidermoid carcinoma of the mouth. Thus, it should be asked to what extent the effect of this compound was selective for cancer cells. This problem was investigated using various hepatic cell lines showing different cyto-biological characteristics. The F_2 and F_3 lines still correspond to apparently normal cells whereas the F_1 line may be considered as malignant transformed cells [6]. The LF line was derived from hepatoma cells. The results of a 16-hr period of ketonucleoside action on cell multiplication of the various cell lines at different intervals of time after seeding are reported in Table 7. They clearly show that the compound inhibited cell growth in apparently normal cells as well as in neoplastic cells. Taking into account the moderate level of inhibition induced at the low concentration used (10 μg/ml), we gave higher doses of the ketonucleoside to three lines corresponding to cultures of normal hepatocytes (F_2), malignant transformed hepatocytes (F_1) and cancerous cells (KB). For each dose (10, 20, or 40 μg/ml) used, there was no significant difference in the action of the

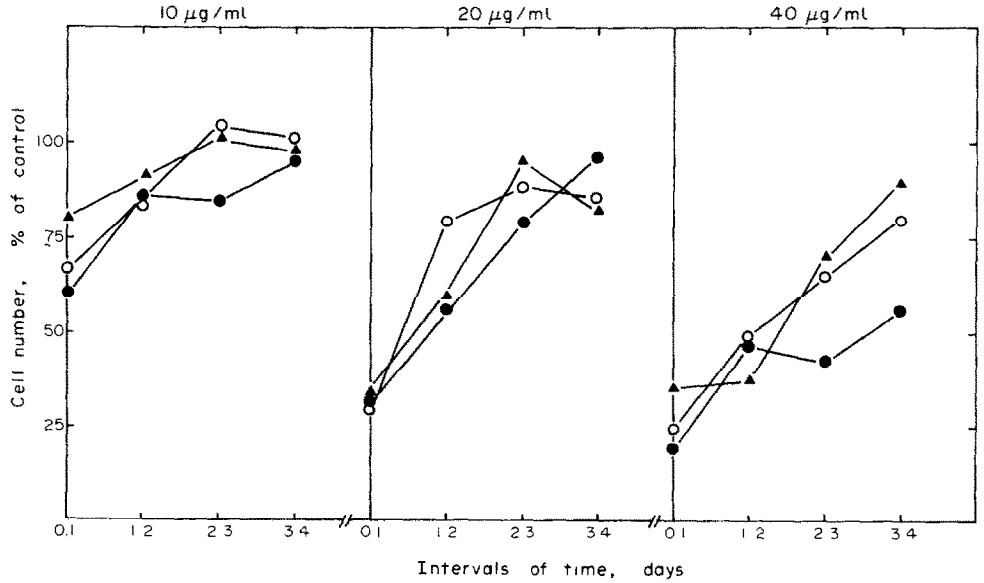

Fig. 2. Kinetics of unsaturated 2'-ketonucleoside action at different concentrations on the multiplication of normal and transformed cells. Abscissa—time expressed as a constant 24-hr period of action considered at different intervals of time after seeding. Ordinate—percentage of the number of cells in treated cultures as compared with control cultures. The concentration of the ketonucleoside is indicated at the top of the graph. The experimental conditions were identical with those described in Table 4 except that the three cell lines were cultured in Williams' medium supplemented with 15% fetal calf serum. ●: F_2 cells; ▲: F_1 cells; O: KB cells.

compound on the three cell lines along the different steps of the cultures (Fig. 2). These findings demonstrate that in cell cultures, the effect of the unsaturated 2′-ketonucleoside is not restricted to cancer cells.

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