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Preclinical report

Time-schedule dependency of S 16020, a new topoisomerase II inhibitor

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S 16020 is a new olivacine derivative which has been shown to intercalate into DNA and to stabilize the cleavable complex formed by DNA and purified topoisomerase II. The aim of the present study was to estimate the impact of time exposure on the *in vitro* activity of S 16020. This was done on seven cancer cell lines of human origin (head and neck, kidney, and ovary). Doxorubicin was used as a reference drug. The cytotoxic activity of S 16020 remained stable during at least 3 h. A loss of activity of about 30% was apparent after 6 or 24 h preincubation. This relative loss of activity reached about 50% after 72 h preincubation. Considering all tested cell lines, the average IC_{50} decrease was 89 ± 8% for S 16020 with incubation times between 1 and 72 h. An exposure index (EI) was calculated to evaluate the effect of time on the cytotoxic efficacy. The reference time was 1 h exposure. The EI values were corrected to take into account the loss of drug activity. For the majority of cell lines EI values were greater than 1 for both drugs, particularly after a 6 h exposure time. This means that, in this case as compared to the shorter exposure (1 h), increasing time has a relative detrimental effect on drug efficacy. For the two cancer cell lines of ovarian origin, EI values remained close to 1 for both drugs whatever the total exposure time. This means that, in this case, time and concentration have symetrical effects on cell survival. The pharmacological information provided by the present study may be useful in designing future clinical trials on this potentially interesting new topoisomerase II inhibitor. As a consequence of these data, 1 and 3 h drug administration schedules are currently tested during phase I trials. [© 1999 Lippincott Williams & Wilkins.]

Key words: S 16020, time-schedule dependency, topoisomerase II.

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Introduction

Topoisomerases are highly conserved nuclear enzymes which control and modify the topological state of DNA.[1] Type I topoisomerases (Topo I) are involved in transcription, whereas type II topoisomerases (Topo II) play a critical role during DNA replication and mitosis. Different anticancer agents like etoposide,[2] anthracyclines[3] and ellipticine derivatives[4] are presumed to exert their cytotoxic activity by stabilizing the cleavable complex formed between DNA and Topo II. Topo II is thus a relevant intracellular target when designing new anticancer drugs. S 16020 is a new olivacine derivative which has been shown to intercalate into DNA, and to stabilize the cleavable complex formed by DNA and purified Topo II.[5] S 16020 has demonstrated marked antitumor activity in various experimental tumor models and was shown to be more efficient than elliptinium acetate.[6,7] S 16020 was recently evaluated at the experimental level in comparison with doxorubicin against 13 human tumors;[7] the antitumor activity of S 16020 was found to be superior to that of doxorubicin (Dox) in five xenografts.

One of the main objectives of preclinical investigations for a new anticancer drug is to supply rational guidelines which may be useful in providing the basis for future clinical trials.[8] Particularly useful could be the time-schedule dependency of the drug's cytotoxic effects. A typical example concerns the widely used 5-fluorouracil, for which the advantages of continuous infusion scheduling was first claimed in the laboratory[9] and several years later in clinical practice.[10] The need for a strong preclinical rationale concerning the impact of the schedule has been underlined for a specific Topo II interfering drug, etoposide, for which the best dose and schedule have still to be established.[11]

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The aim of the present study was to estimate the impact of time exposure on the *in vitro* activity of S 16020. This was done on seven cancer cell lines of human origin (head and neck, kidney, and ovary). Dox was used as a reference anticancer drug acting on Topo II.

Material and methods

Chemicals

S 16020 was obtained as a freeze-dried powder from Servier (Courbevoie, France). Dox was purchased from Sigma (St Louis, MO). S 16020 and Dox were diluted in water at a final concentration of 10^{-2} M, which was dispatched in 50 μl samples and frozen at −20°C. The 3-(4-5 dimethylthiazol-2-yl)-2,5 diphenyl-tetrazolium (MTT) was from Research Organics (Cleveland, OH); it was prepared in phosphate-buffered saline (PBS; Sigma) at a concentration of 5 mg/ml, filtered and kept at 4°C in the dark.

RPMI 1640, fetal calf serum (FCS), L-glutamine, penicillin and streptomycin were from Sigma.

Stability study

S 16020 and Dox were preincubated in cell-free medium for 1, 3, 6, 24 or 72 h in a humidified incubator at 37°C with an atmosphere containing 5% CO_2 in air. Aliquots of these preincubated media were tested against KB cells over 1 h. Cells were then washed twice and incubated at 37°C in 5% CO_2 in RPMI 1640 medium without drug for a total incubation duration of 96 h. The MTT test was performed at 96 h. In addition, S 16020 and Dox concentrations were determined in the preincubated medium by high-performance liquid chromatography with UV detection.

Experimental conditions for the evaluation of the time-schedule dependency of S 16020 and Dox

The seven human tumor cell lines used are described in Table 1.[12-16] Cell lines were free of mycoplasma contamination. Cells were routinely cultured in a humidified incubator at 37°C with an atmosphere containing 5% CO_2 in air. Cell lines were grown in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine and antibiotics (100 U/ml penicillin, and 0.1 mg/ml streptomycin).

Table 1. Human tumor cell lines investigated

Cell line	Tumor type	Doubling time[a] (h)	Initial density (cells/well)
RXF-393	renal	27.5±0.5	1000
RXR-631	renal	25.0±2.0	1000
KB	buccal	18.1±1.0	500
KB-VM4 (teniposide resistant)	buccal	25.5±3.5	500
KB-VP2 (etoposide resistant)	buccal	19.5±0.5	500
SKOV3	ovary	22.0±8.0	2000
IGR OV-1	ovary	13.7±7.0	5000

[a]Mean ± SD from three independent experiments.

Cells were grown in 96-well microtiter plates in the culture medium; 24 h after subculture, cells were exposed to the drugs for the following durations: 1, 3, 6, 24 or 72 h. At the end of the incubation periods, cells were washed twice and incubated at 37°C in 5% CO_2 in RPMI 1640 medium without drug for a total incubation duration of 96 h. The concentrations of S 16020 and Dox applied ranged between 10^{-10} and 10^{-4} M, and between 5×10^{-11} and 10^{-4} M, respectively.

Evaluation of growth inhibition

The growth inhibitory effects were assessed after 96 h of total incubation time by the MTT test previously described.[17] Briefly, 20 μl of 5 mg/ml MTT solution was added to each well and the plates were incubated for 4 h at 37°C. The supernatant was aspirated and the formazan solubilized by 100 μl of DMSO after mild shaking for 30 min. Absorbance was measured at 540 nm (Dynatech MR4000). Two to five wells per concentration were used and three independent experiments were performed. Results were expressed as the relative percentage of absorbance compared to the controls without drug. IC_{50} was defined as the drug concentration causing a 50% reduction in cell survival as compared to controls. The influence of time exposure on drug cytotoxicity was assessed using the following factor: EI (exposure index)=$I[C \times T]_{50}$ at 3, 6, 24 or 72 h/$I[C \times T]_{50}$ at 1 h, where $I[C \times T]_{50}$ was defined as the cumulative drug exposure ($C \times T$, product of the applied concentration by the duration of exposure) causing 50% reduction in growth compared to controls.

For a given drug, EI was corrected taking into account the percentage of the residual activity found during the stability study of this drug (EI corrected= EI × % residual activity).

Statistics

The influence of preincubation time or incubation time on the evolution of the tested parameters was measured by non-parametric Spearman correlation.

Results

Stability of S 16020 and Dox in cell-free medium

The functional stability of both cytotoxic agents, S 16020 and Dox, was evaluated after 1, 3, 6, 24 or 72 h preincubation at 37°C in cell-free medium (Table 2). The cytotoxic activity of S 16020 remained stable for at least 3 h. A loss of activity of about 30% was apparent after 6 and 24 h preincubation. This relative loss of activity reached about 50% after 72 h preincubation. The cytotoxic activity of Dox was stable until 24 h preincubation and showed activity reduced by about 30% after 72 h preincubation.

Cytotoxic effects of S 16020 and Dox on tested cell lines

The tested cell lines showed different sensitivity profiles (Table 3). Both drugs showed comparable cytotoxicity profiles. Both epipodophyllotoxin-resistant cell lines (KB-VM4 and KB-VP2) exhibited relative resistance to S 16020 and Dox (Table 4). As compared to the KB parental cell line, KB-VM4 cells were about 3-fold more resistant to S 16020 and 1.5-fold more resistant to Dox after a 1 h incubation. KB-VP2 cells were about 7-fold more resistant to S 16020 and 3-fold more resistant to Dox after a 1 h incubation. For both drugs, no significant changes in resistance factors were shown when the exposure times were increased.

Time-schedule dependency of S 16020 and Dox

For both drugs, IC_{50} decreased as exposure time increased. Considering all tested cell lines and given a 1–72 h incubation time, the average IC_{50} decrease was $89 \pm 8\%$ for S 16020 and $94 \pm 3\%$ for Dox.

EI was calculated to evaluate the effect of time on the cytotoxic efficacy of both drugs when considering $[C \times T]_{50}$ (Table 5). The reference time was 1 h exposure. EI values were corrected to take into account the loss of drug activity (Table 2). Two groups of cell lines could be identified. For the majority of cell lines, including KB, KB-VM4, KB-VP2, RXF-393 and RXF-631, EI values were greater than 1 for both drugs, particularly after a 6 h exposure time. This means that, in this case as compared to the shorter exposure (1 h), increasing exposure time has a relative detrimental effect on drug efficacy. Figure 1 illustrates the impact of S 16020 exposure time on the survival of KB cells. In contrast, for the two cancer cell lines of ovarian origin, IGROV1 and SK-OV3, EI values remained close to 1 for both drugs whatever the total exposure time. This means that, in this case, time and concentration have a symetrical effect on cell survival. Figure 2 illustrates the impact of S 16020 exposure time on the survival of IGR OV 1 cells.

Discussion

Despite the unavoidable gap between the bench and the bedside, *in vitro* investigations on tumoral cell lines may represent a valuable tool in assessing the impact of drug schedule on optimal cytotoxic action. A striking example is provided by the antimetabolite 5-fluorouracil: had the experimental data demonstrating the superiority of prolonged exposure[9] been available and taken into consideration earlier during drug

Table 2. Impact of drug preincubation on cytotoxic efficacy (KB cells)[a]

Preincubation time (h)	S 16020		Dox	
	IC_{50} (μmol/l)	Residual activity (%)	IC_{50} (μmol/l)	Residual activity (%)
Without	0.56 ± 0.11	–	0.36 ± 0.00	–
1	0.59 ± 0.03	94	0.34 ± 0.07	106
3	0.62 ± 0.02	90	0.40 ± 0.09	90
6	0.82 ± 0.10	68	0.36 ± 0.00	97
24	0.79 ± 0.11	71	0.36 ± 0.02	100
72	1.08 ± 0.17*	52*	0.50 ± 0.04	72

[a]Mean ± SD (two separate experiments. Evolution of the parameter as a function of the increase of preincubation time.
*$p<0.05$.

Table 3. Evolution of IC_{50} as a function of exposure time[a]

Cell lines	Incubation time (h)	IC_{50} (nmol/l)	
		S 16020	Dox
KB	1	557±68	671±206
	3	234±52	192±60
	6	144±22	136±4
	24	143±25	72±4
	72	86±5*	61±6**
KB-VM4	1	1655±445	836±129
	3	614±74	364±73
	6	506±11	254±10
	24	352±1	160±42
	72	192±49**	84±19**
KB-VP2	1	3900±1824	2035±870
	3	2080±1301	1092±293
	6	1440±989	740±101
	24	454±11	346±1
	72	322±59**	167±18**
RXF-393	1	4685±1520	2640±297
	3	3235±714	1265±92
	6	1990±495	679±51
	24	1465±262	258±42
	72	616±77**	194±4**
RXF-631	1	1400±764	2355±488
	3	766±74	872±180
	6	558±138	456±114
	24	177±3	222±89
	72	92±25**	133±61**
IGR OV-1	1	4550±1485	5000±4808
	3	1500±0	3150±2051
	6	1250±354	1700±141
	24	315±92	455±64
	72	130±85**	94±51**
SKOV3	1	1100±141	1100±848
	3	545±431	615±21
	6	130±42	280±28
	24	95±50	69±27
	72	28.5±12**	26.5±2**

[a]Mean IC_{50} ± SD (two or three independent experiments). Evolution of the parameter as a function of the increase in the incubation time. *$p<0.05$; **$p<0.001$.

development, the clinical use of this drug would have probably been improved.[10,18] The present study was focused on the impact of the drug schedule for S 16020, a Topo II inhibitor which is a new ellipticine analog. Dox was taken as a control drug since it shares a common mode of action with ellipticine: Topo II inhibition, intercalation and DNA binding.[19] More precisely, among the three types of curves that relate Topo II inhibitor concentration to cleavable complex formation, ellipticine analogs and anthacyclines exhibit the same bell-shaped curve (an initial increase in complex formation with drug concentration and, at higher drug concentration, a decrease in cleavable complex).[19]

Table 4. Resistance factors to S 16020 and Dox in the two cell lines KB-VM4 and KB-VP2[a]

Cell lines	Exposure time (h)	Resistance factors	
		S 16020	Dox
KB-VM4	1	3±1	1.5±0.5
	3	2.5±0.5	2±1
	6	3.5±0.5	2±0
	24	2.5±0.5	2.5±0.5
	72	2.5±0.5	1±0
KB6VP2	1	7±4	3±0
	3	8.5±3.5	6±0
	6	9.5±4.9	5.5±0.5
	24	3.5±0.5	5±0
	72	3.5±0.5	3±0

[a]Values are mean ± SD from two independent experiments. The resistance factor was calcaulted as the ratio IC_{50} in the resistant cell line at time X/IC_{50} in the parental sensitive cell line at the same time X.

When studying the impact of the drug schedule on drug cytotoxity, the first step is to analyze the stability of the drug itself in the experimental conditions under study. From the present study, it appears that a progressive loss of cytotoxic efficacy occurred for S 16020 after 3 h pre-incubation in the culture medium. This loss of cytotoxic activity of the pre-incubated drug was also observed for Dox but after a 24 h period only. By analogy with other ellipticine derivatives, this loss of activity may be explained by binding to proteins,[20] by the formation of covalent adducts with thiol derivatives[21] or by biochemical transformation into less active compounds.[22] Complementary studies are in progress in order to identify the mechanisms responsible for the loss of S 16020 activity during prolonged contact with the culture medium.

There were two Topo II inhibitor resistant cell lines among the investigated cell lines (KB-VM4 and KB-VP2). It is clear that increasing the exposure time does not significantly change the value of the resistance factor determined for S 16020 and Dox (Table 4).

As initially emphasized by Ludwig *et al.*[23] and by Matsushima *et al.*[24] at the experimental level, and by Powis[25] at the clinical level, the cytotoxic effects of anticancer agents depend on two key parameters, i.e. drug concentration and exposure time. These two factors were taken into account in the present study.

The $C \times T$ values were corrected to take into consideration the loss of active drug over time. Even

though this correction was made, it clearly appears that the growth inhibitory effects generated by the new olivacine derivative S 16020 are essentially dependent on the applied concentration. These concentration values were compatible with pharmacokinetic data obtained during early phase I studies.[26] As clearly shown for five out of seven investigated cell lines at a given $C \times T$ value ($I[C \times T]_{50}$), increasing exposure time leads to a steady increase of the exposure index ($I[C \times T]_{50}$ at a given time superior to 1 h/$I[C \times T]_{50}$ at 1 h). This means that the highest cytotoxicity is generated by the shorter time exposure. In other words increasing exposure time is relatively detrimental to drug cytotoxicity (Table 5).

Similar observations were obtained with Dox. The impact of scheduling on Dox cytotoxicity has been studied by others[27] and us,[28] and there was no consensus regarding schedule dependency of Dox

Table 5 Change in EI values according to the incubation time (mean ± SD from two or three independent experiments)

Cell lines	Incubation time (h)	S 16020		Dox	
		EI[a]	EI corrected[b]	EI[a]	EI corrected[b]
KB	1	–	–	–	–
	3	1.3±0.4	1.2	0.9±0.6	0.8
	6	1.6±0.4	1.1	1.3±0.4	1.3
	24	6.3±1.8	4.5	2.7±0.7	2.7
	72	11.2±0.7**	5.8	7.0±2.7*	5.0
KB-VM4	1	–	–	–	–
	3	1.1±0.2	1.0	1.3±0.5	1.2
	6	1.9±0.5	1.3	1.8±0.4	1.7
	24	5.3±1.4	3.8	4.5±0.5	4.5
	72	8.4±0.1*	4.4*	7.2±0.5*	5.2*
KB-VP2	1	–	–	–	–
	3	1.5±0.3	1.3	1.9±1.2	1.7
	6	2.1±0.5	1.4	2.3±0.7	2.2
	24	3.2±1.5	2.3	4.5±1.9	4.5
	72	6.4±1.9*	3.3*	6.7±3.5*	4.8*
RXF-393	1	–	–	–	–
	3	2.1±0.2	1.9	1.4±0.3	1.3
	6	2.6±0.2	1.8	1.5±0.6	1.4
	24	7.7±1.1	5.5	2.3±0.1	2.3
	72	9.8±2.0*	5.1	5.3±0.5*	3.8*
RXF-631	1	–	–	–	–
	3	1.9±0.9	1.7	1.2±0.5	1.1
	6	2.6±0.8	1.8	1.2±0.5	1.2
	24	3.6±2.0	2.5	2.4±1.4	2.4
	72	6.0±4.6*	3.1*	4.4±2.8*	3.2*
IGF OV-1	1	–	–	–	–
	3	1.0±0.3	0.9	2.4±1.1	2.5
	6	1.8±1.0	1.2	3.6±3.3	3.2
	24	1.8±1.1	1.3	4.3±4.5	4.2
	72	1.9±0.7	1.0	1.8±1.0	1.8
SKOV3	1	–	–	–	–
	3	1.6±1.4	1.4	2.4±1.8	2.5
	6	0.8±0.4	0.5	2.2±1.9	2.0
	24	2.1±1.4	1.5	1.8±0.8	1.7
	72	2.0±1.0	1.0	2.5±2.1	2.5

[a]EI=$I[C \times T]_{50}$ time X/$I[C \times T]_{50}$ time 1 h.
[b]EI value at a given time × residual activity (%) of the drug at this given time.
Evolution of the parameter as a function of the increase in the incubation time.
*$p<0.01$; **$p<0.05$.

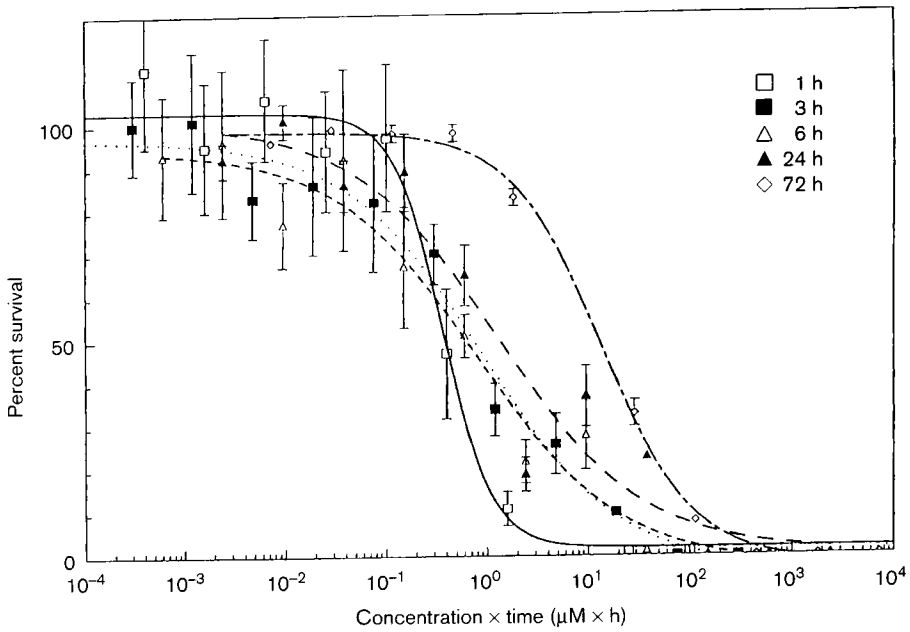

Figure 1. Effect of S 16020 exposure time on the survival of KB cells.

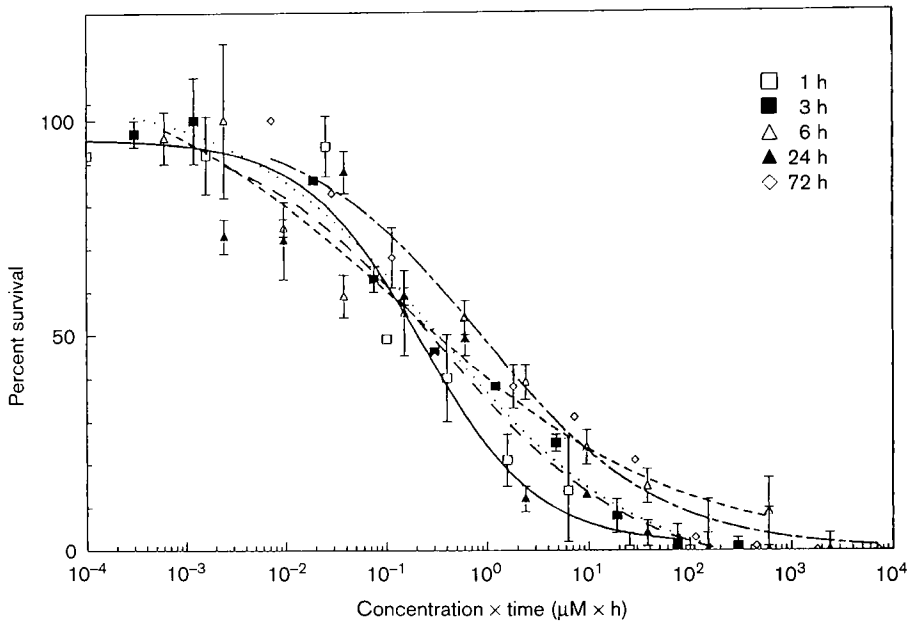

Figure 2. Effect of S 16020 exposure time on the survival of IGR OV-1 cells.

activity against cell lines *in vitro*. The present data argue in favor of the superiority of high-dose short exposures over low-dose long exposures, as observed by other investigators.[29-31] The effect of drug scheduling on S 16020 cytotoxicity may be explained in the light of previous data reported by Dugue *et al.*[32] which concern the covalent binding of elliptinium acetate to nucleic acids; analyzing the kinetics of drug binding to DNA and RNA from L1210 cells, these authors found that the interaction was rapid and already maximal after 8 h. It has been recently shown that elliptinium acetate and S 16020 interact in a similar manner with DNA.[5] The strong and rapid binding of S 16020 to the nucleic acid target may thus explain the fact that short

exposure to this drug generates the greatest cytotoxic effects.

Interestingly, in the present study there were two cancer cell lines of ovarian origin (IGROV1 and SKOV3) which respond differently to the others to drug exposure time, particularly for the drug S 16020. From 1 to 72 h, the drug exposure ratio remained close to 1 meaning that prolonging exposure was less detrimental to cytotoxic activity than for the other studied cell lines. These differences concerning the impact of exposure time on drug cytotoxicity could be explained by different levels of expression of the P-glycoprotein which is responsible for the classic multidrug resistance (MDR) phenotype.[33-35] Recent data suggest that S 16020 is a substrate for P-glycoprotein[36] and, thus, the deleterious effects of a relatively high expression of the P-glycoprotein could be more or less attenuated by prolonged exposure to a MDR relevant drug like S 16020. Investigations are in progress so as to elucidate the mechanisms responsible for these differences of cell sensitivity to S 16020 scheduling.

To conclude, bearing in mind the unavoidable oversimplification of the *in vitro* models as compared to the whole organism, the pharmacological information provided by the present study may be useful in the design of future clinical trials for this promising new Topo II inhibitor. As a consequence of these data, 1 and 3 h drug administration schedules are currently being tested during phase I trials.

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