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Antitumor activity of S 16020-2 in two orthotopic models of lung cancer

N Guilbaud, L Kraus-Berthier, D Saint-Dizier, M-H Rouillon, M Jan, M Burbridge, A Pierré and G Atassi

Institut de Recherches Servier, Division de Cancérologie Expérimentale, 14 rue de la République, 92150 Suresnes, France. Tel: (+33) 1 41 18 22 86; Fax: (+33) 1 41 18 24 40.

S 16020-2, a new olivacine derivative selected on the basis of its cytotoxicity *in vitro* and antitumor activity *in vivo*, was evaluated against the human A549 and the murine Lewis lung tumor models implanted s.c. and i.v. Against Lewis lung carcinoma implanted s.c., S 16020-2 was found to be curative, with an activity and therapeutic index (*TI* = 4) similar to that of cyclophosphamide. S 16020-2 administered weekly demonstrated a high therapeutic efficacy against A549 non-small cell lung carcinoma implanted s.c. in nude mice and induced tumor regression at 80 mg/kg. When A549 tumor cells were injected i.v. in SCID mice, experimental metastases rapidly developed and the progressive invasion of the lung tissue by tumor preceded the death of animals. In this model, S 16020-2 administered at 40 mg/kg i.v. following an early (days 8, 18 and 28) or delayed (days 20, 30 and 40) treatment schedule prolonged the survival of tumor-bearing mice with T/C values of 150 and 145%, respectively. Against the i.v. Lewis lung carcinoma, S 16020-2 was also highly active since when administered at 60 mg/kg on days 5, 9 and 13 it totally inhibited tumor growth and cured up to 89% of mice. When administered on days 11, 15 and 19 to animals with established tumors, S 16020-2 was still active but not curative. In the presented studies, S 16020-2 antitumor activity was superior to that of adriamycin and comparable or superior to cyclophosphamide (used as reference compounds). Our results demonstrate the efficacy of S 16020-2 against these highly agressive and chemoresistant tumor models.

Key words: Lung cancer, orthotopic models, S 16020-2, topoisomerase II, xenografts.

Introduction

Despite recent major advances in the understanding of the biology of lung cancer, this pathology is still the leading cause of death from malignant disease. A number of chemotherapeutic agents now under development show promise in the treatment of non-small cell lung cancer (NSCLC).[1] These encouraging results confirm the potential of chemotherapy and the interest in developing new anticancer agents active against this disease.

Correspondence to G Atassi

S 16020-2 is a highly cytotoxic olivacine analog[2] which was shown to intercalate into DNA and to stabilize the cleavable complex formed by topoisomerase II and DNA.[3] S 16020-2 showed a broad range of antitumor activity against a panel of murine and human tumor models,[4] being particularly active against two tumor models of pulmonary origin: the murine Lewis lung carcinoma and the human NSCLC NCI-H460 both implanted s.c. Since orthotopic tumor models are supposed to be more relevant to the clinical setting than s.c. xenografts,[5] we investigated the antitumor activity of S 16020-2 against Lewis lung carcinoma and the human NSCLC A549 implanted i.v. In these models, tumors rapidly develop in the lungs of the animals, mimicking the spread and growth of tumor in patients. This study was thus undertaken to evaluate the potential activity of S 16020-2 in such pathologies before its clinical development.

Materials and methods

Drugs

S 16020-2, 9-hydroxy-5,6-dimethyl-1-[*N*-[2-(dimethylamino)ethyl]carbamoyl]-6H-pyrido[4,3-*b*]carbazole dichlorhydrate, was synthesized in our institute as described.[6] Reference compounds obtained from various suppliers were ADR (Adriblastine®, Upjohn/Pharmacia, France) and cyclophosphamide (Endoxan®, Sarget, France). Drugs were first dissolved in sterile water and diluted before administration to animals at 0.1 ml/10 g of body weight.

Mice and tumor models

BDF_1 (C57Bl/6 × DBA2) mice were used for the Lewis lung carcinoma model. Swiss athymic nude (*nu/nu*) and CB 17 SCID (*scid/scid*) mice were used for human NSCLC A549 tumor xenografts

implanted s.c. and i.v., respectively. All mice were purchased from Iffa Credo (Lyon, France). They were 4–6 weeks old and weighed 20–22 g at the start of the experiments. The Lewis lung carcinoma was provided by the Division of Cancer Treatment, NCI Tumor Repository (Frederick, MD) and the A549 human cell line was provided by the American Type Culture Collection (Rockville, MD).

To produce pulmonary tumor nodules (experimental metastases) in mice, each animal was given an injection in the tail vein of 0.2 ml of a suspension containing 10^6 (Lewis lung carcinoma) or 2.5×10^6 (A549) viable tumor cells in RPMI 1640 medium (day 0). For s.c. Lewis lung carcinoma, drugs were administered i.p. or i.v. on days 3, 7 and 11. For i.v. Lewis lung carcinoma, drugs were administered i.p. or i.v. on days 5, 9 and 13 (early treatment) or on days 11, 15 and 19 (delayed treatment). For the i.v. A549 tumor model, a 10 day rest between two administrations of cytotoxic drugs was chosen because of the higher sensitivity of SCID mice to the toxic side effects of chemotherapy as compared to conventional or nude mice. Drugs were administered i.p. or i.v. on days 8, 18 and 28 (early treatment) or on days 20, 30 and 40 (delayed treatment). In each experiment, two or three control mice were sacrificed on the first day of treatment in order to confirm the presence of experimental lung metastasis.

The A549 tumor xenograft was also maintained by serial bilaterally transplantation of 2 mm^3 fragments into the flanks of athymic Swiss *nu/nu* mice. For experiments, five mice per group were implanted bilaterally and treatments started when the tumor volumes reached 50 mm^3 (day 0). Drugs were administered i.v. on days 0, 7 and 14.

Evaluation of antitumor activity

Life span. The median survival time (MST) of the treated group (T) was compared with that of the control group (C), results were expressed as T/C:

$$\text{median \%T/C (survival)} = \frac{\text{MST of treated group}}{\text{MST of control group}} \times 100$$

Long-term survivors (LTS) were registered on day 90.

When the %T/C (survival) was used, the therapeutic index (*TI*) of a compound administered following a definite schedule was defined as the ratio of the maximum tolerated dose (i.e. the highest non-toxic dose as determined on the basis of absence of early death) over the minimum active dose (the dose in mg/kg inducing a $T/C > 125\%$).

Metastasis growth inhibition. The lungs of the sacrificed animals were fixed in 10% formaldehye solution and the number of external metastases were quantified using an image analysis system (VIDS IV; Systèmes Analytiques, France). The volume of each metastasis was calculated from two-dimensional measurements:

$$\text{metastasis volume } (mm^3) = \frac{\text{length (mm)} \times \text{width}^2\ (mm^2)}{2}$$

The tumor weight expressed in mg was identical to the tumor volume (mm^3), tumor density being considered to be equal to 1. The total tumor weight per mouse was given as the sum of the metastasis weights of a lung pair. The T/C was calculated as above:

$$\text{median \%T/C (metastases growth)} = \frac{\text{median metastases weight of treated group}}{\text{median metastases weight of control group}} \times 100$$

Tumor growth inhibition. The volume of each s.c. tumor was estimated from two-dimensional tumor measurements performed with a slide caliper following the formula: length (mm) $\times$ width^2 (mm^2)/2. The median tumor volume (MTV) of each treated group was compared with that of the control group and the results were expressed as T/C:

$$\text{median \%T/C (tumor growth)} = \frac{\text{MTV of treated group}}{\text{MTV of control group}} \times 100$$

At the end of the experiment on day 90, the surviving mice were palpated and the numbers of tumor-free animals were registered.

For human tumor xenografts, the relative tumor volume was expressed as the V_t/V_0 index, where V_t is the tumor volume on a given day of measurement and V_0 is the volume of the same tumor at the start of the treatment. For the %T/C calculation the following formula was applied at each day of tumor measurement:

$$\text{median \%T/C} = \frac{\text{median } (V_t/V_0) \text{ treated}}{\text{median } (V_t/V_0) \text{ control}} \times 100$$

Statistical analysis

For s.c. human xenograft studies, data from treated and control groups were analyzed by the Newman–Keuls test.

Results

Subcutaneous Lewis lung carcinoma

S 16020-2 administered i.v. on days 3, 7 and 11 showed a high therapeutic effect against s.c. implanted Lewis lung carcinoma (Table 1). At three doses, i.e. 20, 40 and 80 mg/kg, tumor growth was strongly inhibited and LTS were registered. At 40 mg/kg S 16020-2, 100% of treated mice were considered as cured since six mice out of six were surviving on day 90 with no detectable tumor. The dose of 80 mg/kg was considered to be toxic since one mouse out of six died on day 12. Cyclophosphamide administered i.p. following the same schedule was also highly efficient since tumor growth was totally inhibited at doses ranging from 100 to 400 mg/kg. LTS were scored at 100, 200 and 400 mg/kg, this latter dose of cyclophosphamide being toxic with two early deaths registered just after the last treatment (days 11 and 12). Based on these results, the therapeutic indices of S 16020-2 and cyclophosphamide calculated with T/C (survival) values were similar ($Ti = 4$).

Intravenous Lewis lung carcinoma

In the i.v. implanted Lewis lung carcinoma model, the inoculated cells gave rise to lung meastases resulting in the rapid death of the animals. In the two studies presented in Table 2, the median survival times of the control animals were 19.1 and 16.3 days. In this model, S 16020-2 administered following the early intermittent schedule (days 5, 9 and 13) was highly active (Tables 2 and 3). At 10 mg/kg S 16020-2, the number of metastases and the median metastasis weight were dramatically reduced in comparison with the control values (14.6 and 0.6%). At 60 mg/kg S 16020-2, metastases development was totally inhibited (Table 3). This antitumor effect resulted in a prolongation of survival of the treated animals, S 16020-2 being curative at 30 and 60 mg/kg with 56 and 89% LTS (Table 2 and Figure 1). Adriamycin administered at 8 mg/kg following the same schedule also inhibited the growth of macroscopic metastases detected on day 15 and prolonged the survival of treated animals (T/C = 170%) but resulted in the cure of only 11% of the animals. This observation suggests the presence of non-detectable microscopic lung foci on the day of evaluation (day 15).

In the delayed treatment schedule (days 11, 15 and 19), 10 control mice were sacrificed on day 11 and their lungs were examined in order to confirm the presence of established macroscopic metastases at the beginning of treatment. S 16020-2 was still active in this schedule, since it prolonged the survival time of treated animals, a T/C of 251% being achieved at 60 mg/kg (Table 2). However, S 16020-2 lost its curative property following a delayed treatment schedule. ADR administered i.v. at 10 mg/kg and cyclophosphamide administered i.p. at 120 mg/kg following the same schedule prolonged the survival time of treated animals with T/C

Table 1. Antitumor activity of S 16020-2 against s.c. Lewis lung carcinoma

Experimental group	Schedule and route	Dose (mg/kg)	Median T/C (%) day 21 (tumor growth)	MST (days) (mortality range)	Median T/C (%)	Tumor-free animals[a] day 90
S16020-2	days 3, 7, 11 i.v.	5	100	35.8 (30–43)	125	—
		10	86	44.8 (14–47)	156	—
		20	5	50.0 (46–50)	175	2/2
		40	0	> 90	> 315	6/6
		80	0	> 90 (12–18)	> 315	4/4
Cyclophosphamide	days 3, 7, 11 i.p.	12.5	87	28.8 (10–35)	100	—
		25	93	32.8 (26–37)	114	—
		50	42	41.0 (27–43)	143	—
		100	0	> 90 (41–52)	> 315	3/3
		200	0	> 90	> 315	6/6
		400	0	> 90 (11–12)	> 315	4/4
Control	—	—	100	28.6 (22–38)	100	—

Tumor fragments were implanted s.c. in BDF_1 mice on day 0.
[a]Number of tumor-free animals over LTS scored on day 90.

Table 2. Antitumor activity of S 16020-2 against i.v. Lewis lung carcinoma

Experimental group	Schedule and route	Dose (mg/kg)	MST (days) (mortality range)	Median T/C (%)	LTS[a] day 90
S16020-2	days 5, 9, 13 i.v.	10	29.5 (23–63)	155	0/9
		30	> 90 (49–85)	> 471	5/9
		60	> 90 (9)	> 471	8/9
Adriamycin	days 5, 9, 13 i.v.	8	32.5 (29–36)	170	1/9
Control	—	—	19.1 (17–22)	100	0/20
S16020-2	days 11, 15, 19 i.v.	10	23.8 (20–25)	145	0/10
		30	42.0 (27–46)	257	0/10
		60	41.0 (14–47)	251	0/10
ADR	days 11, 15, 19 i.v.	10	25.0 (16–31)	153	0/10
Cyclophosphamide	days 11, 15, 19 i.p.	120	28.0 (23–34)	171	0/10
Control	—	—	16.3 (14–21)	100	0/30

Lewis lung carcinoma cells (10^6) were inoculated i.v. to BDF_1 mice on day 0.
[a]LTS over total number of mice per group.

Table 3. Effect of S 16020-2 on experimental lung metastases

Experimental group	Schedule and route	Dose (mg/kg)	Median metastasis number (range)	Median metastasis weight (mg)	Median T/C (%)
S16020-2	days 5, 9, 13 i.v.	10	6 (2–19)	0.9	0.6
		30	3 (0–6)	0.42	0.3
		60	0 (0)	0	0
ADR	days 5, 9, 13 i.v.	8	0 (0–4)	0	0
Control	—	—	41 (11–66)	159	100

Lewis lung carcinoma cells (10^6) were inoculated i.v. to BDF_1 mice on day 0. On day 15, nine animals of each treated group and 20 animals of the control group were sacrificed and their lungs removed for metastasis evaluation.

values of 153 and 171%, respectively, but were less active than S 16020-2 at 30 and 60 mg/kg.

Subcutaneous A549 non-small cell lung carcinoma

S 16020-2 administered at 20 mg/kg showed only a moderate antitumor activity against the A549 xenograft (Figure 2). At 80 mg/kg, S 16020-2 induced a significant inhibition of tumor growth (T/C = 10% on day 41) and tumor regressions in all the treated mice, V_t being lower than V_0. At this highest dose, one delayed death was observed on day 40, indicating that the maximal tolerated dose was reached. Differences observed between growth curves of the S 16020-2-treated and control groups were statistically significant from day 21 to 55 ($p < 0.01$). In this model, ADR administered at 10 mg/kg was marginally active (T/C = 43% on day 41).

Intravenous A549 non-small cell lung carcinoma

S 16020-2 antitumor activity was evaluated against A549 non-small cell lung carcinoma implanted i.v. in SCID mice. S 16020-2 administered i.v. at 40 mg/kg following the early intermittent schedule (days 8, 18 and 28) prolonged the survival time of tumor-bearing mice (T/C = 150%) but was not curative (Table 4 and Figure 3). The dose of 60 mg/kg, non-toxic to conventional or nude mice, induced two early deaths on days 12 and 18 and was inactive.

In the second experiment where histological analysis demonstrated that on day 20, the experimental metastases had totally invaded the lungs (data not shown), S 16020-2 was administered at 40 and 60 mg/kg following a delayed intermittent schedule (days 20, 30 and 40). This drug prolonged the survival of the treated mice with a T/C of 145% (Table 4). Cyclophosphamide administered i.p. at

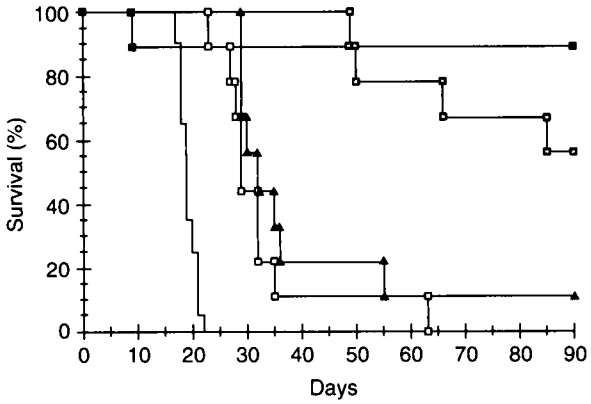

Figure 1. Antitumor activity of S 16020-2 against Lewis lung carcinoma implanted i.v. in B6D2F$_1$ mice. On day 0, control (——) and treated mice were inoculated i.v. (tail vein) with 0.2 ml of RPMI 1640 medium containing 10^6 viable tumor cells as a suspension. Mice were treated i.v. on days 5, 9 and 13 with S16020-2 at 10 (□), 30 (■), 60 (■) mg/kg, and ADR at 8 mg/kg (▲).

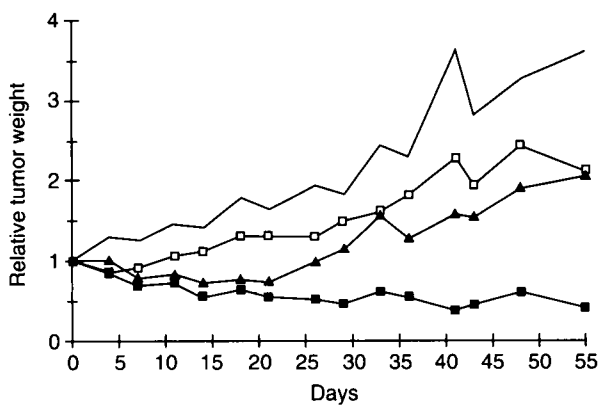

Figure 2. Effect of S 16020-2 on A549 NSCLC tumor growth. Nude mice were implanted bilaterally with tumor fragments and distributed into control (——) or treated groups. Treatment was started 9 days later (day 0). Drugs were administered i.v. on days 0, 7 and 14: S 16020-2 at 20 (□) and 80 (■) mg/kg; ADR at 10 mg/kg (▲).

90 mg/kg following the early or the delayed schedule proved to be totally inactive against this tumor model (T/C = 94 and 101%).

Discussion

S 16020-2 is a new olivacine derivative selected on the basis of its cytotoxicity[2] and antitumor activity *in vivo*.[6] We previously demonstrated that S 16020-2 is a highly active antitumor drug in various murine tumor models and human xenografts, even upon i.v. administration.[4] A good efficacy of S 16020-2 was observed against lung tumor models grafted s.c. In the present study, the antitumor activity of S 16020-2 against s.c. implanted Lewis lung carcinoma was carefully compared with that of cyclophosphamide, using a large range of doses. S 16020-2 and cyclophosphamide were both found highly active and curative in this model, with similar therapeutic indices. S 16020-2 demonstrated also a dose-dependent antitumor activity against the s.c. A549 NSCLC xenograft, superior to that of ADR and induced tumor regressions at 80 mg/kg.

Organ-specific differences in the chemosensitivity of tumor cells have been reported by several authors,[7,8] implicating the need to evaluate new anti-tumor compounds in more relevant tumor models and not only in classical s.c. implanted tumor models.[9,10] Such models allow the propagation of tumors at organ-specific (orthotopic) sites, and were established with human cancers in immunodeficient mice, mainly in SCID mice, in which high rates of tumor growth and metastases are observed.[11,12] Orthotopic lung tumor models have been obtained by intrabronchial instillation, or intrathoracic or i.v. implantation of tumor cell suspensions.[13] In this study we used this latter technique for the A549 tumor cell line with a high tumor take rate in SCID mice. In this model, the progressive invasion of lung tissues by tumor cells observed by histological analysis preceded the death of control animals. Considering the importance of the time of treatment initiation, we have used two different intermittent treatment schedules, an early (days 8, 18 and 28) and a delayed (days 20, 30 and 40) one. In these two schedules, S 16020-2 administered i.v. at 40 mg/kg was efficient in the same manner to prolong the survival of tumor-bearing mice and was more active than cyclophosphamide, a reference molecule frequently included in protocols of chemotherapy for treatment of non-small cell lung carcinoma in the clinic.[14] A more complete evaluation against a large panel of human orthotopic models of different histological types including lung tumor models is in progress in our laboratory.

Against the i.v. implanted Lewis lung carcinoma, a model in which artificial metastasis also rapidly develop in the lungs, S 16020-2 administered at 60 mg/kg on days 5, 9 and 13 (early treatment schedule) was highly effective in inhibiting tumor growth and cured up to 89% of mice, whereas in the same model, ADR also inhibited tumor growth but cured only 11% of mice. When administered following the delayed treatment schedule (days 11, 15 and 19) to animals with established tumors, the super-

Table 4. Antitumor activity of S 16020-2 against i.v. A549 non-small cell lung carcinoma

Experimental group	Schedule and route	Dose (mg/kg)	MST (days) (mortality range)	Median T/C (%)	LTS[a] day 90
S16020-2	Days 8, 18, 28 i.v.	40	70.5 (62–81)	150	0/7
	days 8, 18, 28 i.v.	60	51.5 (12–65)	110	0/7
Cyclophosphamide	days 8, 18, 28 i.p.	90	44.1 (39–52)	94	0/7
Control	—	—	47.0 (42–79)	100	1/11
S16020-2	days 20, 30, 40 i.v.	40	62.5 (59–72)	145	0/5
	days 20, 30, 40 i.v.	60	49.5 (36–63)	115	0/5
Cyclophosphamide	days 20, 30, 40 i.p.	90	43.5 (39–50)	101	0/5
Control	—	—	43.3 (36–48)	100	0/8

Cells (2.5×10^6) were inoculated i.v. on day 0 into five to 11 SCID mice per group.
[a]LTS over total number of mice per group.

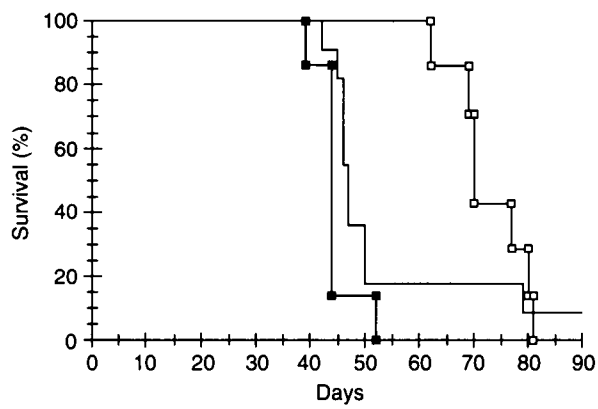

Figure 3. Antitumor activity of S 16020-2 against A549 NSCLC implanted i.v. in SCID mice. On day 0, control (——) and treated mice were inoculated i.v. (tail vein) with 0.2 ml of RPMI 1640 medium containing 2.5×10^6 viable tumor cells as a suspension. Mice were treated on days 8, 18 and 28 i.v. with S16020-2 at 40 mg/kg (□) and i.p. with cyclophosphamide at 90 mg/kg (■).

iority of S 16020-2 over ADR was maintained. The good antitumor activity observed in these s.c. and i.v. implanted tumor models suggests a favorable distribution of this compound, which is interesting information for phase I clinical investigation.

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