The Olivacine S16020 Enhances the Antitumor Effect of Ionizing Radiation without Increasing Radio-induced Mucositis

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ABSTRACT

The combination of a novel topoisomerase II inhibitor, S16020, and ionizing radiation (IR) was investigated with the aim of assessing normal tissue tolerance using a mouse mucosal lip model and antitumor activity in a human carcinoma (HEP2) cell line. No increase of acute mucosal reactions was seen when combining S16020 with IR as compared with IR alone. Using clonogenic cell survival assay, a marked enhancement of HEP2 cell killing was found when S16020 was combined with irradiation. Additional in vivo combination of S16020-IR was able to increase markedly the antitumor efficacy as compared with S16020 or irradiation alone. Interestingly, the radiosensitization effect in vivo was observed at relatively low and nontoxic concentrations of S16020, and no dose-effect relationship was found beyond 30 mg/kg. In conclusion, the combination of IR and S16020 seems promising to enhance antitumor activity without increasing deleterious effect in normal tissues and to provide the basis for a new radio-chemotherapy combination.

INTRODUCTION

S16020 (NSC-659687) is a derivative olivacine that was shown to interact with DNA by intercalation and to stimulate DNA cleavage mediated by topoisomerase II by stabilizing the covalent-enzyme DNA complex. This potent cytotoxic agent demonstrated a broad spectrum of antitumor activity on murine leukemia, Lewis lung carcinoma, B16 melanoma, and M5076 sarcoma) and human (colon, breast, ovary, non-small cell lung, and small cell lung) tumor models (2, 3). This activity compared favorably with other structurally related compounds of the ellipticine family and was comparable with that of the widely used doxorubicin. S16020 also displayed interesting activity on multidrug resistant cell lines (4–6). S16020 is currently being evaluated in Phase II clinical trials in patients with various carcinomas. Preclinical and clinical studies have demonstrated that combining topoisomerase II inhibitors (doxorubicin and etoposide) or other agents such as cisplatin or 5 fluorouracil with radiotherapy can increase tumor-control probability (7–10), whereas S16020 has never been used in combination with IR. A marked increase of toxicity is generally seen when combining some DNA intercalating agent (gemcitabine) or topoisomerase II inhibitor (doxorubicin) with IR (11, 12). On the basis of these observations, it was important to determine both the tolerance of the normal tissues and the antitumor activity when combining irradiation and S16020.

MATERIALS AND METHODS

Reagent. S16020, N-[2-(dimethylamino)ethyl]-9-hydroxy-5,6 dimethyl-6H-pyrido[4,3-b] carbazole-1-carboxamide was provided by SERVIER (France). S16020 was dissolved in its NaCl/NaOH solvent for a stock solution (20 mg/ml) and kept at –20°C. The stock solution was diluted in sterile PBS for the in vitro experiments and in 0.9% NaCl for the in vivo experiments.

Tumor Cell Line. The HEP2 cell line, which was provided by the American Type Culture Collection (Rockville, Maryland) is a human head and neck squamous cell carcinoma with wild-type p53 gene and HPV18 infection. The HEP2 cells were grown in DMEM supplemented with 10% FCS, 0.1% L-glutamine, and 0.2% penicillin-streptomycin at 37°C in a 5% CO2 humidified atmosphere.

Clonogenic Assays. HEP2 cells were seeded in triplicate into T25-cm2 flasks in a range of 200–800 cells/flask according to the condition tested. A single dose of irradiation and/or addition of the drug was done when cells were attached. S16020 was added with the time sequence of 24 h after irradiation. γ-irradiation was delivered by a 137Cs source at a dose rate of 1.97 Gy/min. Cells were cultured up to 12 days in the incubator at 37°C in a 5% CO2 humidified atmosphere.

Animals. Female mice 6–8 weeks of age, (Janvier CERT 53940, Le Genest St. Isle, France) were used: C57 black mice for the mouse mucosal lip reaction model and athymic nude mice for HEP2 tumor xenograft model. The in vivo experiments were carried out at the Institut Gustave Roussy under the Animal Care license C94-076-11 (Ministère de l’Agriculture).

The abbreviation used is: IR, ionizing radiation.
Mouse Mucosal Lip Reaction Model and X-irradiation Conditions. Tolerance of normal tissues was assessed using a mucosal lip reaction model in C57 black mice (13). Mice were fed everyday with a semiliquid food (Renutryl 500 from Nestle Clinical Nutrition, Marne la Vallés, France) to prevent weight loss attributable to difficulties in swallowing subsequent to the irradiation treatment of the mucosal tissue of the lips. S16020 combined with IR was designed in three different ways: (a) S16020 injection 20 days before IR; (b) S16020 injection 20 after IR; and (c) concomitant IR performed within 20 min after the S16020 injection. S16020 was i.v. injected at 50 mg/kg and 80 mg/kg, which corresponded to 50 and 80% of the previously defined maximal tolerated dose. Irradiation was carried out at a dose rate of 0.74 Gy/min with 250 kV RT Phillips X-ray (200 kV; 17 mA; 0.5 mm Cu filter). Six mice were radially located in the X-ray field. A single dose of 16.5 Gy was delivered locally on their snouts. Each experiment was composed of eight groups (control without treatment, solvent alone, S16020 alone, IR alone, and combinations of solvent and IR and S16020 and IR), with six mice/group, and performed at least twice. Mice were weighted and the mucosal lip reactions (erythema and edema) were scored every day during the peak of the acute reactions and every two days outside the peak of reaction (within 3–7 weeks) using the Parkins scoring system (13).

Tumor Xenograft and Assessment of Antitumor Activity of the Combined Treatment. HEP 2 tumor xenografts were obtained by s.c. injection of $3 \times 10^6$ cells in the right flank of nude mice. Xenografts were grown for 3 weeks to a mean tumor volume of $10^7 \pm 27$ mm$^3$. For the concomitant treatment, S16020 was i.v. injected at doses of 30 mg/kg and 60 mg/kg, and within 20 min a single dose of X-rays (15 Gy) was delivered locally on mice xenografts. Irradiation was carried out with 250 kV RT Phillips X-ray at a dose rate of 0.69 Gy/min (220, 20 mA; 0.2 mm Cu filter). Mice were weighed and the tumor size measured twice a week with an electronic caliper. The tumor volume was estimated from two dimensional tumor measurements by the formula:

$$\text{Tumor volume} = \text{length (mm)} \times \text{width}^2 \text{(mm}^2)/2.$$

In each group (six mice/group), the relative tumor volume was expressed as the $V_t/V_o$ ratio (where $V_t$ is the mean tumor volume on a given day during the treatment and $V_o$ is the mean tumor volume at the beginning of the treatment). Treatment efficacy was determined using both criteria: specific growth delay and the optimal percentage of treated versus control according to Langdon et al. (14).

Table 1 Antitumor activity of S16020 combined with IR against human HEP2 head and neck carcinoma xenograft

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Maximum body weight loss % (day)</th>
<th>Optimal treated vs. control growth delay (day)</th>
<th>Efficacy scoring$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR 15 Gy</td>
<td>1.4 (10)</td>
<td>45 (22)</td>
<td>+</td>
</tr>
<tr>
<td>S16020 30 mg/kg</td>
<td>5.3 (3)</td>
<td>67 (14)</td>
<td>0.3</td>
</tr>
<tr>
<td>S16020 30 mg/kg + IR 15 Gy</td>
<td>8.8 (3)</td>
<td>26 (32)$^b$</td>
<td>2.0</td>
</tr>
<tr>
<td>S16020 60 mg/kg</td>
<td>8.0 (7)</td>
<td>44 (32)</td>
<td>1.1</td>
</tr>
<tr>
<td>S16020 60 mg/kg + IR 15 Gy</td>
<td>10.8 (3)</td>
<td>22 (32)$^b$</td>
<td>2.2</td>
</tr>
</tbody>
</table>

$^a$ According to Langdon et al. (14): − inactive; (+) marginally active; ++, ++, ++, +, + active to highly active.

$^b$, $P < 0.05$; $**$, $P < 0.01$ compared with 15 Gy alone.

Statistical Analysis. Statistical significance and comparisons of surviving fraction among treatment groups were analyzed using the two-tailed, unpaired Student-t test for cell survival curves in vitro and the Mann-Whitney nonparametric U test (Statview software) for in vivo relative tumor volume data from the mean of two independent experiments.
Fig. 3  Time course of the erythema (a) and edema (b) reactions for the three tested regimens: 1, concomitant regimen: i.v. injection of S16020 and IR; 2, S16020 i.v. injection 20 days before IR; 3, S16020 injection 20 days after IR in the mucosal lip reaction model in C57 black mice. No significant increase of erythema/edema was seen when combining IR with S16020, as compared with IR alone.
RESULTS AND DISCUSSION

S16020 has been characterized as a new inhibitor of topoisomerase II that stabilized the complex DNA/topoisomerase II enzyme (1). Several studies on human and murine tumor models have shown a more pronounced antitumor activity of S16020 in comparison with other complex-stabilizing inhibitors of topoisomerase II, such as doxorubicin and elliptinium acetate (2, 3, 6). These promising results have led S16020 to be evaluated in Phase I/II clinical trials (ongoing). Radiochemotherapy combinations are increasingly used in the treatment of various carcinomas. The therapeutic gain of these combinations depends on the possibilities available to increase the antitumor efficacy while maintaining acceptable toxicity. With that aim, we have tested the value of combining S16020 with IR in a mouse mucosal model for toxicity evaluation and in a human head and neck squamous cell carcinoma xenograft for antitumor activity.

Our *in vitro*, results combining γ-irradiation and S16020 are plotted in Fig. 1, showing that S16020 led to a marked killing enhancement of HEP2 cells for S16020 concentrations ≤10 nM (IC$_{50}$ = 10 nM; previously estimated in HEP2 cells; data not shown). Different combinations of IR and S16020 were tested, showing that the most efficient one was the sequential exposure of the cells to IR with subsequent S16020 administration. For 5 nM and 10 nM doses of S16020 incubated 24 h after 4Gy, we found that surviving fractions of HEP2 cells were 1.5-fold ($P = 0.003$) and 4.5-fold ($P = 0.0001$), respectively, decreased by the combined treatment as compared with the single irradiation dose of 4Gy. These findings are consistent with several studies which found marked cytotoxicity for cells exposed to doxorubicin or etoposide 24 h after irradiation (7, 8). This enhanced cell-killing effect has been correlated with radiation-induced DSB and impairment of DNA repair process by topoisomerase II inhibitors that stabilize the DNA/enzyme cleavable complex (15).

*In vivo* combination of IR and S16020 in HEP2 xenograft induced a significant decrease in tumor volume as compared with IR alone or S16020 alone, as shown in Table 1. A pronounced antitumor effect of the combined treatment was observed using a relatively low dose (30 mg/kg) of S16020 (Table 1). Importantly, the magnitude of the antitumor effect was not significantly increased when a higher dose (60 mg/kg) was combined with IR, as reported in Fig. 2 and Table 1. This observation was consistent with the intrinsic cytotoxicity of topoisomerase II inhibitors. Indeed, it has been found that ellipticine and anthracyclin derivatives have an optimal dose range to induce DNA strand breaks. Beyond, *i.e.*, at high concentrations of drug, the induction of DNA breaks is suppressed (because of a decrease in cleavable complex) and thus may limit their cytotoxicity (16, 17). Combination was well tolerated by tumor-bearing nude mice, with no toxic death and no more than 10% body weight loss during the course of treatments.

Combined radiochemotherapy are often limited by the tolerance of the normal tissues and especially by acute mucosal reactions. Our results on normal tissue tolerance are plotted in Fig. 3. The acute inflammatory response was first composed of an erythema peak (reaching a maximum degree of severe desquamation with exudation on the days 11 and 12) and subsequently an edema peak. No increase of acute mucosal reaction was scored when S16020 was combined to IR, as compared with IR alone. In addition, no difference was seen regarding the timing of the three distinct combinations used for the combined treatment (IR 20 days before, 20 days after, or concomitant with S16020). However, when irradiation was performed 20 days before S16020 injection, a second minor peak of erythema reaction was seen occurring 7 days after S16020 injection (Fig. 3). The magnitude of this peak (scored 0.5, *i.e.*, doubtful) suggested that there was no clear evidence of a “recall” phenomenon. This is in contrast with other topoisomerase II inhibitors, which may induce a recall phenomenon when injected after irradiation (9). This recall effect has been defined as a reactivation of latent radiation changes in tissues previously irradiated, but which have returned to normal appearance in the time interval. No significant increase in normal tissue toxicity and the absence of recall phenomenon were in contrast with other combinations of topoisomerase II inhibitors and X-irradiation, in particular with doxorubicin. In summary, we have observed that the reactions in mucosal lips were independent of both the S16020 dose and the administration time sequence of the combined treatment as compared with the X-irradiation alone.

In conclusion, combining S16020 with IR to increase significantly the antitumor effect without increasing deleterious reactions on normal tissues was promising. A putative explanation regarding this possible differential effect could be related to the level of expression of topoisomerase II within tissues. Drug activity has been correlated with the level of expression of topoisomerase II (18) and it has been found that topoisomerase II expression was higher in cancer cells compared with normal surrounding cells (19). Another factor that may contribute to this apparent differential effect was that the radiosensitization of tumor could be observed within a low and nontoxic dose range of S16020. However the precise mechanisms involved in this radiosensitization effect remain to be studied.

The findings of this study provide the basis for a new radio-chemotherapy combination that would need to be investigated further for clinical applications.

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