In Vitro and in Vivo Pharmacological Characterizations of the Antitumor Properties of Two New Olivacine Derivatives, S16020-2 and S30972-1

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ABSTRACT

S16020-2, a new olivacin derivative and a topoisomerase II inhibitor, has recently entered clinical trials. New analogues and derivatives have been synthesized from the S16020-2 compound. Preliminary data indicate that S30972-1, one of these S16020-2 derivatives, may exhibit a comparatively higher level of antitumor potency associated with an improved therapeutic index than does S16020-2. The antitumor activities of S16020-2 and S30972-1 were therefore characterized both in vitro and in vivo, with Adriamycin and etoposide chosen as reference compounds. The in vitro data show that S30972-1 is a topoisomerase II inhibitor, mediating its activity through an ATP-dependent mechanism such as S16020-2. The two olivacine derivatives exhibited similar activities in vitro at the levels of the global growth of six human cancer cell lines, of the induction of apoptosis, and of the G0 cell cycle phase arrest. The in vivo antitumor activity characterization included the use of two murine leukemia types (P388-LEU and L1210-LEU), two murine lymphoma-like models (P388-LYM and L1210-LYM), two mammary adenocarcinomas (MXT-HI and MXT-HS), and one melanoma (B16). The data show that S30972-1 is actually more efficient in vivo than S16020-2, a feature that may relate to the fact that S30972-1 is less toxic than S16020-2. The S30972-1 compound exhibited in vivo a level of antitumor activity that was also actually higher than that exhibited by Adriamycin and similar to that exhibited by etoposide.

INTRODUCTION

As emphasized by Burden and Osheroff (1), topoisomerase II is a ubiquitous enzyme that is essential for the survival of all eukaryotic organisms and that plays critical roles in virtually every aspect of DNA metabolism. This enzyme unknots and untangles DNA by passing an intact helix through a transient double-stranded break that it generates in a separate helix (1, 2). Inhibitors of DNA topoisomerase II are therefore widely used as chemotherapeutic agents in cancer treatment (1–3). These inhibitors include DNA-intercalating anthracyclines, anthraquinones, ellipticines, acridines, and non-DNA-intercalating eppodophyllotoxin derivatives (1–3). These topoisomerase-interfering compounds can be divided into poisons and catalytic inhibitors, two general categories based on the mechanism of drug action (4). A peculiarity of DNA topoisomerase poisons is that their action is invariably DNA sequence specific (2, 4); this sequence specificity of the poisons targeting eukaryotic DNA topoisomerase II (including ADR,3 ellipticine, and VP-16) is detailed by Capranico et al. (4). DNA is thus the major target for topoisomerase II inhibitors, and the stabilization of cleavable topoisomerase II-DNA complexes rather than the inhibition of topoisomerase II catalytic activity is essential for drug cytotoxicity (3). Six antineoplastic drugs that target topoisomerase II (i.e., doxorubicin, daunorubicin, idarubicin, mitoxantrone, VP-16, and teniposide) have been currently approved for clinical use in the United States (5). New investigational antitopoisomerase drugs are under analysis. Among them is the S16020-2 olivacin derivative.

S16020-2 (NSC 659687) is a pyridocarbazole derivative characterized by a basic N-dialkylnamiokylcarboxamido group grafted onto an olivacine chromophore (see Fig. 1). It binds through intercalation between adjacent DNA bp, thus inducing an unwinding of the double helix by 10 degrees (6, 7). Although S16020-2 does not interfere with the catalytic cycle of DNA topoisomerase I, it does stimulate the DNA topoisomerase II-mediated DNA cleavage via a strictly ATP-dependent mechanism (7). Quiescent cells demonstrate a significantly lower sensitivity to S16020-2 than proliferating cells, a feature that

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3 The abbreviations used are: ADR, Adriamycin; VP-16, etoposide; ATCC, American Type Culture Collection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTD, maximum tolerated dose; T/C, treated/control; NOS, not otherwise specified.
S16020-2 (6). Koo et al. ras alleles. In fact, activated compounds that are more cytotoxic to tumor cells harboring an i.e., S16020-2) are the only daunorubicin) and NSC 659687 (Antineoplastic Drug Screen, NSC 284682 (3 inhibitors of topoisomerase II tested in the National Cancer Institute’s suggests that topoisomerase II is the main potential target for S16020-2 (6). Koo et al. (3) have shown that among the inhibitors of topoisomerase II tested in the National Cancer Institute’s In Vitro Antineoplastic Drug Screen, NSC 284682 (3’-hydroxydaunorubicin) and NSC 659687 (i.e., S16020-2) are the only compounds that are more cytotoxic to tumor cells harboring an activated ras oncogene than to tumor cells bearing wild-type ras alleles. In fact, activated ras oncogenes appear to enhance the sensitivity of human tumor cells to topoisomerase II inhibitors by potentiating an apoptotic response (3).

S16020-2 was selected on the basis of its in vitro cytotoxicity and its in vivo antitumor activity against P388 leukemia and the colon 38 adenocarcinomas (8). S16020-2 was then shown on a large panel of murine and human tumors to be as active as, or more active than, several chemotherapeutic agents including cyclophosphamide (9) and ADR (10), which are routinely used in hospitals. Due to its antitumor activity in experimental models, its favorable pharmacokinetic characteristics, and its acceptable toxicity (9, 10), S16020-2 is currently being studied in clinical trials (11). New analogues and derivatives have been synthesized from the S16020-2 compound, with some of these new drugs exhibiting comparatively higher levels of antitumor potency with an improved therapeutic index (12).

Among these newly synthesized S16020-2 derivatives is the S30972-1 compound (the structure of which is illustrated in Fig. 1). The aim of the present work is therefore to provide an extensive pharmacological characterization of both the in vitro and the in vivo antitumor activities of S30972-1 in comparison with S16020-2, from which it derives. ADR and VP-16 have been chosen as reference compounds. The in vitro characterization of antitumor activity includes investigations conducted at the level of global growth, cell proliferation, apoptosis, and topoisomerase II targeting. The in vivo characterization includes the use of murine leukemias, lymphomas, mammary adenocarcinomas, and melanomas.

MATERIALS AND METHODS

In Vitro Determination of Global Growth. Six human tumor cell lines were obtained from the ATCC, Manassas, VA, and included two glioblastomas (U373 and U87) and two non-small cell lung (A549 and A427) and two colon (HCT-15 and LoVo) cancer models. The ATCC numbers of these cell lines are HTB 14 (U87), HTB 17 (U373), CCL 185 (A549), HBT 53 (A427), CCL 225 (HCT-15), and CCL 229 (LoVo). The cells were cultured at 37°C in sealed (airtight) Falcon plastic dishes (Nunc, Life Technologies, Inc., Merelbeke, Belgium) containing Eagle’s minimal essential medium (MEM, Life Technologies, Inc.) supplemented with 10% FCS. All of the media were supplemented with a mixture of 0.6 mg/ml glutamine, 200 IU/ml penicillin, 200 IU/ml streptomycin, and 0.1 mg/ml gentamicin (all from Life Technologies, Inc.). The FCS was heat inactivated for 1 h at 56°C.

The six cell lines were incubated for 24 h in 96-microwell plates (at a concentration of 10,000 cells/ml culture medium) to ensure adequate plating before cell growth determination, which was carried out by means of the colorimetric MTT assay as detailed previously (13). This assessment of cell population growth is based on the capability of living cells to reduce the yellow product MTT (Sigma, St. Louis, MO) to a blue product, formazan, by a reduction reaction occurring in the mitochondria. The number of living cells is directly proportional to the intensity of the blue, which is quantitatively measured by spectrophotometry on a DIAS microplate reader (Dynatech Laboratories, Guyancourt, France) at a 570 nm wavelength (with a reference of 630 nm). Each experiment was conducted in sextuplicate. We validated the MTT-related data using two alternative techniques, namely the direct cell counting and the genomic incorporation of [3H]thymidine (data not shown).

Nine concentrations ranging from 10⁻³ to 10⁻⁵ M were assayed for each of the four drugs under study, i.e., the two olivacine derivatives S16020-2 and S30972-1 (see Fig. 1) and the two antitopoisomerase II reference compounds ADR and VP-16.

In Vitro Determination of Cell Kinetics. The influence of S16020-2 and S30972-1 on the cell kinetics (the distribution of the cells into the various phases of the cell cycle) of two human tumor cell lines, i.e., the fast-growing human U373 glioblastoma (cell cycle doubling time = 20 ± 2 h) and the slow-growing human COLO 205 colon cancer (cell cycle doubling time = 74 ± 3 h; ATCC CCL 222) models was assayed at two distinct concentrations (10 and 1000 nM). The experimental

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Fig. 1 Chemical structures of olivacine, the natural isomer of ellipticine, and of two synthetic derivatives of olivacine, i.e., S16020-2 and S30972-1.
schedule was identical with that previously described (14). Briefly, 40,000 cells/ml medium were taken in a logarithmic phase of growth for each of the 2 cell lines under study and plated in 35-× 10-mm Petri dishes (Becton Dickinson), each of which contained an 18-× 18-mm glass coverslip on its bottom and 3 ml of MEM (Life Technologies, Inc.). The two cell lines were incubated for 24 h before the addition of the drugs (or their nonaddition in the control condition) to ensure adequate plating. The experiments were stopped (by the fixation of the coverslips during 30 min in buffered formalin) 72 h after the addition of the drugs to the culture medium. The coverslips supporting the tumor cells were then submitted to the Feulgen reaction, as detailed elsewhere (15). The nuclear DNA content of each cell nuclei was quantitatively determined by means of computer-assisted microscopy (the SAMBA 2005 system; Samba Technologies Inc., Grenoble, France), thus enabling a DNA histogram to be drawn up for each experimental condition. The percentages of cells in the G1, S, G2, and M phases were then computed from each DNA histogram (16). Six hundred cell nuclei were analyzed for each experimental condition, and each experimental condition was performed in sextuplicate.

In Vitro Determination of Apoptosis Level. The levels of apoptosis were determined in the human U373 glioblastoma and COLO 205 colon cancer cell lines by means of a photometric enzyme immunoassay (Cell Death Detection ELISA PLUS; Boehringer-Mannheim, Brussels, Belgium). This assay permitted the quantitative in vitro determination of the cytoplasmic histone-associated DNA fragments in the cell cultures exhibiting cell deaths occurring through apoptotic features (17). The experimental protocol was the same as described previously (18).

Topoisomerase II Targeting. Restriction endonucleases and DNA polymerase I (Klenow fragment) were purchased from New England BioLabs, Ltd. (Hitchin, United Kingdom) and proteinase K from Merck (Darmstadt, Germany). Human DNA topoisomerase IIa was provided by TopoGEN, Inc. (Columbus, OH). Closed circular pSP65 plasmid and yeast DNA topoisomerase II were prepared as described previously (7). Circular pSP65 DNA was cleaved with EcoRI restriction endonuclease and labeled at its 3' end with [α-32P]dATP (ICN Biomedical, Costa Mesa, CA) using DNA polymerase I, Klenow fragment (7). The labeled DNA was cleaved with restriction endonuclease HindIII into two fragments (2976 and 29 bp), each labeled at one end.

The cleavage reactions were conducted for 10 min at 30°C with yeast DNA topoisomerase II or at 37°C with human DNA topoisomerase IIa. The incubation mixture (15 μl) contained labeled pSP65 DNA (8 × 104 dpm) and either 19 nm yeast DNA topoisomerase II or 2 units of human DNA topoisomerase IIa in the cleavage buffer [10 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM MgCl2, 0.1 mM EDTA] with or without 1 mM ATP. The reaction was stopped by the addition of SDS and proteinase K to the final concentrations of 0.8% and 63 μg/ml, respectively, and the mixtures were incubated for an additional hour at 50°C. After the addition of 2 μl of loading buffer (150 mM EDTA, 50% glycerol, 0.4% bromphenol blue, 0.4% xylene cyanol), the samples were heated at 65°C for 2 min and then analyzed by electrophoresis (2 V/cm) for 20 h in a 1.2% agarose gel containing 0.1% SDS in 90 mM Tris-borate, 2.5 mM EDTA buffer. The gel was dried and autoradiographed.

In Vivo Determination of Antitumor Activity. The S16020-2- and S30971–1-mediated antitumor activities were characterized on seven different murine tumor models including the P388 and L1210 leukemia types, the P388 and L1210 lymphoma-like models, the MXT-HI and MXT-HS mammary carcinomas, and the B16 melanoma.

The P388 and L1210 leukemia types were developed at the beginning of the 1950s (19). In our present experiments, we used them for primary in vivo screening because we had observed that they exhibit distinct sensitivities to topoisomerase I (20) and II (21) inhibitors. The P388 (P388-LEU) and L1210 (L1210-LEU) leukemias are maintained in our laboratory by weekly transplantations of an inoculum of 106 cells i.p. into 6-week-old female DBA/2 mice (Ifa Credo, L’Arbresle, France) for the P388 model and into 6-week-old female C57BL × DBA/2 F1 (B6D2F1) mice (Ifa Credo) for the L1210 model. Without treatment, the animals die between 9 and 14 days after the injection. For the experiments under discussion, we used 6-week-old female CDF1 mice (Ifa Credo).

As detailed in “Discussion,” we developed two lymphoma-like models from these P388 and L1210 leukemia types. Briefly, we established solid s.c. tumors by inoculating 106 P388 or L1210 leukemic cells under the skin of the mice. These lymphoma-like models had been maintained for 2 years in our laboratory preceding the experiments under discussion. The transfer from one passage to the next was performed every 3 weeks for the L1210 lymphoma-like model (L1210-LYM) and every 2 weeks for the P388 lymphoma-like one (P388-LYM). At each transfer three L1210 or P388 tumors were minced into 10-mm pieces or larger, and these pieces were randomly s.c. inoculated into the right flanks of three “new bank” mice by means of a trochar (13-gauge). The same type of mouse was used for the lymphoma-like models as for the leukemia models.

The MXT-HS model described here is a hormone-sensitive form of the MXT model, whereas the MXT-HI model is a hormone-insensitive variant. The way in which we obtained these HS and HI variants from the original MXT tumor is detailed in “Discussion.” The MXT-HI and MXT-HS tumors are maintained in our laboratory by monthly s.c. transplantations into 6-week-old female B6D2F1 mice (Ifa Credo). The same procedure is used to maintain the B16 melanoma on B6D2F1 mice (Ifa Credo).

We determined the MTD for the S16020-2 and S30972-1 compounds. This MTD determination was conducted by defining the maximum dose of the drug that can be administered acutely (i.e., in one i.p. single dose) to healthy animals (B6D2F1), i.e., not grafted with tumors. The survival and weight of the animals were recorded for up to 14 days postinjection. Five different doses of each drug (10, 20, 40, 80, and 160 mg/kg) were used for the MTD index determination with each experimental group being composed of three mice for this purpose. All of the mice died that had received single 160-mg/kg i.p. injections of either S16020-2 or S30972-1, whereas all mice survived with a single i.p. injection of 80 mg/kg of each of these two compounds. The MTD for both olivacine derivatives was therefore defined as 80 mg/kg in the present study. In the case of each of the seven tumor models under study, three
doses were assayed [MTD/2 (40 mg/kg), MTD/4 (20 mg/kg) and MTD/8 (10 mg/kg)] in comparison with control. Each experimental group contained nine mice. The MTD values for ADR and VP-16 were 10 and 40 mg/kg, respectively.

The mice inoculated i.p. at day 0 with either $10^6$ P388 or L1210 cells received one i.p. injection per day of either 0.2 ml of saline (control group) or the antitumor drug for 4 consecutive days, from day 1 to day 4.

The mice were grafted with the P388 lymphoma-like tumors at day 0 and treated 9 times (for the three following weeks) at days 5, 7, 9, 12, 14, 16, 19, 21, and 23 postgraft. A similar experimental protocol was adopted for the L1210 leukemia model, but the first drug administration started at day 7, with the following drug administrations performed at days 9, 11, 14, 16, 18, 21, 23, and 25 postgraft. The experimental schedule for the MXT-HI and MXT-HS mammary carcinomas and the B16 melanoma was identical with that for the L1210 model.

All of the animals were kept in plastic cages in a room with a controlled temperature (22 ± 1°C), light exposure (from 6 a.m. to 6 p.m.), and 40–70% relative humidity. Food (AO4; Usine Alimentaire Rationnelle, Villemoisson, France) and water were provided ad libitum. The present experiments were conducted with Animal Use Approval provided by the local Animal Ethic Commitee of the Faculty of Medicine of the Université Libre de Bruxelles.

Solid tumor (the P388 and the L1210 lymphoma-like models, the MXT-HI and MXT-HS mammary carcinomas, and the B16 melanoma models) sizes were measured weekly by means of a calliper and expressed as an area (mm$^2$) by multiplying together the two largest perpendicular diameters.

The S16020-2 and S30972-1-induced influence on the survival periods of the tumor-bearing animals was evaluated by means of the T/C index [the ratio of the average survival time of the treated mice (the death of the fifth mouse in a given treated group of nine mice) to the average survival time of the control mice (the death of the 5th control mouse) multiplied by 100 (20, 21)]. On the basis of this evaluation, a compound is considered to be significantly active if it increases the T/C index by at least 30% (T/C = 130%). If the T/C index is higher than 300%, the compound is considered as leading to the “long term survival” (if not the cure) of the animals. If the T/C index is <70%, the compound is considered to be toxic.

Statistical Analysis. Results are presented as the mean ± SEM. The statistical comparisons of the data were conducted by means of the Fisher F (one-way ANOVA for more than two groups) or the Student t (for two groups) tests after a check of the equality of variance by means of the Levene test, and of the fit of the data to a normal distribution by means of the $x^2$ test of goodness-of-fit. When these parametric conditions were not satisfied, the nonparametric Kruskall-Wallis (for more than two groups) or the Mann-Whitney (for two groups) tests were conducted. All of the statistical analyses were carried out with Statistica (Statsoft, Tulsa, OK).

RESULTS

In Vitro Determination of Global Growth. Table 1 shows that the two olivacine derivatives, S16020-2 and S30972-1 (see Fig. 1), exhibited significantly ($P < 0.05$ to $P < 0.0001$) higher cytotoxic activities than VP-16 on all six human cancer cell lines under study. The differences in cytotoxic activities between these two olivacine derivatives and ADR were less marked than what was observed with respect to VP-16 (Table 1).

In Vitro Determination of Cell Kinetics. We assessed the drug-induced influences at the level of cell kinetics by determining the percentage of cells in the G2 phase of the cell cycle. We chose this cell kinetic marker because actual topoisomerase II inhibitors (see below) induce an increase in the G2 phase length due to the fact that cells treated by such drugs try to repair their damaged DNA during this G2 phase before undergoing mitosis. The level of DNA damage is therefore indirectly reflected by the length of the G2 phase and, consequently, by the percentage of cells engaged in this specific phase of the cell cycle. Fig. 2 shows that both the S16020-2 and the S30972-1 compounds induced marked increases in the percentages of U373 cells arrested in the G2 phase of their cell cycles. In slowly proliferating COLO 205 colon cancer cells, the S30972-1 compound induced significantly higher G2 arrest features than the S16020-2 compound while at the same time exhibiting activities similar to those of the S16020-2 compound in highly proliferating U373 glioblastoma cells (Fig. 2). The kinetics of G2 phase arrest induced by either S16020-2 or S30972-1 were not the same, a feature that should be compared with the fact that these two compounds did not display similar in vivo antitumor activities (see below).

In Vitro Determination of Apoptosis Level. Of the four topoisomerase II inhibitors under study, i.e., the two olivacine derivatives (S16020-2 and S30972-1) and the two reference compounds (ADR and VP-16), the two olivacine derivatives induced the most marked apoptotic features in the highly proliferating U373 human glioblastoma cells (Table 2). In the

### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>ADR</th>
<th>VP-16</th>
<th>S16020-2</th>
<th>S30972-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT-15</td>
<td>240</td>
<td>960</td>
<td>72</td>
<td>NS</td>
</tr>
<tr>
<td>LoVo</td>
<td>40</td>
<td>260</td>
<td>39</td>
<td>NS</td>
</tr>
<tr>
<td>U373</td>
<td>430</td>
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<td>310</td>
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<tr>
<td>U87</td>
<td>85</td>
<td>270</td>
<td>36</td>
<td>110</td>
</tr>
<tr>
<td>A549</td>
<td>260</td>
<td>440</td>
<td>340</td>
<td>337</td>
</tr>
<tr>
<td>A427</td>
<td>84</td>
<td>210</td>
<td>67</td>
<td>NS</td>
</tr>
</tbody>
</table>
slowly proliferating cells (the human COLO 205 colon cancer model), S30972-1 appeared to be more effective than S16020-2 (Table 2).

**Topoisomerase II Targeting.** Fig. 3 shows that both compounds (S16020-2 and S30972-1) induced the formation of cleavable complexes. In this experiment, the yeast enzyme was more efficient on DNA cut effects than the human DNA topoisomerase II. However, it was not possible to compare the two enzyme concentrations.

The data shown in Fig. 3 confirm a previous report showing that the cuts induced by topoisomerase II in presence of S16020-2 are ATP dependent (7). The same observation can be made for S30972-1 (Fig. 3). However, the S30972-1 effects induced on topoisomerase II cuts of DNA were lower than the S16020-2 effects and considerably less efficient than the etoposide effects (Fig. 3). Lastly, the cut induction was biphasic with respect to the S30972-1 concentration, as it was with the S16020-2. However, the effects were maximum at \( \sim 150 \mu M \) S30972-1, in contrast to the optimal S16020-2 concentration, which was 20 times lower (Fig. 3).

**In Vivo Determination of Antitumor Activity.** The 2 leukemia models appeared to be less selective than the five solid tumor models in discriminating between the antitumor activities of the four topoisomerase II inhibitors under study. Indeed, the four compounds cured a high number of leukemic mice in several different experimental protocols (Table 3). In contrast, the five solid tumor models enabled a clear-cut distinction to be drawn between the antitumor activity of these four anticancer
drugs. VP-16 and S30972-1 displayed the highest antitumor activity for the P388 lymphoma-like model (Table 3). S30972-1 and VP-16 displayed very significant antitumor activity over all seven tumor models under study. All four topoisomerase II inhibitors displayed significant antitumor activity for the L1210 lymphoma-like tumor (Table 3). S30972-1 and VP-16 displayed the highest antitumor activity for the aggressive HI variant of the mammary adenocarcinoma. However, S30972-1 and VP-16 did not increase the survival periods of the melanoma-bearing mice (Table 3). S30972-1 was very effective against the melanoma-bearing mice (Table 3). These two compounds were able to cure several MXT-HI mammary carcinoma-bearing mice (Table 3). S30972-1 and VP-16 displayed very significant antitumor activity for the L1210 lymphoma-like tumor (Table 3). Thus, of the four topoisomerase II inhibitors under study, the S30972-1 olivacine derivative definitively exhibited the highest antitumor activity over all seven tumor models under study. All of the significant drug-induced increases in the survival periods of the mice bearing the five solid tumor models under study were accompanied by significant drug-induced decreases in tumor growth (data not shown).

### DISCUSSION

Olivacine is the natural isomer of ellipticine (see Fig. 1) and has been extracted from a plant (Aspidosperma olivaceum) in 1958 (22) and synthesized in 1966 (23). Although its antitumor activity has been described since 1966, this activity has been studied less than ellipticine activity. This led the Bisagni team to synthesize different structural analogues of olivacine in the hope of modulating its antitumor activity (8). Despite the lack of studies on the relations between the structure and the activity of olivacine derivatives, some data have been obtained from the results produced by different research teams studying ellipticine.

**Table 2** S16020-2- and S30972-1-induced effects at apoptosis level

The data are expressed as mean percentages of cytoplasmic histone-associated-DNA fragments (as compared with the control value = 100%) ± SE (n = 4).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Drug</th>
<th>Concentration (μM)</th>
<th>Drug exposure (h)</th>
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<tr>
<td></td>
<td></td>
<td>6</td>
<td>24</td>
</tr>
<tr>
<td>U373</td>
<td>S16020-2</td>
<td>0.1</td>
<td>137 ± 22</td>
</tr>
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<td></td>
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<td>10.0</td>
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<tr>
<td></td>
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<td>1000.0</td>
<td>129 ± 19</td>
</tr>
<tr>
<td>S30972-1</td>
<td>0.1</td>
<td>104 ± 15</td>
<td>166 ± 14</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>114 ± 12</td>
<td>189 ± 17</td>
</tr>
<tr>
<td></td>
<td>1000.0</td>
<td>134 ± 15</td>
<td>270 ± 25</td>
</tr>
<tr>
<td>ADR</td>
<td>0.1</td>
<td>140 ± 15</td>
<td>115 ± 16</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>168 ± 11</td>
<td>141 ± 19</td>
</tr>
<tr>
<td></td>
<td>1000.0</td>
<td>320 ± 24</td>
<td>387 ± 34</td>
</tr>
<tr>
<td>VP-16</td>
<td>0.1</td>
<td>111 ± 10</td>
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<td>135 ± 15</td>
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<td>S16020-2</td>
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<td>101 ± 10</td>
<td>95 ± 15</td>
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<td>89 ± 10</td>
<td>71 ± 8</td>
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<tr>
<td>S30972-1</td>
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<td>101 ± 10</td>
<td>168 ± 11</td>
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<td>1000.0</td>
<td>104 ± 10</td>
<td>284 ± 15</td>
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</table>

a-b Levels of statistical significance as compared to the control values: *P < 0.05; **P < 0.01.

**Fig. 3** A, yeast DNA topoisomerase II-mediated DNA cleavage in the presence or absence of ATP. Lane 1, pSP65 DNA substrate; Lane 2, cleavage reaction in the absence of drug; Lanes 3 and 4, 10 μM etoposide; Lanes 5 and 6, 8 μM S16020-2; Lanes 7–12, 4, 10, 30, 50, 100, 150, and 200 μM S30972-1 in the presence of ATP; Lanes 14–20, 4, 10, 30, 50, 100, 150, and 200 μM S30972-1 in the absence of ATP, which was present in Lanes 2, 3, and 6 and absent in Lanes 4 and 5. B, comparison of yeast and human DNA topoisomerase II-mediated DNA cleavage in the presence of ATP. Lanes 1, 3, 5–8, and 13–16, yeast DNA topoisomerase II; Lanes 2, 4, 9–12, and 17–20, human DNA topoisomerase II; Lanes 1 and 2, no inhibitor; Lanes 3 and 4, 100 μM etoposide; Lanes 5–8 and 9–12, 2, 10, 20, and 40 μM S16020-2 (in duplicate); Lanes 13–16 and 17–20, 2, 10, 20, and 40 μM S30972-1 (in duplicate).

Hence, hydroxylation in position 9 increases the affinity of the compound for DNA (24), favors the stabilization of the cleavable complex (25) and a quinonimine formation (26), and increases the cytotoxicity of the compound and, consequently, its antitumor activity (27). However, some methoxylated derivatives in position 9 may be more efficient in vivo than their hydroxylated equivalents, which are more toxic generally (28). The addition of a lateral dialkylaminoalcane-type basic chain to an ellipticine (28) or an acridine (29) chromophore markedly increases the cytotoxicity of the compound and its antitumor activity. Nitrogen methylation in the indol chromophore theoretically decreases the risk of producing alkylating quinonimine derivatives in vivo, and thus seems to decrease the toxicity of the compound so synthesized (30). By applying these kinds of structure/activity relations to olivacine, Jaztold-Howorko et al. (8) and Guillonneau et al. (12) were able to synthesize the S16020-2 compound (NSC 659687) and different analogues including the S30972-1 derivative illustrated in Fig. 1. Three
S16020-2 substitution sites were selected to obtain new derivatives, i.e., the carbon atoms in positions 10 and 11 (methylolation) and the hydroxyl function in position 9 (the addition of a hydrolyzable function) (12). Methylation in position 10 caused a slight increase in vivo toxicity. Of the hydrolyzable derivatives, the esterification (see Fig. 1) and the hydroxyl function in position 9 (the addition of a hydroxyl group) were selected to obtain new derivatives like the human COLO 205 colon cancer model), a feature that would relate to their antitopoisomerase II activity as demonstrated by the topoisomerase II targeting data. Both compounds induced marked apoptotic features in human cancer cells at a higher intensity than that observed in the reference compounds. Thus, most of the in vitro data show that the two olivacine derivatives, S16020-2 and S30972-1, which are topoisomerase II inhibitors, have a higher level of antitumor activity than VP-16 and also, but to a lesser extent, than ADR. This greater efficiency of the olivacine derivatives (in relation to the reference compounds) also manifests itself in vivo, but with very different activity profiles of the S16020-2 and S30972-1 compounds. However, the detection of this difference requires the use of solid tumor models which mimic clinical reality, to a certain extent at least. The leukemia models do not enable this distinction to be made. This is why the National Cancer Institute (Bethesda, MD) abandoned this kind of model for its primary drug discovery screening, after using it for about 30 years (19).

When leukemic P388 cells of lymphoblastic origin (19) are grafted s.c. instead of i.p., they develop as biologically very aggressive anaplastic lymphomas. This biological aggressiveness can be evidenced histologically in the form of a dramatic local invasion (i.e., the dermis and surrounding muscles of the peritoneal cavity) and a metastatic process occurring first in the liver (as early as the third day postgraft), and then in the lungs (occurring around the 7th day postgraft) and, occasionally, in the kidneys (around the 15th day postgraft if the animals are still alive). Mice suffering from P388 lymphoma-like models usually die about 2 weeks after the cell injection. Data from the present study clearly indicate that none of the four compounds tested here was able to cure P388 lymphoma-bearing mice. The two most efficient compounds in this model were VP-16 and S30972-1, and the least efficient one was ADR.

Despite the fact that they are also of lymphoblastic origin (19), L1210 leukemia cells develop much less aggressive anaplastic lymphomas than the P388 lymphoma model. Indeed, the L1210 lymphoma-like model does not invade adjacent tissues or spread to either the cytotoxic activity of the original compound or the production of hydroxyl derivatives after hydrolysis by intracellular or extracellular esterases. The glutaric ester of S16020-2 is more hydrolyzable in vitro than in the case of the 11-methyl derivatives. This means that 9-substitution is much more favorable in the case of S16020-2 than in the case of the 11-methyl derivatives. The activity of the different esters can be attributed to either the cytotoxic activity of the original compound or the production of hydroxyl derivatives after hydrolysis by intracellular or extracellular esterases. The glutaric ester of S16020-2 is more hydrolyzable in vitro than in the case of the 11-methyl derivatives. This means that 9-substitution is much more favorable in the case of S16020-2 than in the case of the 11-methyl derivatives. The activity of the different esters can be attributed to either the cytotoxic activity of the original compound or the production of hydroxyl derivatives after hydrolysis by intracellular or extracellular esterases. The glutaric ester of S16020-2 is more hydrolyzable in vitro than in the case of the 11-methyl derivatives. This means that 9-substitution is much more favorable in the case of S16020-2 than in the case of the 11-methyl derivatives. The activity of the different esters can be attributed to either the cytotoxic activity of the original compound or the production of hydroxyl derivatives after hydrolysis by intracellular or extracellular esterases. The glutaric ester of S16020-2 is more hydrolyzable in vitro than in the case of the 11-methyl derivatives. This means that 9-substitution is much more favorable in the case of S16020-2 than in the case of the 11-methyl derivatives. The activity of the different esters can be attributed to either the cytotoxic activity of the original compound or the production of hydroxyl derivatives after hydrolysis by intracellular or extracellular esterases. The glutaric ester of S16020-2 is more hydrolyzable in vitro than in the case of the 11-methyl derivatives. This means that 9-substitution is much more favorable in the case of S16020-2 than in the case of the 11-methyl derivatives. The activity of the different esters can be attributed to either the cytotoxic activity of the original compound or the production of hydroxyl derivatives after hydrolysis by intracellular or extracellular esterases. The glutaric ester of S16020-2 is more hydrolyzable in vitro than in the case of the 11-methyl derivatives. This means that 9-substitution is much more favorable in the case of S16020-2 than in the case of the 11-methyl derivatives. The activity of the different esters can be attributed to either the cytotoxic activity of the original compound or the production of hydroxyl derivatives after hydrolysis by intracellular or extracellular esterases. The glutaric ester of S16020-2 is more hydrolyzable in vitro than in the case of the 11-methyl derivatives. This means that 9-substitution is much more favorable in the case of S16020-2 than in the case of the 11-methyl derivatives. The activity of the different esters can be attributed to either the cytotoxic activity of the original compound or the production of hydroxyl derivatives after hydrolysis by intracellular or extracellular esterases. The glutaric ester of S16020-2 is more hydrolyzable in vitro than in the case of the 11-methyl derivatives. This means that 9-substitution is much more favorable in the case of S16020-2 than in the case of the 11-methyl derivatives. The activity of the different esters can be attributed to either the cytotoxic activity of the original compound or the production of hydroxyl derivatives after hydrolysis by intracellular or extracellular esterases. The glutaric ester of S16020-2 is more hydrolyzable in vitro than in the case of the 11-methyl derivatives. This means that 9-substitution is much more favorable in the case of S16020-2 than in the case of the 11-methyl derivatives. The activity of the different esters can be attributed to either the cytotoxic activity of the original compound or the production of hydroxyl derivatives after hydrolysis by intracellular or extracellular esterases. The glutaric ester of S16020-2 is more hydrolyzable in vitro than in the case of the 11-methyl derivatives. This means that 9-substitution is much more favora...
metastasize. Untreated animals suffering from this L1210 lymphoma die 4 to 5 weeks postgraft. Once more, the two most efficient compounds with respect to this L1210 lymphoma model were VP-16 and S30972-1, and the least efficient one was ADR.

More than 80% of female breast cancers are invasive intraduct carcinomas, i.e., NOS (31). We are therefore using the MXT tumor as an experimental model to reproduce this clinical reality because this tumor originates in the galactophorous ducts (like NOS cancers) and not in the glandular acini, as is the case of most murine mammary tumors (32, 33). Mammary cancers of the galactophorous ducts have a hormone sensitivity profile very different from the profile of glandular acini cancers; whereas the former are essentially “steroidosensitive,” the latter are “prolactin-sensitive” (31–34). An experimental protocol was thus developed in our laboratory to differentiate hormone-insensitive MXT-HI tumor strains into hormone-sensitive MXT-HS strains (35). Data from the present study show that ADR and S30972-1 exhibited the highest levels of antitumor activity on the MXT-HS strain whereas S30972-1 and VP-6 actually appeared to be effective against the very aggressive MXT-HI strain (Table 3).

Experimental melanomas set up in mice by painting their skins with a carcinogen have little in common with human melanomas. In fact, even if these experimental melanomas display certain morphological characteristics close to those of human melanomas, they are actually less aggressive biologically (36). Nevertheless, there does exist an experimental model in the shape of the B16 murine melanoma which displays numerous similarities with human melanomas (37, 38). Of the four compounds under study, the two olivacine derivatives were effective against the B16 melanoma strain that we used, whereas the two reference compounds were not.

In conclusion, olivacine derivatives are promising new antitumor agents which belong to the class of topoisomerase II inhibitors. One of these (S16020-2) recently entered clinical trials. The present study shows that the glutaric ester of S16020-2, i.e., S30972-1, exhibits an in vitro antitumor activity similar to S16020-2. In sharp contrast, S30972-1 is more efficient in vivo than S16020-2, a feature that could relate to the fact that S30972-1 is less toxic than S16020-2. S30972-1, exhibits an antitumor activity (on a panel of seven distinct tumor models) that was actually greater than the activity displayed by ADR and etoposide.

REFERENCES


In Vitro and in Vivo Pharmacological Characterizations of the Antitumor Properties of Two New Olivacine Derivatives, S16020-2 and S30972-1

Hugues Malonne, Sophie Farinelle, Christine Decaestecker, et al.


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