F14512, a Potent Antitumor Agent Targeting Topoisomerase II Vectored into Cancer Cells via the Polyamine Transport System

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Abstract

The polyamine transport system (PTS) is an energy-dependent machinery frequently overactivated in cancer cells with a high demand for polyamines. We have exploited the PTS to selectively deliver a polyamine-containing drug to cancer cells. F14512 combines an epipodophyllotoxin core-targeting topoisomerase II with a spermine moiety introduced as a cell delivery vector. The polyamine tail supports three complementary functions: (a) facilitate formulation of a water-soluble compound, (b) increase DNA binding to reinforce topoisomerase II inhibition, and (c) facilitate selective uptake by tumor cells via the PTS. F14512 is 73-fold more cytotoxic to Chinese hamster ovary cells compared with CHO-MG cells with a reduced PTS activity. A decreased sensitivity of L1210 leukemia cells to F14512 was observed in the presence of putrescine, spermidine, and spermine. In parallel, the spermine moiety considerably enhances the drug-DNA interaction, leading to a reinforced inhibition of topoisomerase II. The spermine tail of F14512 serves as a cell delivery vehicle as well as a DNA anchor, and this property translates at the cellular level into a distinct pharmacologic profile. Twenty-nine human solid or hematologic cell lines were used to characterize the high cytotoxic potential of F14512 (median IC_{50} of 0.18 μmol/L). Finally, the potent antitumor activity of F14512 in vivo was evidenced with a MX1 human breast tumor xenograft model, with partial and complete tumor regressions. This work supports the clinical development of F14512 as a novel targeted cytotoxic drug and sheds light on the concept of selective delivery of drugs to tumor cells expressing the PTS. [Cancer Res 2008;68(23):9845–53]

Introduction

Natural polyamines, such as spermine and spermidine, are ubiquitous polycationic molecules that play crucial roles in a number of cell processes, including cell proliferation and differentiation. They are involved in numerous steps of protein synthesis, affect the biological activity of proteins, stabilize nucleic acids, and contribute to cell growth (1). For these reasons, the need for polyamines in tumor cells is crucial, and high levels of polyamines have been detected in many tumor types (2, 3). Such an accumulation might be a good marker for defining patients with high risk of progression, as shown with colon cancer (4) or renal cell carcinoma (5).

The critical role of polyamines in cell proliferation has stimulated considerable interest in polyamine metabolism as a new target for chemotherapy. Several polyamine derivatives have been synthesized to inhibit polyamine biosynthesis enzymes, to perturb polyamine homeostasis by activation of polyamine catabolism, or to inhibit polyamine uptake (6, 7). These approaches are currently the subject of an active research (8, 9).

In addition to the endogenous synthesis of polyamines, the activity of the polyamine transport system (PTS) was also found higher in proliferating cells than in resting cells (10) and the uptake of polyamine by tumor tissues was superior to normal tissues (11). The high specific activity of the PTS in tumor cells is thought to be associated with the inability of biosynthetic enzymes to provide sufficient levels of polyamines to sustain rapid cell division. These bioproduction constraints are partially offset by scavenging polyamines from exogenous sources and many tumor types have been shown to contain elevated levels of polyamines resulting from an active PTS for importing exogenous polyamines. An active PTS has been characterized in a large number of tumor cell lines from different origins (12). In terms of molecular knowledge, although much is known about the PTS in bacteria, yeast (13), and leishmania (14), the mammalian PTS is the source of models (15) but remains to be molecularly described. Nevertheless, there has been a number of studies demonstrating that the selectivity of the PTS is not restricted to natural polyamines (12, 16). Most of these have involved alteration to the polyamine molecule itself to adjust the chain length, spacing between charges, extent of alkylation, etc. Based on this, polyamine vectorization was proposed as a valuable strategy to increase the selectivity of anticancer agents. The literature reports several examples of polyamine conjugates with cytotoxic drugs, such as chlorambucil (17), nitroimidazoles (18), aziridines (19), acridines (20), enediyenes (21), anthracenes (16), naphtoquinones (22), camptothecin (23), and protoberberine (24). All these conjugated drugs are DNA-interacting agents used to develop a general strategy by exploiting both the transport mechanism and the high affinity of polyammonium cations for DNA. Nevertheless, only few studies report in vivo experiments and none of them presented enough benefit to be developed because the increased cytotoxicity of these compounds were, when tested in vivo, correlated to an increased toxicity and/or a minor increase.
of antitumor efficacy (19, 23, 25). We report here the first successful design of novel, highly potent polyamine-containing drug exploiting the PTS for tumor cell delivery.

Materials and Methods

Chemicals and Drugs
F14512 and etoposide were provided by Pierre Fabre Médicament. The design and synthesis of F14512 have been patented (WO 2005/100363) and the structure-activity relationships will be described elsewhere. F14512 and etoposide were dissolved in DMSO and used in cellular assays at a maximal final concentration of 0.1%. Putrescine, spermidine, spermine and 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma. [14C]Spermidine (112 mCi/mmol) was purchased from Amersham.

Cell Culture and Cell Growth Inhibition
All cell lines were propagated by standard tissue culture procedures in the medium suggested by the supplier (Supplementary Data). The antiproliferative activity of test compounds was measured after 72 h of incubation using the ATPlite assay (Perkin-Elmer) on a comprehensive panel of 29 cell lines. EC_{50} values were determined with curve-fitting analysis (nonlinear regression curve, variable hill slope coefficient), performed with the algorithm provided by GraphPad Software.

Evaluation of Antiproliferative Activity against Chinese Hamster Ovary Cell Lines
Chinese hamster ovary (CHO) cells were chosen along with a mutant cell line (CHO-MG) to determine how the test compounds gain access to cell. The CHO-MG cell line is polyamine transport deficient and was isolated after selection for growth resistance to methylglyoxal-bis(guanylhydrazone), using a single-step selection after mutagenesis with ethylmethanesulphonate (26). CHO cells were cultured in monolayers using α-MEM supplemented with 10% FCS, fungizone (1.25 μg/mL), and penicillin-streptomycin (100 units/100 μg/mL).

CHO-MG cells were plated at 2 × 10^3/mL for 24 h and then the antiproliferative activity of test compounds was measured, in the presence of 2 mmol/L aminoguanidine, using the ATPlite assay as described above. Controls were carried out to verify that, under our experimental conditions, aminoguanidine was not cytotoxic to CHO and CHO-MG cells.

Antiproliferative Activity in the Presence of Additional Exogenous Natural Polyamines
The antiproliferative effects of F14512 and etoposide have been evaluated according to a procedure described previously (20). Briefly, murine leukemia L1210 cells were cultivated in 96-well plates in the presence of F14512 or etoposide at appropriate concentrations and 500 μmol/L putrescine, 100 μmol/L spermidine, 50 μmol/L spermine, or physiologic saline as control in a medium containing 2 mmol/L aminoguanidine. After 48 h, cell growth was determined using MTT as described previously.
Spermidine Uptake Inhibition

The ability of F14512 and etoposide to compete with \[^{14}C\]spermidine uptake was determined in L1210 cells in vitro according to a procedure previously described (27). $K_i$ values for inhibition of spermidine uptake were determined using the Cheng Prussoff equation from the IC$_{50}$ values derived by iterative curve fitting of the sigmoidal equation describing the velocity of spermidine uptake of the respective competitor.

Topoisomerase II Inhibition

Cleavage of plasmid DNA. The experimental procedure has been previously detailed (28).

Figure 2. Effect of exogenous polyamines on the antiproliferative effect of F14512 (A) or etoposide (B) against L1210 murine leukemia cells, determined by a MTT assay after 48 h of incubation. L1210 cells were incubated with the test drug alone (○) or in the presence of 50 μM/L spermine (×), 100 μM/L spermidine (■), or 500 μM/L putrescine (▲). C, transport of \[^{14}C\]spermidine (\[^{14}C\]SPD) into L1210 cells in the presence of increasing concentrations of F14512 (■) or etoposide (▲). $K_i$ values are indicated.

Cleavage of a radiolabeled DNA fragment. Ten nanograms of 5'-end radiolabeled DNA probe (a 374-bp fragment of c-myc between positions 65 and 425 of its genome) was incubated for 5 min at 37°C with 1 μL (2 units) of purified human topoisomerase IIa (TopoGen) in the presence of test compound or 2.5% DMSO as control and treated as previously described (29). We defined for each assay the maximum tolerated concentration of DMSO to avoid any solvent effect. DMSO (3-5%, depending on the batch of enzyme) can be used with these topoisomerase II assays. In addition, 10 ng of a longer fragment of c-myc (401 bp) were added as control of precipitation efficacy. Dried gels were numerized for quantification using a Molecular Imager FX apparatus and its Quantity One software (Bio-Rad).

Comet Assay

The comet assay was adapted from a published procedure (30). A549 human lung cancer cells were seeded into culture dishes, at $1 \times 10^6$ cells per dish, and cultured for 24 h before treatment at 37°C for 1, 2, 4, 6, or 24 h with the solvent or with the test drug. The comets, stained with 2.5 μg/mL picogreen (Molecular Probes), were then analyzed using the tail moment (TM) parameters (31) calculated by the Comet Assay III software (Perceptive). Fifty cells were analyzed per slide and each experimental point resulted from two independent experiments.

Differential Cytotoxicity with CHO-K1 and xrs-6 Cell Lines

These two cell lines were cultivated as previously described (32). The drug cytotoxicity was evaluated using the ATPlile assay as mentioned above. IC$_{50}$ values were determined after 48 h of drug treatment to calculate the IC$_{50}$(test)/IC$_{50}$(ref) ratio. A ratio of >2 reflects a major contribution of DNA double strand breaks (DSB) in the cytotoxic action of the drug.

Tumor Growth Inhibition

Homogenous female athymic nude mice (Ico: Swiss-nu/nu, Charles River) were implanted with MX-1 human breast tumor xenografts and treated as previously described (33). Animals were handled and cared for in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and the European Directive EEC/86/609, under the supervision of authorized investigators. Two evaluation criteria were used in parallel: (a) growth inhibition, calculated as the ratio of the median tumor volumes of drug-treated versus control groups, T/C, % = (median tumor volume of drug-treated group on day $x$/median tumor volume of control group on day $x$) × 100, the optimal value, being the minimal T/C ratio that reflects the maximal tumor growth inhibition achieved (34); (b) tumor regression, defined as partial if the tumor volume decreased by 50% or less of that at the start of treatment, without dropping below measurable size, or defined as complete if the tumor burden became impalpable (35). Maximum weight loss or gain, expressed as a percentage of the initial body weight of the experimental animals, was used to provide an assessment of the toxicity of test compounds. According to National Cancer Institute (NCI) criteria, a dose is considered toxic if the induced body weight loss is higher than 20% of the initial mouse body weight (36).

Results

Rational Design of F14512

Etoposide is arguably one of the most specific and potent inhibitor of topoisomerase II. This old drug remains extensively used for the treatment of different types of malignancies, including small-cell lung cancers, testicular tumors, and non–Hodgkin’s lymphomas. The glycosidic moiety of etoposide at the C4 position (Fig. 1A) plays little or no role in the interaction with the topoisomerase II target (37) but it subtly alters DNA interactions (38). Based on these considerations, we designed a topoisomerase II inhibitor that preserves the aglycone part of etoposide, substituted on the C4 position by a polyamine tail susceptible to provide water solubility, DNA interaction, and recognition by the PTS expressed on tumor cells. The structure of the polyamine was optimized, as well as the linker between the two parts of the
molecule, to finally select F14512 composed of an epipodophyllotoxin core linked to spermine via an acetamide linker (Fig. 1B).

**PTS-Dependent Cytotoxicity of F14512**

Selective activity against a CHO cell line proficient for PTS. CHO cells were chosen along with a mutant CHO-MG cell line to study the selective transport of F14512 via the PTS. CHO-MG cells, selected for its resistance to the antitumor agent methylglyoxalbis(guanylhydrazone), a cytotoxic analogue of spermidine which is substrate of the PTS, are deficient in polyamine uptake (26). These cells have been routinely used to define the molecular requirements for the selective delivery of polyamine conjugates into cells containing active polyamine transporters (27, 39). Comparison of drug cytotoxicity in these two cell lines provided an important screen to detect cell entry via the PTS. In perfect agreement with the design strategy, we observed that F14512 was 73-fold more cytotoxic against CHO with an active transport system compared with CHO-MG cells (Fig. 2B). The IC$_{50}$ values for F14512 were 0.12 and 8.7 µmol/L in CHO and CHO-MG cells, respectively, which strongly suggest that F14512 enters into CHO cells via the PTS. In contrast, etoposide produced a similar cytotoxicity against both CHO and CHO-MG cells, characterized by IC$_{50}$ values of 1.1 and 0.9 µmol/L, respectively (Fig. 2A).

**Competition of F14512 with natural polyamines.** The antiproliferative effect of F14512 against L1210 murine leukemia cells was characterized by an IC$_{50}$ value of 20 nmol/L (Fig. 3A). Protection was observed by the addition of exogenous native polyamines because the antiproliferative activity of F14512 was massively reduced from 10- to 30-fold. In contrast, the addition of polyamines in the culture medium had no significant effect on the antiproliferative activity of etoposide (Fig. 3B).

To confirm whether the intracellular accumulation of F14512 was dependent on the PTS, we determined its ability to compete with $^{14}$C-spermidine for uptake in L1210 cells. F14512 competitively inhibited the transport of $^{14}$C-spermidine in L1210 with a $K_i$ value of 0.34 ± 0.04 µmol/L. This value corresponds to a low calculated $K_i$ compared with other polyamine conjugates (27, 40). In contrast, addition of etoposide up to 150 µmol/L did not inhibit the uptake of $^{14}$C-spermidine, preventing the determination of a $K_i$ value. The IC$_{50}$ and $K_i$ values of F14512 obtained with L1210 cells are entirely consistent with the cytotoxicity determined with the CHO/CHO-MG pair of cell lines and, collectively, these data show that F14512 enters into cancer cells via the PTS.

**DNA Binding and Damaging Activity of F14512**

The stabilization of DNA topoisomerase II covalent complexes by etoposide leads to DNA DSBs but the drug shows minimal, if any, interaction with DNA in the absence of the enzyme. In sharp contrast, F14512 is a solid DNA binder, capable of protecting DNA from heat denaturation. At a drug/DNA nucleotide ratio of 0.5, F14512 increases the melting temperature of calf thymus DNA and poly(dAT)$_2$ by 6°C and 17°C, respectively, and the interaction was also characterized by UV spectroscopy (hypochromatic at bathromic shifts at 285 nm) and circular dichroism (induction of a positive circular dichroism at 285 nm upon binding to DNA, suggesting minor groove binding; data not shown). No effect was observed with a non–polyamine-conjugated etoposide derivative. This interaction is directly attributable to the cationic spermine moiety binding to the DNA phosphate backbone and/or within the minor groove of the double helix.

Two complementary approaches were deployed to investigate inhibition of topoisomerase II. First, a plasmid cleavage assay was used to evidence the induction of DNA DSBs. As shown in Fig. 3A, supercoiled plasmid DNA was treated with human topoisomerase II in the presence of increasing concentrations of etoposide or F14512. A 401-bp radiolabeled DNA fragment was incubated in the presence of 1, 10, or 100 µmol/L etoposide or F14512. A 374-bp radiolabeled DNA fragment was incubated with 4 units topoisomerase II in the absence (lane Topo II) or presence of increasing concentrations (5, 10, 20, or 50 µmol/L) of F14512 or etoposide. DNA samples were separated by electrophoresis on a 1% agarose gel containing 1 µg/mL ethidium bromide. Gels were photographed under UV light. Nck, nicked; Sc, supercoiled; Lin, linear. The arrow points to the DSBs induced predominantly by F14512 compared with etoposide. B, a 374-bp radiolabeled DNA fragment was incubated in the presence of 1, 10, or 100 µmol/L etoposide or F14512. A 401-bp radiolabeled c-myc probe was used as control for DNA precipitation (T1) and was added to the 374-bp radiolabeled DNA fragments in the absence of topoisomerase II (T2) or incubated with this enzyme (T3). Cleavage patterns were visualized after electrophoresis on denaturing polyacrylamide gels and numerization. Plain arrows, major sites of cleavage. Dotted arrows, two sites detected principally with F14512, not with etoposide.

Figure 3. Inhibition of topoisomerase II. A, a plasmid DNA was incubated with the drug and strand breaks were visualized on agarose gels. In brief, supercoiled pLAZ (130 ng, lane DNA) was incubated with 4 units topoisomerase II in the absence (lane Topo II) or presence of increasing concentrations (5, 10, 20, or 50 µmol/L) of F14512 or etoposide. DNA samples were separated by electrophoresis on a 1% agarose gel containing 1 µg/mL ethidium bromide. Gels were photographed under UV light. Nck, nicked; Sc, supercoiled; Lin, linear. The arrow points to the DSBs induced predominantly by F14512 compared with etoposide. B, a 374-bp radiolabeled DNA fragment was incubated in the presence of 1, 10, or 100 µmol/L etoposide or F14512. A 401-bp radiolabeled c-myc probe was used as control for DNA precipitation (T1) and was added to the 374-bp radiolabeled DNA fragments in the absence of topoisomerase II (T2) or incubated with this enzyme (T3). Cleavage patterns were visualized after electrophoresis on denaturing polyacrylamide gels and numerization. Plain arrows, major sites of cleavage. Dotted arrows, two sites detected principally with F14512, not with etoposide.
consistent and establish that F14512 is a highly potent topoisomerase II poison in vitro. Its high capacity to stabilize cleavable complexes is most likely attributable to its reinforced DNA binding capacity via the polyamine tail.

**DNA Damage in Cells**

The DNA-damaging activity was also evaluated in cellulo using A549 human lung cancer cells, which present an active uptake system for polyamines (40). Cells were incubated 1 to 24 h in the presence of F14512 or etoposide at 50 or 500 nmol/L, respectively. These concentrations are close to the IC\(_{50}\) values of antiproliferative activities (Fig. 5) and were chosen to compare the two compounds at roughly equicytotoxic concentrations. Interestingly, although F14512 was much more potent than etoposide in terms of DNA damages in vitro, a lower amount of DNA strand breaks was observed in cellulo (Fig. 4A and B). Typical cell images from the Comet assay are presented in Fig. 4A and the variation of the TM as a function of the time of incubation is presented (Fig. 4A). TM values of 5.2 ± 1.0 were measured with 50 nmol/L F14512 after 4 to 6 h incubation, whereas etoposide at 500 nmol/L induced a larger amount of DNA strand breaks, characterized by a TM value of 11.5 ± 3.6. The kinetics of action of the two molecules are distinct. The comet formed rapidly with etoposide after 1 h of treatment and then decreased, with a minor TM value after 6 h, whereas the comet formed progressively with F14512 and persisted after 6 h. F14512 is 35 times more cytotoxic than etoposide against A549 cells but apparently less DNA damages are sufficient to produce this cytocidal effect. The balance between strand breaks formation and DNA repair may be different for the two compounds. These data indicate that F14512 functions as a DNA-damaging agent in cells and that its mode of action is not entirely superimposed to that of etoposide.

The contribution of DNA damages (topoisomerase II–induced DSBs) to drug-induced cell death was evaluated using a pair of cell lines with a normal or a deficient DNA repair machinery. The mutant xrs6 cell line (41) bears a mutated form of the Ku80 subunit of DNA-PK, thus providing a hypersensitivity to ionizing radiation and drugs producing DNA DSBs repaired by nonhomologous end joining (NHEJ) recombination (42). IC\(_{50}\) values for the wild-type (CHO-K1) and mutated (xrs6) cell lines are indicated in Fig. 4C along with the IC\(_{50}\) \(\text{CHO-K1}/\text{IC}_{50}^{\text{xrs6}}\) ratio, which reflects the contribution of DNA DSBs (repaired by NHEJ) in the cytotoxic action of the drug. As expected, etoposide was more cytotoxic to xrs6 cells than to CHO-K1 cells (IC\(_{50}\) \(\text{CHO-K1}/\text{IC}_{50}^{\text{xrs6}}\) ratio = 4.64), in agreement with previous data reported with different pairs of DSB repair deficient and proficient cell lines (43, 44). Interestingly, F14512 was 160-fold more cytotoxic to xrs6 cells compared with etoposide and the IC\(_{50}\) \(\text{CHO-K1}/\text{IC}_{50}^{\text{xrs6}}\) ratio with F14512 was about 10 times higher than that of etoposide. This result confirms that F14512 induces deletional DSBs and suggests that the nature of lethal DNA damages induced by etoposide and F14512 are different. The DNA repair pathway activated by F14512 remains to be elucidated at this point.

**In vitro Antiproliferative Activity**

The antiproliferative effect of F14512 on 29 human solid or hematologic cancer cell lines were determined (Fig. 5A) and compared with etoposide (Fig. 5B). After 72 h of incubation, F14512 inhibited the growth of these cells with a median EC\(_{50}\) value of 0.18 µmol/L that was about 8-fold lower than that of etoposide with a median EC\(_{50}\) of 1.4 µmol/L. The antiproliferative activity of F14512 was superior to that of etoposide against 21 of 29 cell lines (Fig. 5; Supplementary Data). The superiority of F14512 was particularly important with MX-1 (breast), LOX-IMV1 (melanoma), HL-60 (leukemia), SW872 (liposarcoma), or MESA (sarcoma) cell lines because EC\(_{50}\) values of F14512 was 23 to 88 lower than that of etoposide. On the other hand, eight cell lines were more sensitive to etoposide than to F14512 and this is consistent with the idea that the mechanism of action of the two compounds might be not strictly identical.

It is worth to mention that all these experiments were performed without aminoguanidine. To verify that the batch of serum used in these experiments did not contain a high level of serum amine oxidase (45), F14512 was restested in the same conditions (identical medium and serum, 72 h of incubation) but in the presence of 2 mmol/L aminoguanidine against a subpanel of five cell lines (BxPC3, LOVO, Nanimala, SKOV3, and SKBR3); no significant variation of EC\(_{50}\) values was observed (data not shown). Therefore, a cytocidal action resulting from an oxidization of spermine moiety of F14512 by serum amine oxidase is not considered.

**In vivo Antitumor Activity**

As mentioned above, MX-1 breast tumor cells were found to be much more sensitive to F14512 than to etoposide (IC\(_{50}\) values of 57.1 nmol/L and 1.31 µmol/L, respectively; Fig. 5). The corresponding in vivo model was available; therefore, we compared the antitumor activity of the two molecules in this xenograft model. The median tumor volumes of the MX-1 breast tumor-bearing mice treated over 2 weeks with a range of doses of F14512 are shown in Fig. 6. F14512 shows an outstanding activity in this model. Indeed, a high activity against these MX-1 tumors, with treatments starting 7 days after tumor implantation, was obtained at 1.25 mg/kg/injection, with evidence of partial and complete tumor regressions. At 1.25 mg/kg injection (Fig. 6C), 7 of 10 mice exhibited complete tumor regression, with 4 mice showing no tumor regrowth (cures) before the end of the experiment (day 46), whereas tumor regrowth was detected in the two other cases from days 36 or 39 (Fig. 6B and C). Optimal T/C values of <10%, namely 0% to 9%, obtained at 0.63 to 1.25 mg/kg/injection, were also indicative of a high level of activity according to the criteria of Corbett and colleagues (36). Furthermore, the lower dose of 0.32 mg/kg/injection also resulted in a significant antitumor activity, reflected by a T/C ratio of 26% (Fig. 6D). The lowest dose tested (i.e., 0.16 mg/kg/injection) proved to be inactive, whereas cures were observed for 14 of 15 mice with F14512 at 2.5 mg/kg/injection but this dose was associated with toxicity (data not shown). Overall, these data showed the superior antitumor activity of F14512 to etoposide, which showed only marginal in vivo activity against MX-1 tumor xenografts (Fig. 6D).

For etoposide, the dose of 20 mg/kg/injection proved to be inactive, according to the NCI criteria, with T/C values ranging from 49% to 52%. A higher activity was obtained at 30 mg/kg/injection, with evidence of 30% partial tumor regressions; however, this marked antitumor activity was associated with toxicity (data not shown). These in vivo data leave no room for doubt that F14512 is a highly potent antitumor agent in this MX-1 breast human tumor xenograft model when administered i.p. as multiple injections over 2 weeks. This spermine-linked epipodophyllotoxin derivative is considerably more potent than etoposide in this breast cancer model.

**Discussion**

F14512 was designed with the goal to combine the potent cytotoxic properties of epipodophyllotoxin derivatives and a vectorization to cancer cells via the PTS. In addition, we reasoned that the polyamine moiety, acting as a substrate for the PTS, could
serve as a DNA anchor and would provide water solubility. A family of polyamine-epipodophyllotoxin conjugates, with different linkers and lengths of the polyamine chain, was designed and synthesized. The spermine derivative F14512 was selected on the basis of multiple in vitro and in vivo experiments. F14512 was considerably (73-fold) more cytotoxic against PTS-proficient CHO cells compared with CHO-MG cells. This high ratio clearly speaks for a specific recognition of F14512 by the PTS. The fact that natural polyamines have no effect on the cytotoxicity of etoposide but decreased the cytotoxicity of F14512 against L1210 murine leukemia cells, known for their active PTS (46), corroborated the conclusion of a specific uptake of F14512 by the PTS. The competition of F14512 for the PTS was then confirmed by the inhibition of $\text{[14C]}$spermidine uptake, which was characterized by a sub-μmol/L $K_i$ value. Altogether, these data show that F14512 exhibits a high affinity for the PTS.

In addition to the vectorization via the PTS, the spermine moiety of F14512 can be exploited for the high affinity of polyammonium cation for DNA (47). This property has already been used to strengthen the activity of several DNA-damaging agents, including topoisomerase II poisons (20, 48). In this context, one can expect to increase the stability of epipodophyllotoxin/DNA/topoisomerase II ternary complexes by a stronger interaction of DNA with the positively charged polyamine chain. However, this idea is not so trivial because it requires a careful design to link the DNA-interacting group to the molecule without reducing accessibility to and inhibition of topoisomerase II. The C4-linked epipodophyllotoxin aglycone is an optimal candidate for this strategy when one takes into account the mode of interaction between etoposide and the DNA topoisomerase II complex (37, 38). The data presented here clearly establish that at the DNA topoisomerase II level, our design strategy was also correct. F14512 is a 10-fold more potent topoisomerase II poison than etoposide in vitro and this is most likely a direct consequence of its increased affinity for DNA. The spermine moiety does not alter the location of the cleavage sites on DNA. Similar observations have been reported previously with other polyamine-based DNA-binding conjugates, such as a chlorambucil conjugate (49). Our fine tuning of the podospermine

**Figure 4.** A, representative images of cellular DNA damages, as seen by the comet assay, observed in the presence of etoposide (500 nmol/L) and F14512 (50 nmol/L), after treatment of A549 cells for 2 h. B, variation of the TM (calculated as described in Materials and Methods) as a function of the time of incubation of A549 cells with etoposide or F14512. C, evaluation of in vitro cytotoxicity of F14512 or etoposide against CHO-K1 and xrs6 cell lines after 48 h of incubation.
hybrid has been successful to simultaneously address the issues of cell delivery and DNA topoisomerase II targeting.

At a cellular level, it is interesting to notice that F14512 and etoposide, when used at the equicytotoxic concentrations, did not show a comparable potency for inducing DNA damage. F14512 is ~10-fold more cytotoxic than etoposide against A549 cells but induced around 5-fold less DNA strand breaks. We are convinced that the spermine moiety of F14512 is not only a cell delivery vector but also plays a direct role in the mechanism of action leading to the antiproliferative activity. At this stage, we cannot exclude the

Figure 5. Antiproliferative activity of F14512 (A) compared with etoposide (B). Inhibition of proliferation was assessed using the ATP-lite (Perkin-Elmer) assay on a panel of 29 cell lines after 72 h of incubation. EC_{50} values were determined with a curve-fitting analysis (nonlinear regression curve, variable hill slope coefficient). The representation of the EC_{50} values was compared with a cutoff concentration chosen at 1 μmol/L. Vertical dotted bars, median of EC_{50} to display the general antiproliferative potency of each compound. *, for KB cells treated with F14512, EC_{50} > 100 μmol/L.

Figure 6. Effects of F14512 administered i.p. over 2 wk at 0.32 mg/kg/injection (A), 0.63 mg/kg/injection (B), or 1.25 mg/kg/injection (C), on MX-1 breast human tumor s.c. implanted in mice (Median tumor volume). The best response obtained with etoposide when administered according the same procedure at 20 mg/kg/injection is presented as comparison (D). Each condition has been evaluated against a total of 10 to 15 tumor-bearing mice in two to three separate experiments, each using five mice. Data relative to control and treated animals are represented by dotted and solid lines, respectively.
possibility that F14512 presents a cell uptake or subcellular distribution profile distinct from that of etoposide. The implication of another biological target, interacting with the polyamine tail, may also be considered to account for the high antiproliferative activity of the conjugate. A similar finding was reported previously with anthracene or acridine polyamine conjugates and topoisomerase inhibition (48). Another plausible hypothesis is that F14512 produces specific DNA damages at distinct genomic loci, which are not easily repaired and more lethal for tumor cells. The mechanism of DNA damages and repair remains to be investigated with this compound.

The antiproliferative activity of F14512 was evaluated against a panel of 29 human solid or hematologic cancer cell lines and compared with that etoposide. The superior antiproliferative activity of F14512 was shown with 21 of 29 cell lines. This observation likely reflects the degree of PTS activity in these 21 cancer cell lines and how its use for guided drug delivery can be an advantageous. Interestingly, the overall profiles of F14512 and etoposide are not strictly similar (with a Pearson coefficient of correlation of 0.75), suggesting here again that the spermine moiety of F14512 plays a significant role in its efficacy. The superiority of F14512 was particularly important with cell lines from breast cancer, liposarcoma, melanoma, and leukemia.

The high potential of F14512 was confirmed in vivo using the MX-1 human breast tumor xenograft model. This cell line exhibits a 23-fold higher sensitivity to F14512 compared with etoposide in vitro and this trend was also seen in vivo. F14512 displays a marked antitumor activity when administered i.p. as multiple injections over 2 weeks. A marked antitumor activity without toxic side effects was observed over a wide range of doses of 0.32 to 1.25 mg/kg/injection. The optimal T/C values <10% at 0.63 and 1.25 mg/kg/injection were judged as representative of a high level of activity according to standard criteria (36) and were associated with partial or complete tumor regression. F14512 proved to be considerably more potent than etoposide against MX-1 tumor xenografts, with apparently a much larger therapeutic index. Indeed, etoposide exerts an activity against this model at 30 mg/kg/injection, but this dose was associated with toxicity because 30% of treated mice at this high dose exhibited weight loss of >20%. The higher dose of 40 mg/kg/injection was associated with definite toxicity, as reflected by a maximal median body weight loss of 20.5% and 20% early deaths. Under identical conditions, F14512 proved much more active and well tolerated at a range of active doses. Therefore, although F14512 is structurally related to etoposide, its pharmacologic profile appears to be distinct. To our knowledge, this is the first time that a highly pronounced superiority is shown with a polyamine-containing derivative of an established drug. In summary, we described here the properties of F14512, the leading compound of a novel class of targeted cytotoxic agents that exploit the PTS to deliver a drug selectively into cancer cells. F14512 functions as a highly active topoisomerase II poison that produces DNA damage. Its spermine moiety contributes to the uptake into cancer cells via the PTS and plays a role in mediating cytotoxic activity. The drug is significantly more cytotoxic than etoposide and highly active in vivo in the MX-1 human breast tumor model. This study provides a strong rationale for the clinical development of F14512 and to further exploit the concept of selective delivery of drugs to tumor cells expressing the PTS.

Disclosure of Potential Conflicts of Interest


Acknowledgments

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