CPI-0004Na, a New Extracellularly Tumor-Activated Prodrug of Doxorubicin: In Vivo Toxicity, Activity, and Tissue Distribution Confirm Tumor Cell Selectivity

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ABSTRACT

The search for cancer therapies that are more selective for tumor cells and spare normal sensitive cells has been very active for at least 20 years. The extracellularly tumor-activated peptidic prodrug of doxorubicin (Dox) CPI-0004Na (N-succinyl-β-alanyl-L-leucyl-L-alanyl-L-leucyl-Dox) is potentially such a treatment. Here, we report the results of lethality studies performed with this compound in the mouse, showing that it is up to 4.6 times less toxic than Dox-HCl by the i.v. route and up to 16.2 times after i.p. administration. Pharmacokinetics and tissue distribution data indicate that this reduced toxicity is attributable to a lower uptake of Dox in normal tissues after treatment with CPI-0004Na than after the administration of an equimolar dose of Dox-HCl. For example, heart exposure to Dox is reduced >10-fold. Because of this reduced toxicity, higher doses of CPI-0004Na than of the parent drug could be used to treat nude mice bearing s.c. human breast (MCF-7/6) and colon (LS-174-T and CXF-280/10) tumors. In all three models, the prodrug showed a much improved efficacy as compared with Dox-HCl. Particularly, LS-174-T tumors that do not respond to Dox were inhibited by 68% after treatment with CPI-0004Na. Tissue distribution studies performed with MCF-7/6 tumor-bearing nude mice and comparing CPI-0004Na and Dox-HCl confirmed that the improved activity of the prodrug is actually the result of selective generation and uptake of Dox at the tumor site. Dox levels in tumor tissue were 2-fold higher after treatment with CPI-0004Na than after treatment with an equimolar dose of Dox-HCl, whereas normal tissue levels were reduced 1.4–29-fold.

INTRODUCTION

Despite the development of new strategies for the treatment of metastatic diseases, such as vaccination (1, 2), inhibition of tumor angiogenesis (3–5), or of telomerase (6), immuno or gene therapy (7–10), the primary systemic treatment for most patients remains chemotherapy. Its overall efficacy is, however, still impaired by the severe dose-limiting toxicities of all available cytotoxic agents (11), and it is imperative to develop selective release systems for these effective drugs, which would spare normal cells and reduce toxicity. Such selective delivery would also allow the use of higher doses of the drugs and could eventually lead to a certain efficacy against some resistant tumors (12–15).

One of the most direct approaches to achieve such an objective consists in the design of less toxic prodrugs of chemotherapeutic agents that can be converted to the active agent only when they reach the vicinity of a tumor cell or mass. A number of strategies have been proposed, but as of today, very few led to really encouraging preclinical results (16). One way to design a tumor-selective prodrug of an intracellularly acting cytotoxic agent is to prevent its entry into cells, for example through conjugation to a moiety that can be cleaved off in conditions prevailing in the extracellular environment of tumor cells. The simplest moieties that have been considered are peptides, and recently published studies described oligopeptidic derivatives of Dox that are supposedly activated by the peptidases prostate-specific antigen (17, 18) and plasmin (19).

We also developed oligopeptidic derivatives of Dox that are unable to enter cells, are stable in whole blood in vitro, and reactivated by an extracellular tumor peptidase (20). Our lead candidate for further development is CPI-0004Na (21). Exposure of this compound to enzymes released by cancer cells liberates L-Dox, a derivative that freely diffuses inside cells, where it is further cleaved to yield active Dox. Here, we show the reduced toxicity and increased efficacy of CPI-0004Na, as compared with Dox-HCl in animal models, and provide pharmacokinetic evidence supporting the anticipated mechanism of action.

MATERIALS AND METHODS

Drugs and Animals. Dox-HCl was purchased from Meiji Seika Pharma International (Tokyo, Japan). CPI-0004Na was synthesized as detailed elsewhere (21) from Dox-HCl and N-(9-fluorenylmethoxycarbonyl)β-alanyl-L-leucyl-L-alanyl-L-leucyl-L-leucine (Abbott Laboratories, Chicago, IL). Quenching of the amine deprotection reaction with succinic anhydride yielded the desired compound CPI-0004Na. All of the animals used were obtained from Iffa-Credo (Brussels, Belgium).

Lethality Studies. OF-1 male mice were used to compare the LD₅₀ of Dox-HCl and CPI-0004Na. Sterile solutions of the drugs in 0.9% (w/v) NaCl were administered i.p. or i.v. in the lateral tail vein (10 μg/l of body weight) on day 0 or on 5 consecutive days starting on day 0. Body weight and mortality were recorded daily for 28 days. LD₅₀ were estimated from sigmoidal regressions of cumulative mortality on day 28 versus dose curves.

Human Tumor Xenograft Studies. Fragments (~3 mm³ in size) of MCF-7/6 human breast tumors or of LS-174-T colon tumors were implanted s.c. in the flanks of 5-week-old BALB/c nu/nu or Swiss nu/nu mice, respectively. MCF-7/6 tumor growth was supported by estrone administered at 1 mg/l in the animals’ water supply. Treatments were administered i.v. once a week for 5 consecutive weeks, starting when tumors reached a mean diameter of at least 6 mm. Drug solutions were prepared for the lethality studies. Tumor volume was determined twice a week from caliper measurements [length × (width)²/2]. The minimal ratio of relative tumor volume in treated versus control groups was used to evaluate treatment efficacy. Treatment toxicity was assessed based on clinical signs and body weight evolution. The CXF-280/10 (human colon tumor) efficacy study was performed by Oncostest GmbH (Freiburg, Germany) using a similar protocol.

Tissue Distribution Studies. OF-1 normal male mice or female BALB/c nu/nu mice bearing s.c. MCF-7/6 human breast tumors (~150 mm³) were treated with 86.2 μmol/kg of Dox-HCl or CPI-0004Na as i.v. bolus injections or as 2-h infusions. Thirty min before the initiation of infusion, mice received 60 μl (i.p.) of a 5 mg/ml solution of diazepam (Valium injection; Roche, Basel, Switzerland). Dosing solutions (1.6 ml) were then injected through wing
needles maintained in the lateral tail vein using a compact infusion pump (Harvard Apparatus, Millis, MA) and a flow of 13 μl/min. At designated time points, animals were sacrificed by ether anesthesia, and plasma, heart, liver, spleen, kidneys, lungs, and tumors were collected, snap frozen in liquid nitrogen, and stored at −80°C until further processing. All tissues were processed immediately after thawing and maintained in an ice-bath throughout the procedure. After homogenization, drugs and metabolites were extracted immediately after alkalinization, as well as after acidification in the case of samples collected from CPI-0004Na-treated animals. Samples (in a final volume of 0.5 ml) were added to 1.8 ml of a 4:1 mixture of chloroform and methanol. A freshly prepared 3.45-μm internal standard solution (0.1 ml; N-(N-tert-poly-2)-phenylalanine-phenylalanine for the alkaline extractions, N-(N-succinyl-L-lysyl-L-lysyl)-phenylalanine for the acidic extractions) was also added, followed by 600 μl of either 0.5 M, pH 9.8 borate or 0.5 M, pH 3.0 citrate buffer. The tubes were immediately vortexed, and after a 10-min centrifugation at 2000 × g, the organic layers were recovered, and solvent was evaporated. Residues were dissolved by a 10-s ultrasonication at 100 W in 0.5 ml of a 70:30 mixture of 0.1% ammonium formate (pH 4.0) and acetonitrile. All samples were analyzed using 4.6 × 100 mm (2 μm particle size) TSK Super-ODS reverse phase HPLC columns (TosoHaas, Stuttgart, Germany) under isocratic conditions (30% acetonitrile, 0.1% trifluoroacetic acid in water) with a flow rate of 1.5 ml/min for 6.5 min. Fluorescence detection (λex = 235 nm; λem = 560 nm) was used, and drugs and metabolites were identified according to their respective relative retention times as determined from a set of standards. Calculated concentrations were corrected for recovery at the extraction step using previously determined correction factors. For tissues, concentrations were further corrected for blood contamination of the organs as described elsewhere (22), and results were expressed as the concentration of drug or metabolite per mg of tissue. The theoretical concentrations were further corrected for recovery at the procedure. After homogenization, drugs and metabolites were extracted processed immediately after thawing and maintained in an ice-bath throughout.

### RESULTS

**Reduced in Vivo Toxicity of CPI-0004Na as compared with Dox.** As an extracellularly tumor-activated prodrug of Dox, CPI-0004Na should be significantly less toxic *in vivo*. This is of course a major requirement that would allow treatment with increased dose levels and/or more frequent treatments. We therefore estimated the LD50 of CPI-0004Na and Dox-HCl in the normal mouse. We tested both the i.p. and i.v. routes and also checked the effect of dose fractionation.

Whatever the dosing protocol, CPI-0004Na effectively appears to be less toxic than Dox-HCl (Table 1). Because it is slightly more toxic when given i.v. with an estimated LD50 of 100 μmol/kg as compared with 155 μmol/kg by the i.p. route and because, on the contrary, Dox-HCl is more toxic when given i.p., the difference in toxicity between CPI-0004Na and its parent drug ranges from 3.3- to 6.9-fold by the i.v. and i.p. routes, respectively. Interestingly, when CPI-0004Na is administered on 5 consecutive days rather than as a single injection, the cumulative LD50s are increased by both routes to reach 336 μmol/kg i.p. and 207 μmol/kg i.v. (Table 1). No effect of dose fractionation on the toxicity of Dox-HCl is observed by the i.p. route, and the difference by the i.v. route is much less pronounced than in the case of CPI-0004Na. This results in a prodrug that is up to 4.6 times safer than Dox-HCl by the i.v. route and up to 16.2 times safer by the i.p. route.

**CPI-0004Na Is More Active Than Dox in Human Tumor Xenograft Models.** To be a useful prodrug, CPI-0004Na not only has to be less toxic than Dox-HCl, but it must also be more active when used at well-tolerated dose levels. To check this, we performed a number of experimental chemotherapy studies in different human tumor xenograft models. In all studies, mice bearing established tumors (~150 mm3 upon initiation of treatment) received i.v. bolus injections of the drugs once a week for 5 consecutive weeks. Different dose levels were always used for each drug. MCF-7/6 human breast tumors (Fig. IA) did respond to Dox-HCl at 7.6 μmol/kg with a maximum growth inhibition of 50% reached on day 52 after initiation of treatment. On the other hand, CPI-0004Na at 46.5 μmol/kg, a dose that was well tolerated based on clinical signs and on the evolution of body weight (not shown), yielded a maximal inhibition of tumor growth of 72% on day 63. The lower 34.5 μmol/kg dose used also inhibited tumor growth by 63% on day 63. Therefore, a good efficacy dose response was observed for CPI-0004Na at doses that were well tolerated. CPI-0004Na also proved more active than Dox-HCl in the LS-174-T colon carcinoma model (Fig. 1B). This tumor is particularly insensitive to the action of Dox, although the same dose did slow down the

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**Table 1** Estimated LD50 of Dox and CPI-0004Na in OF-1 male mice

<table>
<thead>
<tr>
<th>Schedule</th>
<th>Doxorubicin/HCl (μmol/kg)</th>
<th>CPI-0004Na (μmol/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.p. 1X</td>
<td>22.6</td>
<td>155</td>
</tr>
<tr>
<td>i.p. 5X</td>
<td>4.14 [20.7]</td>
<td>67.2 [136]</td>
</tr>
<tr>
<td>i.v. 1X</td>
<td>30.5</td>
<td>100</td>
</tr>
<tr>
<td>i.v. 5X</td>
<td>8.96 [44.8]</td>
<td>41.4 [207]</td>
</tr>
</tbody>
</table>

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Fig. 1. Experimental chemotherapy with CPI-0004Na. A, BALB/c nu/nu mice bearing s.c. MCF-7/6 human breast tumors (~150 mm3) received one i.v. bolus injection/week for 5 consecutive weeks of saline (0), 7.6 μmol/kg Dox-HCl (●), 34.5 μmol/kg CPI-0004Na (○), or 46.5 μmol/kg CPI-0004Na (□). B, Swiss nu/nu mice bearing s.c. LS-174-T human colon tumors were treated similarly with saline (0), 7.6 μmol/kg Dox-HCl (○), or 62.1 μmol/kg CPI-0004Na (□). C, a similar protocol was used by Oncotest GmbH (Freiburg, Germany) on CFX-280/10 human colon tumors (●; saline, ×; 5.7 μmol/kg Dox-HCl, ■; 46.5 μmol/kg CPI-0004Na). The graphs present the evolution of the median relative tumor volumes (RTV; 8–12 tumors/dose group) starting from the day treatment was initiated (day 0).
growth of MCF-7/6 tumors. Conversely, starting from day 10 after the initiation of treatment, CPI-0004Na stabilized tumor volume to allow a maximal tumor growth inhibition of 68% on day 30. Again, only a minor weight loss (maximum, 7%) and no particular clinical sign of toxicity were observed for the 62.1 μmol/kg CPI-0004Na treatment.

Finally, CPI-0004Na was evaluated in another colon carcinoma model, CXX-280/10, by Oncostest GmbH (Freiburg, Germany). CXX-280/10 tumor is more responsive to DoxHCl than LS-174-T (Fig. 1C). DoxHCl dosed at 7.6 μmol/kg was found to be toxic in the CXX-280/10 model, whereas 5.7 μmol/kg was well tolerated. CPI-0004Na, at the well-tolerated 46.5 μmol/kg dose, greatly inhibited tumor growth over the 50-day study period and induced transient regression of some tumors between 20 and 49 days after the initiation of treatment. Therefore, CPI-0004Na was much more efficacious inhibiting CXX-280/10 tumor growth than Dox at well-tolerated dose levels.

**Tissue Distribution and Pharmacokinetics Studies.** To explain the decreased toxicity of CPI-0004Na as compared with Dox, we performed tissue distribution studies in normal mice and compared the results obtained after treatment with equimolar dose levels of both compounds. When Dox is administered as an i.v. bolus injection at the dose of 86.2 μmol/kg, its plasma concentration quickly drops to reach a level of about 1 nmol/ml after 1 h (Fig. 2A). It then remains relatively stable (very slow decrease) at least up to 24 h. In the case of CPI-0004Na (Fig. 2B), the initial decrease in the plasma concentration of the intact prodrug is much more progressive, but continuous. As a result, although the elimination half-life of CPI-0004Na is, at 2.2 h, much lower than that of Dox (9.4 h), its AUC is twice higher (274.15 nmol × h/ml compared with 134.5 in the case of DoxHCl). Similarly, the values obtained for the distribution volume and clearance of CPI-0004Na (331.2 ml/kg and 584.4 ml/h × kg, respectively) are much lower than those obtained in the case of DoxHCl (34046 ml/kg and 2924 ml/h × kg). Interestingly, the intact prodrug is the major constituent found during the first 4 h after administration. The metabolites A-L-Dox, L-Dox, and Dox are generated rapidly. They reach their maximal concentration within 1 h after treatment, ~5 nmol/ml for A-L-Dox and L-Dox and ~0.7 nmol/ml for Dox. Their concentration then decreases to reach a plateau-like level of ~0.1 nmol/ml between 6 and 9 h (Fig. 2B). As a result, the AUC of Dox, the active agent, in plasma is 40 times lower after treatment with CPI-0004Na than with DoxHCl (Table 2).

In tissues of animals treated with CPI-0004Na, Dox is the major metabolite. Its maximal tissue concentration is generally quickly reached, within 1 to 6 h after treatment. It then slowly decreases (not shown). Interestingly, heart is the tissue whose Dox concentration is the lowest. Kidneys and spleen are the organs that are the most exposed to Dox. Kidney is also the organ in which the concentration of the intact prodrug and the two other metabolites are and remain the highest. We compared the AUC of Dox in tissues after an i.v. bolus injection of DoxHCl or CPI-0004Na (Table 2). In animals treated with DoxHCl, spleen is the most exposed tissue, followed by kidney, and heart has the lowest AUC. When CPI-0004Na is used, the highest AUC value is of course observed in kidney followed by spleen, and heart is once again the least exposed tissue. However, the AUCs obtained for all tissues are much lower than after DoxHCl treatment. Decreases ranging from 80% for lung to 93% in the heart are observed. Administering the prodrug as a 2-h infusion rather than as a bolus injection further reduces (1.5–4-fold) the AUCs obtained in all tissues with the exception of heart, which is not affected (not shown). Interestingly, the most important effect is observed in kidney, the most exposed organ after a bolus injection.

To confirm the anticipated mode of action of CPI-0004Na, i.e., that it allows an enhanced uptake of the active drug Dox in tumors relative to normal tissues, we also performed tissue distribution studies with tumor-bearing mice. Because we observed, in the normal mouse, that a slow infusion dosing protocol might be valuable to further decrease toxicity, we conducted these new experiments by infusing equimolar dose levels (86.2 μmol/kg) of DoxHCl or CPI-0004Na during 2 h in athymic mice bearing MCF-7/6 human breast tumors. In tumors as well as in normal tissues, Dox is the major metabolite detected up to 96 h after initiation of treatment (Fig. 3). Significant amounts of the intact prodrug are detected only in liver and kidneys, the latter containing the highest levels of L-Dox as well.

After treatment with DoxHCl, the observed AUC is the lowest in tumors, spleen being by far the most exposed tissue with a value that is 40 times higher (Table 3). When Dox is administered in the form of the prodrug CPI-0004Na, the AUCs are reduced in all tissues, except in tumors that appear to be more exposed to the active agent. Actually, Dox AUC in tumors of mice treated with CPI-0004Na is almost doubled (192%) as compared with the value obtained when DoxHCl

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**Table 2 Tissue exposure to Dox of normal mice after treatment with DoxHCl or CPI-0004Na**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Dox AUC</th>
<th>CPI-0004Na AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>1843</td>
<td>130.3 (7.07%)</td>
</tr>
<tr>
<td>Liver</td>
<td>3900</td>
<td>373.2 (9.57%)</td>
</tr>
<tr>
<td>Spleen</td>
<td>13484</td>
<td>1111 (8.39%)</td>
</tr>
<tr>
<td>Kidney</td>
<td>11928</td>
<td>2125 (17.82%)</td>
</tr>
<tr>
<td>Lung</td>
<td>4701</td>
<td>916.0 (19.48%)</td>
</tr>
<tr>
<td>Plasma</td>
<td>134.5</td>
<td>5.05 (2.2%)</td>
</tr>
</tbody>
</table>

* AUC values in pmol × h/ing.

* Percent of value after DoxHCl treatment.

* AUC values in nmol × h/ml.

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*Fig. 2. Plasma pharmacokinetics of CPI-0004Na and DoxHCl. Plasma concentration versus time curves of Dox or CPI-0004Na and its metabolites after treatment of OF-1 normal mice with an i.v. bolus injection of 86.2 μmol/kg of DoxHCl (A) or CPI-0004Na (B). Mean concentrations (bars; SD; six animals/time point) are presented. Drugs and metabolites were extracted from plasma samples and quantified by HPLC analysis as described in “Materials and Methods.” ×, Dox; □, CPI-0004Na; ○, A-L-Dox; ◊, L-Dox.*
is used at the same dose level (Table 3). At the same time, heart exposure is reduced by 90%. Dox AUC is decreased by 96% in spleen and >80% in lungs and liver. As mentioned above, kidney is the least affected tissue with a reduction of the AUC value of only 27%. The AUC of L-Dox in kidneys also represents half that of Dox.

**DISCUSSION**

On the basis of estimated LD₅₀ₐₕ, the toxicity of our new candidate prodrug CPI-0004Na is reduced more than 3–16-fold in the mouse as compared with the parent drug. This difference depends on the administration route and on dose fractionation. This latter observation is interesting because the dose intensity of DoxHCl that can be administered to a patient is constant whatever the dosing schedule (23), something we also observed in the animal studies reported here. Theoretically, this reduced toxicity results from the inability of the prodrug to enter cells along with its relative stability in blood and normal body fluids (20, 21). Our pharmacokinetics and tissue distribution results partly confirm this. Indeed, CPI-0004Na remains the major Dox derivative in plasma for the first 4 h after an i.v. bolus injection, and although it is eliminated faster than Dox, the much reduced distribution volume and clearance could be indicative of the expected poor uptake of the prodrug by tissues. The plasma Dox AUC of animals treated with the prodrug is 40 times lower than that of animals treated with an equimolar dose of DoxHCl, and the plasma pharmacokinetic parameters we determined for DoxHCl are in good agreement with those published previously (24).

Normal tissue exposure to Dox is also considerably reduced when CPI-0004Na is used, up to 93% for heart tissue. This is particularly important because, besides the side effects usually encountered with most anticancer drugs, the therapeutic efficacy of anthracyclines is impaired by the high risk of developing congestive heart failure when cumulative doses exceed 500–550 mg/m², a dose that can often be reached when tumors are still responsive (25, 26). Because the development of cardiotoxicity after treatment with anthracyclines appears to be related to cardiac muscle content of active drug or metabolites (27, 28), it is likely that CPI-0004Na will also be much less cardiotoxic. Among the organs tested, spleen and kidneys are the most exposed to Dox, and kidneys, together with the lungs, are less affected organs when CPI-0004Na is used instead of DoxHCl with only an 85% decrease of the Dox AUC. However, we showed that this could be improved by administering the prodrug as a 2-h infusion rather than as a bolus injection, which allowed a further 4-fold decrease. Other tissues except the heart were also affected by the increase of the duration of administration.

Because of its reduced toxicity, higher dose levels of CPI-0004Na could be used in in vivo efficacy studies compared with DoxHCl. Significantly better tumor growth inhibition was observed with the candidate prodrug as compared with the parent compound in breast and colon carcinoma models, at equally well tolerated dose levels. We observed a very good response (68% inhibition) in the LS-174-T model in which Dox displayed no activity at all at its maximal tolerated dose in the treatment protocol used. Although LS-174-T cells are not particularly resistant in vitro, this is an indication that certain forms of resistance could be overcome by our approach. As evidenced by the tissue distribution studies performed with mice bearing MCF-7/6 tumors, the improved efficacy could be attributable to an increased accumulation of Dox in tumors. If a doubling of Dox AUC in tumors can be observed when an equimolar dose of CPI-0004Na is used instead of DoxHCl, at equitoxic dose levels, the difference must be much more important.

**Table 3 Tissue exposure of MCF-7/6 tumor-bearing mice after i.v. treatment with DoxHCl or CPI-0004Na**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>DoxHCl</th>
<th>CPI-0004Na</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>1933</td>
<td>2.163</td>
</tr>
<tr>
<td>Spleen</td>
<td>47184</td>
<td>62.10</td>
</tr>
<tr>
<td>Liver</td>
<td>4315</td>
<td>2.030</td>
</tr>
<tr>
<td>Kidney</td>
<td>6433</td>
<td>132.6</td>
</tr>
<tr>
<td>Lung</td>
<td>4680</td>
<td>141.8</td>
</tr>
<tr>
<td>Tumor</td>
<td>1138</td>
<td>137.0</td>
</tr>
<tr>
<td>Plasma</td>
<td>21.18</td>
<td>5.777</td>
</tr>
</tbody>
</table>

* AUC values in pmol × h/mg.
* ND, not detected.
* AUC values in mmol × h/ml.
* Nothing detected starting from 4 h.
* Nothing detected starting from 16 h.
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