New Angiopep-Modified Doxorubicin (ANG1007) and Etoposide (ANG1009) Chemotherapeutics With Increased Brain Penetration

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This report describes the synthesis and preliminary biological characterization of 2 (ANG1007) and 3 (ANG1009), two new chemical entities under development for the treatment of primary and secondary brain cancers. 2 consists of three doxorubicin molecules conjugated to Angiopep-2, a 19-mer peptide that crosses the blood–brain barrier (BBB) by an LRP-1 receptor-mediated transcytosis mechanism. 3 has a similar structure, with the exception that three etoposide moieties are conjugated to Angiopep-2. Both agents killed cancer cell lines in vitro with similar IC50 values and with apparently similar cytotoxic mechanisms as unconjugated doxorubicin and etoposide. 2 and 3 exhibited dramatically higher BBB influx rate constants than unconjugated doxorubicin and etoposide and pooled within brain parenchymal tissue. Passage through the BBB was similar in Mdr1a (−/−) and wild type mice. These results provide further evidence of the potential of this drug development platform in the isolation of novel therapeutics with increased brain penetration.

Introduction

Doxorubicin and etoposide exhibit excellent therapeutic activity against a variety of solid tumors.1-4 Doxorubicin, a cytotoxic anthracycline antibiotic that intercalates into DNA, is thought to promote cytotoxicity by inhibiting DNA and RNA polymerases and by interacting with topoisomerase II to form DNA-cleavable complexes.5 Etoposide, a semisynthetic derivative of podophyllotoxin used for different malignancies and as first line treatment in small cell lung cancer,6 is thought to promote cytotoxicity by disrupting cell cycle progression, presumably by promoting DNA strand breaks in combination with DNA topoisomerase II.7 Chemotherapy for malignant brain tumors has limited efficacy, largely due to restricted blood–brain barrier (BBB)8 permeability for chemotherapeutic drugs.8 Intercellular tight junctions between capillary endothelial cells, a continuous (i.e., nonfenestrated) capillary endothelium, multiple intracellular efflux pumps with broad substrate specificity, and an array of intracellular and extracellular degradative/metabolic enzymes all combine to produce the restrictive diffusion barrier characterizing the BBB.9-12 Penetration of both doxorubicin and etoposide into brain tissue is dramatically inhibited by the BBB.13,14

We have developed a novel drug-development technology called the engineered peptide compound (EPC) platform that exploits the endogenous LRP-1 receptor-mediated transcytosis system. This platform is based on a proprietary 19-amino-acid sequence, called Angiopep-2, that crosses the BBB by an LRP-1 mediated mechanism. 1 (ANG1005),15 a new chemical entity (NCE) under development for the treatment of primary and secondary brain cancers, is the first agent to reach clinical trials based on this platform.16 1 (Figure 1) is composed of one Angiopep-2 peptide conjugated to three molecules of paclitaxel, a broad-spectrum antitumor agent that inhibits reorganization of microtubules during interphase and mitosis. In mice, 1 inhibited growth of orthotopic human glioblastoma (U87 MG) brain tumors more potently than paclitaxel alone and significantly increased animal survival rates.16 In phase I clinical trials in humans, 1 reached therapeutic concentrations in brain tumors and produced significant antitumor responses in patients with primary gliomas or secondary brain metastases who had failed prior standard therapy.17,18 The present study describes the synthesis and preliminary biological characterization of two new Angiopep-modified doxorubicin and etoposide derivatives with increased brain penetration.

Chemistry

Synthesis. The first derivative 2 consists of three molecules of doxorubicin conjugated via a cleavable ester bond to one Angiopep-2 peptide (Figure 1). For its synthesis (Scheme 1), the primary amine in the sugar of doxorubicin was first protected by an Fmoc group to provide intermediate 4 in

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†Abbreviations: LRP1, low-density lipoprotein receptor-related protein; pAb, polyclonal antibody; PBS, phosphate-buffered saline; BBB, blood–brain barrier; DMSO, dimethylsulfoxide; UPLC, ultra performance liquid chromatography; FA, formic acid; DIEA, N,N-diisopropylethylamine; FmocOSu, N-(9-fluorenylmethoxycarbonyloxy)succinimide; DCM, dichloromethane; TBTU, O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate; RPC, reverse phase chromatography; DMAP, 4-dimethylaminopyridine.
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Figure 1. Chemical structure of 1, 2, and 3. 1, 2, and 3 are each composed of three molecules of paclitaxel, doxorubicin, and etoposide, respectively, linked by cleavable ester to the Angiopep-2 peptide.

Scheme 1. Synthesis of 2

Scheme 2. Synthesis of 3
good yield (80%), which was then reacted with succinic anhydride to afford, primarily, the key acid 5. Some bis-acetylated side-products, via the two hydroxyls, were also formed (< 15%) and removed by SiO₂ purification. The free acid 5 was activated by TBTU prior to in situ conjugation with Angiopep-2. Under standard Fmoc deprotection conditions, the desired product 2 was obtained in 17% yield after purification on an AKTAexplorer system (GE Healthcare Life Sciences, Baie d’Urfe, Québec, Canada; see Experimental Section).

The second derivative 3 consists of three molecules of etoposide conjugated via a cleavable ester bond to the Angiopep-2 peptide (Figure 1). For the synthesis of 3 (Scheme 2), a dimethyl amino acetyl group was first introduced by acetylation at the 4’ position of the phenol of etoposide to generate intermediate 6 in 67% yield, which was then reacted with glutaric anhydride in the presence of DMAP to produce, primarily, semiglutarate 7 at the 2’ position in 40% yield. Some regioisomer 8 at the 3’ position was also isolated in 5% yield. The assignment of regioisomers 7 and 8 was performed by proton chemical shifts (Table 1) and confirmed by correlation spectroscopy (COSY) (data not shown). Regioisomer 7 was conjugated in situ with Angiopep-2 as previously described to provide 3 in 26% yield.

3 was soluble in dextrose 5% in water (D5W) up to 20 mg/mL. Similarly, the HCl salt of 2 could be dissolved at the same concentration in D5W. The high solubility of 2 and 3 facilitated their use in vitro and in vivo studies.

Analysis of End-Products. HPLC profiles of the final synthetic products exhibited single peaks with retention times for 2 (4.8 min) and 3 (5.4 min) that were distinct from unconjugated Angiopep-2 (4.2 min) (Figure 2). The masses of the HPLC-purified final products, as determined by mass spectrometry, were consistent in each case with three drug moieties conjugated to a single Angiopep-2 peptide. Thus, the observed mass of purified 2 (LC-HRMS, ESI, m/z 2089.9674 [M + 2], 1393.2419 [M + 3], and 1045.4395 [M + 4]) was consistent with the calculated mass of C₁₉₇H₂₄₂N₅₂O₇₀ (4177.6428) (Figure 3A). Similarly, the observed mass of purified 3 (m/z 2305.9327 [M + 2], 1537.6443 [M + 3], 1153.7463 [M + 4], and 922.7970 [M + 5]) was consistent with the calculated mass of C₂₁₉H₂₈₁N₃₂O₉₁ (4610.8955) (Figure 3B).

Results and Discussion

In Vitro Cytotoxicity. The in vitro cytotoxic activities of 2 and 3 were evaluated using a thymidine incorporation assay. To do so, human tumor cell lines were incubated for 48 h in the presence of increasing concentrations of 2, 3, doxorubicin, or etoposide. After aspirating off the old media, cells were pulse-labeled for 2 h in fresh media containing [³H]-thymidine. Uptake of tritium was evaluated in a β counter, and the drug concentrations required to inhibit cell proliferation by 50% (IC₅₀) were calculated. Results from these experiments showed that, in general, 2 and 3 had cytotoxic activities that were comparable to their parental drugs (doxorubicin and etoposide) in U87 MG glioblastoma, SK-Hep-1 hepatocarcinoma, and NCI-H460 lung carcinoma cell lines (Table 2), albeit 3 appeared to be somewhat less potent than etoposide in glioblastoma and lung carcinoma cells.

For 2 and 3, as well as the earlier agent 1, conjugation of the individual anticancer drugs to Angiopep-2 was achieved via a cleavable ester bond. When doxorubicin was conjugated to Angiopep-2, the Angiopep-2-doxorubicin conjugate lost its antiproliferative activity, indicating that doxorubicin needed to be released from the peptide to be active in cancer cells (data not shown). To evaluate their in vitro half-lives, a release study was performed on 2 and 3 by incubating the drugs in human serum for 0–4 h and quantifying the release of doxorubicin.
or etoposide by HPLC or LC/MS analysis. On the basis of these results, the estimated half-lives for 2 and 3 were 24 and 63 min, respectively.

**Cell Cycle Effects.** Doxorubicin and etoposide arrest cells in the G2 phase of the cell cycle, although the mechanism may be different for both agents. The cytotoxic effect of doxorubicin on malignant cells is thought to be related to nucleotide base intercalation and cell membrane lipid binding. DNA intercalation of doxorubicin inhibits the action of DNA and RNA polymerases and interacts with topoisomerase II to form DNA-cleavable complexes, resulting ultimately in a cell-cycle pause in G2. The role of cellular membrane binding in this process remains unclear. Etoposide is thought to produce breaks by either interacting directly with topoisomerase II or by participating in the formation of free radicals, which again pauses cells in G2. To analyze whether 2 and 3 had similar cell cycle effects, U87 MG glioblastoma cells were treated with 2 (33 nM), doxorubicin (100 nM), 3 (1 μM), or etoposide (3 μM) for 48 h and cellular DNA content was determined by flow cytometry (Table 3).

In the experiments comparing doxorubicin to 2, cells prior to drug addition were distributed across the cell cycle in a...
normal logarithmic growth pattern, with 27% of the cells in the G2/M phase of the cell cycle (Table 3). Addition of doxorubicin increased the proportion of cells in G2/M to 59.9%, as expected from earlier published reports. Addition of the Angiopep-2-doxorubicin derivative 2 also increased the percentage of cells in G2/M to nearly the same extent, 57.3%, as unconjugated doxorubicin. Similarly, the proportion of cells in G2/M rose from 38% in drug-naive cells to 87% and 70.8% after treatment with etoposide and the Angiopep-2-etoposide derivative 3, respectively. Thus, treatment with doxorubicin and 2, as well as etoposide and 3, produced very similar cell cycle delays in G2/M, consistent with the conjugated and unconjugated drugs having similar molecular effects.

**Blood – Brain Barrier Influx Rate Constants.** We used an in situ brain perfusion method previously adapted in our laboratory, to measure the transport of 2 and 3 into brain tissue in mice (Experimental Section). In brief, [125I]-2, [14C]-doxorubicin, [125I]-, or [3H]-etoposide were perfused into situ into the carotid artery for 0.5, 1, 2, or 4 min, followed by a tracer-free washout period of 60 s. After perfusion, the animals were sacrificed, the brain was surgically removed and homogenized, and radioactivity in total brain tissue was quantified. In the total brain homogenate, Vd increased in a linear fashion for 2 (Figure 4A) and 3 (Figure 5A), exhibiting no evidence of saturation after 4 min of perfusion for either agent. By comparison, the Vd values for doxorubicin and etoposide were low and remained relatively constant over the same period, indicating little uptake of unconjugated drugs into total brain tissue. The slopes of the curves, which correspond to the BBB influx rate constants (Kin), are presented in Table 4. The Kin for 2 (31 ± 0.7 × 10−3 mL/g/s) was 12.4-fold greater than unconjugated doxorubicin (2.5 ± 0.1 × 10−4 mL/g/s), while the Kin for 3 (22 ± 0.2 × 10−4 mL/g/s) was 24.4-fold greater than unconjugated etoposide (0.9 ± 0.1 × 10−4 mL/g/s). Thus, both new Angiopep-2 drug conjugates exhibited dramatically higher BBB influx rate constants than their unconjugated precursors, consistent with an enhanced ability to cross the BBB. The observed Kin values for 2 and 3 were similar to 1 (33 ± 0.2 × 10−4 mL/g/s) (Table 3), suggesting similar influx kinetics for the three drugs. In control experiments, [14C]-inulin, which is normally excluded from brain tissue in vivo, was perfused in the presence of unlabeled 2 and 3 to verify the physical integrity of the BBB (data not shown).

**Parenchymal Uptake.** To formally analyze whether 2 and 3 enter brain parenchyma or remain associated with the brain capillary endothelium, we estimated the apparent volume of distribution (Vd) for the two agents in total brain tissue, brain capillaries, and brain parenchyma after in situ perfusion and brain capillary depletion. For the brain capillary depletion, the brain was collected and homogenized as described above and then subjected to differential centrifugation through 35% Dextran 70. The radioactivity was then quantified in the capillary (pellet) and parenchymal (supernatant) fractions. At the 2 min time point, both 2 (Figure 4B) and 3 (Figure 5B) were present in parenchymal tissue at levels significantly higher than unconjugated doxorubicin and etoposide, indicating a higher rate of passage across the BBB for both agents relative to their parental precursors. By comparison, the Vd values for doxorubicin and etoposide were low and remained relatively constant over the same period, indicating little uptake of parental drugs into parenchymal tissue or total brain tissue. Significant

### Table 3. Effects of 2 and 3 on the Cell Cycle

<table>
<thead>
<tr>
<th>molecules</th>
<th>G0/G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>58.2</td>
<td>14.1</td>
<td>27</td>
</tr>
<tr>
<td>doxorubicin</td>
<td>30.8</td>
<td>8.9</td>
<td>59.9</td>
</tr>
<tr>
<td>2</td>
<td>32.3</td>
<td>10.1</td>
<td>57.3</td>
</tr>
<tr>
<td>control</td>
<td>56.8</td>
<td>5.1</td>
<td>38</td>
</tr>
<tr>
<td>etoposide</td>
<td>8.9</td>
<td>4.2</td>
<td>87</td>
</tr>
<tr>
<td>3</td>
<td>25.7</td>
<td>3.3</td>
<td>70.8</td>
</tr>
</tbody>
</table>

*a* U87 MG glioblastoma cells were treated for 24 h with no drug (control), doxorubicin, 100 nM, or product 2, 33 nM. U87 glioblastoma cells were also treated for 24 h with no drug (control), etoposide, 3 μM, and product 3, 1 μM. DNA cellular content was analyzed by flow cytometry. Results are presented as the percentage of cells in each cell cycle phase (%). One representative experiment of three is shown.

### Table 2. In Vitro Cytotoxicity of 2 and 3

<table>
<thead>
<tr>
<th>molecules</th>
<th>IC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>glioblastoma (U87 MG)</td>
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</tr>
<tr>
<td>doxorubicin</td>
<td>6.0</td>
</tr>
<tr>
<td>etoposide</td>
<td>8.9</td>
</tr>
<tr>
<td>control</td>
<td>56.8</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td>doxorubicin</td>
<td>10</td>
</tr>
<tr>
<td>etoposide</td>
<td>48</td>
</tr>
<tr>
<td>control</td>
<td>58.2</td>
</tr>
<tr>
<td>3</td>
<td>330</td>
</tr>
<tr>
<td>doxorubicin</td>
<td>10</td>
</tr>
<tr>
<td>etoposide</td>
<td>48</td>
</tr>
<tr>
<td>control</td>
<td>58.2</td>
</tr>
<tr>
<td>etoposide</td>
<td>145</td>
</tr>
<tr>
<td>control</td>
<td>62</td>
</tr>
</tbody>
</table>

*Values are means of 3–5 experiments (n = 8).*

Figure 4. In vivo brain uptake of 2. (A) Time course of brain uptake of [125I]-2 (filled circles) and [14C]-doxorubicin (open circles) measured by in situ brain perfusion. Results were expressed as the apparent volume of distribution (Vd) for the radiolabeled 2 or doxorubicin in total brain homogenate. Lines represent best fits to the data by least-squares regression. (B) After a 2 min perfusion of [125I]-2 (filled bars) and [14C]-doxorubicin (open bars), brain capillary depletion was performed and radioactivity was quantified in total brain homogenate, brain capillary fractions, and brain parenchymal fractions (Experimental Section). Results were expressed as the apparent volume of distribution (Vd) for the radiolabeled drugs found in these brain compartments. All data represent mean ± SD (n = 3–6 per time point).
anticancer agents, including doxorubicin and etoposide. The role of ABCB1 in the BBB can be conveniently assayed using Mdr1a (−/−) mice, which lack this transporter and consequently exhibit greater brain accumulation of many peripherally administered drugs. Consistent with the demonstrated role of this pump in the efflux of doxorubicin and etoposide at the BBB, mice bearing homozygous mutations of Mdr1a exhibited >1.3- and 2-fold increased brain penetration of unconjugated doxorubicin (Figure 6A) and etoposide (Figure 6B) relative to wild type and mutant mice, respectively. These increases in doxorubicin and etoposide brain uptake are similar to previous published data. By comparison, brain parenchymal uptake of doxorubicin and etoposide for 2 min prior to brain capillary depletion and quantification of radioactivity (Experimental Section). Results are expressed as the apparent volume of distribution (Vd) for the radiolabeled drugs found in these brain compartments. All data represent mean ± SD (n = 3–6 per time point).

**Table 4. Blood–Brain Barrier Influx Rate Constants for New Angiopep-2 Modified Anticancer Drugs and Their Respective Unmodified Analalogues**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Vd Brain (mL/100g/2min)</th>
<th>Vd Capillaries (mL/100g/2min)</th>
<th>Vd Parenchyma (mL/100g/2min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>doxorubicin</td>
<td>2.5 ± 0.1</td>
<td>22 ± 0.2</td>
<td>33 ± 0.2</td>
</tr>
<tr>
<td>etoposide</td>
<td>0.9 ± 0.1</td>
<td>6.9 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>pacitaxel</td>
<td>31 ± 0.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

levels of 2 and 3 were also observed in the capillary fraction, as expected from the extraordinarily high density of brain capillaries.

**Transport of 2 and 3 in Mdr1a (−/−) mice.** Brain capillary endothelial cells contain multiple efflux pumps that protect the brain from unwanted chemical assaults and contribute to the intrinsic or acquired multidrug resistance (MDR) that can arise after prolonged chemotherapy. In humans, one of the most important efflux pumps expressed at the BBB is the ATP-binding cassette (ABC) transporter protein B1 (ABCB1) also known as MDR1 or P-glycoprotein (P-gp). At the BBB, this efflux pump limits the entry to the brain of a vast array of anticancer agents, including doxorubicin and etoposide.

In mice, ABCB1 is encoded by three Mdr genes, and previous studies have demonstrated that the Mdr1a isoform is expressed at the BBB in mice. By comparison, brain parenchymal uptake of doxorubicin and etoposide for 2 min prior to brain capillary depletion and quantification of radioactivity (Experimental Section). Results are expressed as the apparent volume of distribution (Vd) for the radiolabeled drugs found in these brain compartments. All data represent mean ± SD (n = 3–6 per time point).

**Figure 5.** In vivo brain uptake of 3. (A) Time course of brain uptake of [125I]-3 (filled circles) and [3H]-etoposide (open circles) measured by in situ brain perfusion. Results were expressed as the apparent volume of distribution (Vd) for the radiolabeled 3 or etoposide in total brain homogenate. Lines represent best fits to the data by least-squares regression. (B) After a 2 min perfusion of [125I]-3 (filled bars) and [3H]-etoposide (open bars), brain capillary depletion was performed and radioactivity was quantified in total brain homogenate, brain capillary fractions, and brain parenchymal fractions (Experimental Section). Results were expressed as the apparent volume of distribution (Vd) for the radiolabeled drugs found in these brain compartments. All data represent mean ± SD (n = 3–6 per time point).

**Figure 6.** In situ brain perfusion in wild-type and Mdr-1a (−/−) knockout mice. Brain parenchymal uptake of (A) [125I]-2 and [14C]-doxorubicin (B) [125I]-3 and [3H]-etoposide measured by in situ brain perfusion in wild-type (open bars) and Mdr1a (−/−) mice (closed bars). Mice were perfused with 250 nM of [125I]-2, 750 nM [14C]-doxorubicin, 250 nM of [125I]-3, or 250 nM [3H]-etoposide for 2 min prior to brain capillary depletion and quantification of radioactivity (Experimental Section). Results are expressed as the apparent volume of distribution (Vd) for the radiolabeled drugs found in the brain parenchyma. Data represent the means ± SD obtained for at least three mice. *P = 0.023, Mdr1a (−/−) mice vs wild type in the doxorubicin-treated animals.
been reported to be substrates for BCRP. Results are expressed as the % of injected dose per gram of tissue. The identification of new neurotherapeutics that can cross the BBB may also remain after conjugation of neuroactive peptides, antibodies, and siRNAs to Angiopep-2. Protein (BCRP or ABCG2), which is also expressed at the ABCB1 efflux pump at the BBB may be modified with chemical modification appears to be flexible. Angiopep-2 is a 19-amino-acid sequence derived from the Kunitz-type domain present on some LRP-1 ligands, such as aprotinin, bikunin, amyloid precursor protein, and tissue factor pathway inhibitor. We now have evidence that each of which carries three separate drug moieties, cross the BBB efficiently. The molecular mechanism explaining how these modifications to Angiopep-2 affect or modulate LRP-1 binding remains to be established. It should also be noted that doxorubicin, etoposide, and paclitaxel, although different in their chemical properties and hydrophilicities, are nonetheless relatively small. Currently, modification of Angiopep-2 with larger biologicals, including therapeutic peptides, monoclonal antibodies, and siRNA, is under investigation in our laboratory.

Finally, no evidence to date would suggest that conjugation of the Angiopep-2 sequence to anticancer agents dramatically inhibits their activities. In this and earlier studies, the in vitro cytotoxic activities of each of which were similar to those of their unconjugated precursors, doxorubicin, etoposide, and paclitaxel, respectively. Moreover, I is currently in phase I clinical trials and has shown promising activity against primary and secondary brain cancers. The prior data are consistent with our recent release studies, which indicate that the anticancer moieties are likely cleaved in vivo from Angiopep-2 conjugates prior to performing their anti-proliferative activities. Again, the generality of these conclusions will require an analysis of more agents, including larger biologics, but preliminary evidence indicates that functionality may also remain after conjugation of neuroactive peptides, antibodies, and siRNAs to Angiopep-2.

Conclusions

The identification of new neurotherapeutics that can cross the BBB has proven difficult in the past, and novel methodologies for enhancing cross-BBB transport have long been needed. The EPiC platform is based on a 19-amino-acid peptide (Angiopep-2) that shows high transport across brain capillary endothelial cells via an LRP1-mediated mechanism. Using Angiopep-2, new chemical entities were created with enhanced transport across the BBB. The first agent developed with this platform was I, which carries three paclitaxel molecules conjugated to Angiopep-2. This study examined two new agents, 2 and 3, which carry three molecules of doxorubicin and etoposide, respectively, conjugated...
to Angiopep-2. Both agents killed cancer cell lines in vitro with similar IC_{50} values and with apparently similar cytotoxic mechanisms as unconjugated doxorubicin and etoposide. 2 and 3 also exhibited dramatically higher BBB influx rates than unconjugated doxorubicin and etoposide and pooled within brain parenchymal and tumor tissue. Passage through the BBB for both agents was not increased in mice lacking the ABCB1 efflux pump, implying their transport bypasses this pump system, which distinguishes the new agents from unconjugated doxorubicin and etoposide. In total, these results provide evidence that the novel drug development platform described here can be used broadly to isolate a range of small-molecule neurotherapeutics with increased brain penetration.

**Experimental Section**

**Reagents.** Doxorubicin hydrochloride and etoposide were purchased from Enzo Life Sciences (Plymouth Meeting, PA). All other reagents and anhydrous solvents were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. NMR (1H, 13C) spectra were recorded on Varian AS600 spectrometers (Palo Alto, CA) in CDCl3, CD2OD, or DMSO with solvent resonance as the internal standard. Low- and high-resolution mass spectra were recorded on Bruker microTOF spectrometers (Billerica, MA) using electron spray ionization (ESI-TOF). The purity of the conjugate target compounds was determined to be >95% by UPLC/MS on a Waters Acquity BEH phenyl 1.7 μm (4.6 mm x 50 mm) using a gradient of 10% to 90% MeCN in water and 0.1% FA to give 4 as a red powder 100 mg, 33%. UPLC purity, 95%. 1H NMR (600 MHz, CDCl3) δ (ppm) 7.96 (m, 1H), 7.74 (m, 2H), 7.56 (m, 2H), 7.36 (m, 2H), 2.97 (m, 2H), 2.79 (m, 2H), 2.05 (m, 2H), 2.15 (m, 2H), 1.8 (m, 1H), 1.31 (d, J = 6.2 Hz, 3H). 13C NMR (150 MHz, CDCl3) δ (ppm) 215.0, 187.53, 187.04, 163.16, 161.58, 156.48, 156.15, 155.85, 144.41, 141.85, 136.46, 135.95, 130.60, 133.95, 128.27, 127.61, 125.58, 121.11, 120.50, 120.38, 119.02, 112.08, 111.76, 77.0, 69.85, 67.18, 66.14, 62.64, 57.25, 47.48, 37.11, 36.15, 34.41, 32.07, 17.36. LC-HRMS (ESI, MicroTOF), m/z calcd for C26H43NO3, 421.2921, found 421.2847 (M + Na+).

**N-Fmoc Doxorubicin Hemisuccinate (5).** DIEA (0.17 mL, 1.0 mmol) was added dropwise to a solution of N-Fmoc doxorubicin hydrochloride (0.28 g, 0.366 mmol) and succinimide (0.11 g, 1.1 mmol) in DMF (20 mL) under stirring. The mixture was stirred at room temperature and monitored by UPLC. After two days, the reaction did not progress any more. The solvent was removed, and the resulting residue was purified using Silicycle silaflash 40 g cartridge on a Biotage system (2% to 15% MeOH in DCM) to give 5 as a red powder 100 mg, 33%. UPLC purity, 95%. 1H NMR (600 MHz, CDCl3) δ (ppm) 7.96 (m, 1H), 7.74 (m, 2H), 7.56 (m, 2H), 7.36 (m, 2H), 2.97 (m, 2H), 2.05 (m, 2H), 2.15 (m, 2H), 1.8 (m, 1H), 1.31 (d, J = 6.2 Hz, 3H). 13C NMR (150 MHz, CDCl3) δ (ppm) 207.75, 187.53, 187.15, 175.67, 172.59, 172.49, 165.56, 155.56, 144.12, 141.88, 136.30. 14.5, 136.84, 135.65, 133.97, 128.24, 128.23, 127.60, 125.60, 121.23, 120.49, 120.36, 111.89, 112.13, 111.78, 101.18, 98.54, 70.32, 69.99, 67.93, 66.95, 66.32, 62.48, 57.21, 47.81, 36.0, 33.94, 30.89, 30.66, 29.46, 29.35, 29.27, 17.26. LC-HRMS (ESI, MicroTOF), m/z calcd for C46H34NO16 865.2582, found 888.2701 (M + Na+).

**Synthesis of 2.** DIEA (0.25 mL, 1.44 mmol) was added dropwise to a solution of 5 (0.79 mg, 0.692 mmol) and TBTU (213 mg, 0.72 mmol) in DMF (21 mL) under stirring. The mixture was stirred at room temperature for 50 min, thus a solution of Angiopep-2 (671 mg, 0.229 mmol) in DMSO (2 mL) and DMF (12 mL) was added. The mixture was stirred at room temperature for 20 min. HPLC showed the reaction was complete. After stirring for another 10 min, the solvent was removed. The residue was purified using Biotage SNAP cartridge KP-C18- HS 120 g (40% to 80% MeCN in water with 0.05% TFA) to give Fmoc protected conjugate as a red powder 200 mg, UPLC purity, 95%. To a solution of above Fmoc protected conjugate (260 mg, 0.053 mmol) in DMSO (1 mL) and DMF (12 mL) was added piperidine (20% in DMF, 1.5 mL). The solution became blue. After stirring for 10 min, the solution was cooled to 0 °C and treated with FA (0.5 mL in DMF, 6 mL) to get a clear solution. The solution was removed under reduced pressure. The resulting residue was triturated with Et2O (3 × 10 mL) and EtOAc (3 × 10 mL). The resulting red solid was purified using 30 RPC column on AKTAexplorer (10–40% MeCN in water and 0.15% FA) to give 2 as a pink powder 82 mg, 17% in two steps. UPLC purity, 95%. LC-HRMS (ESI, MicroTOF), m/z calcd for C19H25NO25 1477.6428, found 1480.7964 (2+), 1393.2419 (3+), 1045.4395 (4+).

**Etoposide 4’-Dimethylglycine (6).** A mixture of etoposide (235 mg, 0.4 mmol) and DMAP (73 mg, 0.6 mmol) in DMF (4 mL) was stirred at room temperature for 20 min, then N,N-dimethylacetyl chloride (96 mg, 0.52 mmol) was added in one pot under stirring. After 30 min, the reaction was complete according to HPLC: FA (1 M in DMF, 0.5 mL) was added, and the solvent was concentrated under reduced pressure. The solution was loaded to a 30 RPC column on AKTAexplorer for purification (gradient 10–30% MeCN in H2O with 0.1% FA). After lyophilization, 6 (180 mg, 67%) was obtained as a colorless powder. 1H NMR (600 Hz, CD3OD) δ (ppm) 7.01 (1H, s), 6.56 (1H, s), 6.39 (2H, s), 5.98 (2H, d, J = 2.9 Hz), 5.05 (1H, d, J = 3.4 Hz), 4.77 (1H, q, J = 4.9 Hz), 4.68 (1H, d, J = 5.4 Hz), 4.66 (1H, d, J = 7.8 Hz), 4.46 (2H, s), 4.45 (1H, dd, J = 10.3, 8.8 Hz), 4.31 (1H, td, J = 8.0 Hz), 4.17 (1H, dd, J = 10.3, 4.9 Hz), 3.68 (6H, s), 3.56 (1H, q, J = 10.0 Hz), 3.54 (1H, td, J = 9.3 Hz), 3.52 (1H, dd, J = 14.2, 5.6 Hz), 3.32 (1H, m), 3.26 (1H, dd, J = 9.1, 4.1 Hz), 3.24 (1H, dd, J = 9.2, 5.4 Hz), 3.02 (6H, s), 2.96 (1H, m), 1.33 (3H, d, J = 4.9 Hz). 13C NMR (150 Hz, CD3OD) δ (ppm) 40.27
solution was loaded to a 30 RPC column on AKTAexplorer glutaric anhydride (222 mg, 1.94 mmol). The reaction mixture (0.25 mL, 1.46 mmol) were added consecutively, followed by chloroform (11 mL) was cooled to 0 °C. Iodination of peptide derived anticancer drugs was initiated by addition of 250 μCi of [125I]-Na (1 mCi) was added to the bead suspension for 5 min at room temperature. Iodination of peptide derived anticancer drugs was initiated by the addition of 250 μCi of 2 or 3 (100−150 μL) diluted in 0.1 M PBS, pH 6.5. After incubation for 10 min at room temperature, Ido-beads were washed and the supernatants were collected by a 12×column and purified by HPLC to remove free iodine. After iodination, radiolabeled products were reanalyzed by HPLC and results show that more than 95% of the radioactivity was associated to 2 or 3.

In Vitro Cytotoxic Activity. For the thyminde uptake assay, tumor cells were cultured in 96-well plates at a density of 5000 cells per well. After incubation of cells with anticancer drugs for 48 h, the medium was aspirated and cells were pulse labeled for 2 h at 37 °C with a medium containing 2 μCi/mL [methyl-3H]-thymidine (GE Healthcare). Cells were harvested and placed in a MicroBeta counter (1450 MicroBeta liquid scintillation and luminescence Counter; Perkin-Elmer) for determination of tritium uptake. Incorporated [3H]-thymidine was plotted as a function of drug concentration. Concentration values required to get a 50% inhibition (IC50) were estimated using GraphPad software (La Jolla, CA).

In Vitro Half-Life Analysis. The transport of [125I]-2, [123I]-3, and [125I]-doxorubicin or [125I]-etoposide in mouse brain was measured using the in situ brain perfusion method adapted in our laboratory for the study of drug uptake in the mouse brain (Dugenas et al.25). In situ brain perfusion of [125I]-inulin in the prepuaratus of unbanded 2 and 3 was also performed to verify the physical integrity of the BBB. Briefly, the right common carotid artery of mice anasthetized with ketamine/xyazine (140/8 mg kg−1, ip) was exposed and ligated at the level of the bifurcation of the common carotid, rostral to the occipital artery. The common carotid artery was then catheterized rostrally with polyethylene tubing filled with heparin (25 U/mL) and mounted on a 26-gauge needle. The syringe containing the perfusion fluid (radiolabeled molecules at the appropriate concentrations in Krebsbicarbonate buffer (128 mM NaCl, 24 mM NaHCO3, 4.2 mM KCl, 2.4 mM NaH2PO4, 1.5 mM CaCl2, 0.9 mM MgCl2 and 9 mM d-glucose) gassed with 95% O2 and 5% CO2 to obtain a pH of 7.4, and warmed to 37 °C in a water bath) was placed in an infusion pump (Harvard pump PHD 2000; Harvard Apparatus, Saint-Laurent, QC, Canada) and connected to the catheter. Prior to the perfusion, the contralateral blood flow contribution was eliminated by severing heart ventricles. The brain was perfused for 5 min at a flow rate of 1.15 mL/min. After perfusion, the brain was further perfused for 60 s with Krebs buffer to wash out the excess of radiolabeled molecules. Mice were then decapitated to terminate perfusion and the right hemisphere was quickly isolated on ice before being subjected to capillary depletion. Briefly, for capillary depletion, the mice brain was homogenized on ice in Ringer’s HEPES buffer with 0.1% BSA in a glass homogenizer. Brain homogenate was then mixed thoroughly with 35% Dextran 70 (50:50) and centrifuged at 5400g for 10 min at 4 °C. The supernatant composed of brain parenchyma and the pellet...
representing capillaries were then carefully separated. Aliquots of homogenates, supernatants, pellets, and perfusates were taken to measure their contents in radiolabeled molecules. Aliquots of the perfusion fluid were also collected and weighed to determine tracer concentrations in the perfusate. [125I]-2 and [125I]-3 samples were counted in a Wizard 1470 automatic γ counter (Perkin-Elmer Inc., Woodbridge, ON). [3H]-Etoposide and [14C]-doxorubicin samples were digested in 2 mL of Solvable (Packard) at 50 °C and mixed with 9 mL of Ultima gold XR scintillation cocktail (Packard). Radioactivity was counted in a Packard Tricarb model 1900 TR.

Brain Tissue Distribution. The tissue distribution of [125I]-2, [125I]-3, [14C]-doxorubicin, or [3H]-etoposide was measured in nu/nu mice bearing orthotopic U87 glioma tumors. Briefly, female athymic nude mice (Crl:Nu/Nu-nu-mBR; 20–25 g, 4–6 weeks old) (Charles River Canada, St. Constant, QC) were maintained in a pathogen-free environment. Intracerebral tumors were established by stereotactic inoculation of 5 × 10^5 U87 cells in mice brain as described. Sixteen days after inoculation, when first presenting with significant body weight loss, the mice were used for the tissue distribution studies. [125I]-2 and [14C]-doxorubicin were administered at doses of 15 and 6 mg/kg, respectively, by intravenous bolus injection. [125I]-3 and [3H]-etoposide were also administered by intravenous bolus injection at doses of 20 and 8 mg/kg, respectively. Thirty min after injection, mice were anesthetized with ketamine/xylazine (140/8 mg/kg, ip), whole blood was collected, and animals were then perfused by the heart with cold saline for 15 min at a flow rate of 5 mL/min. At the end of the perfusion, major organs were dissected and weighed. Brain hemispheres were separated, and the brain tumor mass was carefully dissected and weighed. The contralateral hemisphere served as the normal brain control. [125I]-2 and [125I]-3 tissue samples were counted in a Wizard 1470 automatic γ counter (Perkin-Elmer Inc., Woodbridge, ON). [3H]-etoposide and [14C]-doxorubicin tissue samples were digested in 2 mL of Solvable (Packard, Boston, MA) at 50 °C and mixed with 9 mL of Ultima gold XR scintillation cocktail (Packard, Boston, MA). Radioactivity was counted in a Packard Tri-Carb liquid scintillation counter model 1900 TR.

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References

(23) Dagenais, C.; Rousseille, C.; Pollock, G. M.; Scherrmann, J. M. Development of an in situ mouse brain perfusion model and its


(45) Gabathuler, R.; Demeule, M.; Regina, A.; Che, C.; Beliveau, R.; Castaigne, J. P. Development of a new Engineered Peptide Compound (EPiC) platform for the transport of small and large therapeutics to the CNS. Poster presented at: Neuroscience 2009, Chicago, IL, October 18, 2009.