Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 306 (2005) 122–131
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Enhancement of brain distribution of anticancer agents using ΔG, the 12 kDa active fragment of ZOT

Divya Menon [a], Chetan S. Karyekar [c], Alessio Fasano [b], Ruliang Lu [b], Natalie D. Eddington [a,*]

[a] *Pharmacokinetics Biopharmaceutics Laboratory, Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, 20 Penn Street, HSF II 543, Baltimore, MD 21201-6808, USA*
[b] *Center for Vaccine Development, School of Medicine, University of Maryland, Baltimore, MD 21201-6808, USA*
[c] *Department of Clinical Pharmacokinetics, Abbott Laboratories, Abbott Park, IL, USA*

Received 9 June 2005; received in revised form 16 August 2005; accepted 13 September 2005

Abstract

Objective: The objective of this study was to evaluate the ability of ΔG, the 12 kDa active fragment of ZOT, to increase the brain distribution of MTX and paclitaxel, two commonly used anticancer agents with poor distribution into the brain.
Methods: As part of dose estimation of ΔG, [^{14}C]-sucrose (40 μCi/kg), a hydrophilic paracellular marker, was co-administered with ΔG (0, 400 and 800 μg/kg) with and without protease inhibitors to male Sprague–Dawley rats ($n = 3$ per group) via an intracarotid cannula. MTX (50 mg/kg) and [^{3}H]-paclitaxel (120 μCi/kg) were co-administered with the effective doses of ΔG determined from the above study via the intracarotid cannula. Animals were euthanized by carbon dioxide asphyxiation at the specified time periods and brain and plasma samples were analyzed for the respective drug.
Results: The brain distribution of [^{14}C]-sucrose was significantly enhanced at both doses of ΔG. A fold enhancement in the *B*/*P* ratios of 1.88 and 2.68 was observed at the 400 and 800 μg/kg doses respectively, when the protein was protected from metabolic degradation with PIs. ΔG significantly increased the brain distribution of MTX at each of the doses administered, with over a seven-fold increase at the 600 μg/kg dose. [^{3}H]-paclitaxel brain $AUC_{0-60\,min}$ was significantly higher in the presence of ΔG (800 μg/kg with PIs) with a 2.5-fold enhancement in brain exposure.
Conclusions: ΔG significantly enhances the brain distribution of MTX (hydrophilic) and paclitaxel (lipophilic) and has the potential to be further developed as adjunct therapy to increase delivery of poorly permeable chemotherapeutic and other CNS targeted compounds.

Keywords: ZOT; ΔG; Blood–brain barrier; Tight junctions; Brain distribution; Permeation enhancer

1. Introduction

Effective drug delivery in disease states of the central nervous system (CNS) poses a continuing

* Corresponding author. Tel.: +1 410 706 6710; fax: +1 410 706 6580.
E-mail address: neddingt@rx.umaryland.edu (N.D. Eddington).

0378-5173/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2005.09.006

challenge to the successful treatment of these disorders. The principle reason for this is the presence of the blood–brain barrier (BBB) formed by the highly specialized capillary endothelial cells (Bradbury, 1984). This anatomical barrier is formed by complex tight junctions, contains few pinocytotic vesicles and is devoid of fenestra (Bradbury, 1984; Rubin and Staddon, 1999). Tight junctions (TJ) are considered to be the major determinant of paracellular permeability across membranes (Artursson, 1990) and restrict the paracellular diffusion of polar molecules. Small lipid soluble molecules with a molecular weight <500 Da can cross the BBB via lipid mediated free diffusion (Pardridge, 2001). However, if the surface area of the molecule is greater than 50–100 $Å^2$, in vivo permeability does not increase proportionately to an increase in lipophilicity (Fischer et al., 1998). In addition, a number of active efflux transporters are expressed at the BBB which function to reduce the intracellular accumulation of their substrates (Golden and Pollack, 2003). Consequently only a small number of low molecular weight (<500 Da), lipophilic compounds can permeate the BBB (Levin, 1980; Unger et al., 1985; Pardridge et al., 1990).

Most of the serious disorders of the brain including, human inmmunodeficiency virus type-1 (HIV-1) neuroinvasion and brain tumors, do not respond to treatment with large molecules due to their inability to achieve and maintain effective concentrations in the CNS (Pardridge, 2003b). The large molecular weight lipophilic (e.g. saquinavir in HIV-1 infection) or hydrophilic (e.g. methotrexate (MTX) in primary CNS lymphoma) compounds that are effective systemically are unable to attain pharmacologically significant amounts in the brain (Reggev and Djerassi, 1988; Yazdanian, 1999).

Drug delivery to brain tumors has been a controversial subject and is therefore of particular interest. Brain tumors can vary in malignancies but even the so-called benign tumors can be lethal due to their infiltrating properties and their ability to undergo malignant transformations over time. Gliomas and primary CNS lymphomas (PCNSL) are the most commonly observed forms of brain tumor in adults (Behin et al., 2003). The incidence of gliomas has been approximated to 5–10 per 100,000 general population (Legler et al., 1999) and that of PCNSL to 4–5 per 1000 persons in patients with AIDS and 0.3 per 100,000 persons among the immunocompetent (Cote et al., 1996). Once considered rare, the incidence of PCNSL has increased by about 7–10% in both immunocompromised and immunocompetant populations (Cote et al., 1996; Corn et al., 1997). Current management of gliomas involve surgical resection for immediate relief followed by radiation therapy. MTX based chemotherapy along with radiation therapy has had a significant impact on the management of PCNSL in the immunocompetant, but severe neurotoxic effects persist. The current understanding is that inadequate drug delivery to brain tumors is responsible for the unsatisfactory response of chemosensitive brain tumors (Louis et al., 2000; Siegal and Zylber-Katz, 2002).

Zonnula ocludens toxin (ZOT) is a 45 kDa protein isolated by Fasano et al. (1991) from *Vibrio cholerae*. ΔG is its 12 kDa active fragment identified and isolated by deletion studies with ZOT (Di Pierro et al., 2001). Both ZOT and ΔG have been shown to bind to a surface receptor and cause TJ opening via intracellular PKC mediated events (Fasano et al., 1995). In studies conducted with ZOT and insulin in BB/*Wor* diabetic male rats, Fasano et al. have shown that co-administration of ZOT with oral insulin resulted in enhanced insulin transport across the intestinal tight junctions resulting in blood glucose levels comparable to that achieved by the conventional subcutaneous route (Fasano and Uzzau, 1997a,b). More recently, in vitro studies in Caco-2 and bovine brain microvessel endothelial cells (BBMEC) cells as well as in vivo oral studies from this lab have demonstrated that both ZOT and ΔG are capable of transiently and reversibly increasing the paracellular transport in a dose dependant manner, of a number of hydrophilic molecular weight markers (mannitol, sucrose, PEG 4000 and inulin) and therapeutic compounds (acyclovir, paclitaxel, doxorubicin) (Cox et al., 2001, 2002; Karyekar and Eddington, 2002a,b). In a previously reported in vivo study in male Sprague–Dawley rats it was shown that co-administration of ΔG along with protease inhibitors (PI) with [^{14}C]-mannitol resulted in a 2.75-fold increase in its intestinal absorption (bioavailability) (Salama et al., 2003). It was therefore expected that this observed effect of ΔG on the bioavailability of mannitol will translate into an increased distribution of therapeutic agents across the BBB when administered along with ΔG. Hence, the purpose of this study was to examine the effect of ΔG on the brain dis-

tribution of MTX (MW 454.4) and paclitaxel (MW 853.9), two extensively used anticancer agents with different physicochemical properties and poor permeability into the brain (Neuwelt et al., 1991; Huennekens, 1994; Fellner et al., 2002). The hydrophilic paracellular marker sucrose (MW 342.3) was used to assess an effective dose range of ΔG in this study.

2. Materials and methods

2.1. Materials

MTX, xylazine, leupeptin, bestatin, captopril, acetonitrile and Tris–HCl were purchased from Sigma Chemical Co. (St. Louis, MO). All chemicals used were of analytical grade. Ketamine HCl Injection USP was purchased from Bedford laboratories (Bedford, OH). [^{14}C]-sucrose (100 μCi/mmol), [^{3}H]-*o*-benzamido-paclitaxel (37 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). Phosphate buffered saline (PBS), sterile saline (0.9N sodium chloride), and glacial acetic acid were purchased from Fischer Scientific (Newark, DE). The tissue solubilizer, Solvable®, and scintillation fluid, Ultima Gold®, were purchased from Packard BioScience (Groningen, Netherlands). ΔG was isolated and purified as described previously (Di Pierro et al., 2001) and was obtained from Dr. Fasano. Polyurethane tubing (MRE 033) was purchased from Braintree Scientific (Braintree, MA).

2.2. Methods

2.2.1. Brain distribution studies with ΔG in carotid cannulated rats

Male Sprague–Dawley rats weighing 225–275 g were purchased from Harlan (Indianapolis, IN). The animals were housed in an AAALAC accredited facility run on a 12 h light and dark cycle. The animals were allowed unrestricted access to food and water. The rats were anesthetized with an intraperitoneal dose of ketamine (80 mg/kg) and xylazine (12 mg/kg) and the carotid artery was isolated and cannulated using polyurethane tubing in a retrograde manner. The cannula was exteriorized at the back of the neck. The animals were allowed 2 days to recover from the surgery. The patency of the cannulas were maintained by daily flushing with heparinized saline. The protocol for the animal studies was approved by the School of Pharmacy, University of Maryland IACUC.

For the dose estimation studies with sucrose, animals ($n = 3$ per group) were administered [^{14}C]-sucrose (40 μCi/kg) and ΔG (0, 400 and 800 μg/kg) with and without PI (bestatin 30 mg/kg, leupeptin 67 mg/kg and captopril 30 mg/kg) via the intra carotid cannula. This cocktail of PI was previously used to protect ΔG from possible metabolism (Salama et al., 2003). In all the experiments where PIs were used, it was administered as a mixture immediately before the administration of ΔG and the radiolabeled compound. All solutions were freshly prepared in saline on the day of dosing. The animals were euthanized by carbon dioxide asphyxiation 5 min post dosing. For the MTX and paclitaxel brain distribution studies, the animals ($n = 3$ per group) were dosed with the drug (MTX, 50 mg/kg and [^{3}H]-paclitaxel, 120 μCi/kg, respectively), and ΔG (0, 200, 400 and 600 μg/kg) for MTX and (0 and 800 μg/kg) for paclitaxel, respectively with and without PIs. All doses were administered in a total volume not exceeding 1.2 mL. Animals were euthanized by carbon dioxide asphyxiation at the specified time points (2, 5 and 10 min for MTX and 5, 10, 15, 30 and 60 min for paclitaxel, respectively). Blood was collected by cardiac puncture immediately after euthanization, centrifuged at 2500 rpm for 10 min and plasma separated form the samples. Brains were removed within a minute of blood collection, blotted dry and weighed. All samples were stored at −80 °C until analyzed.

The radiolabeled tissue and plasma samples (sucrose and paclitaxel) were prepared for analysis as previously outlined (in the manufacturer's instructions). Briefly, the tissue samples were homogenized with equal volumes of phosphate buffered saline (PBS). An amount of 200 μL of the homogenate was added to 1 mL of Solvable® (alkali tissue solubilizer) and digested at 50 °C in a water bath for 2 h. At the end of the 2 h period, the samples were cooled to room temperature. An amount of 50 μL of glacial acetic acid (bleaching agent) was then added to the samples and further digested at 50 °C for 1 h. At the end of the 1 h period, the samples were cooled to room temperature and 10 mL of the liquid scintillation fluid, Ultima Gold® was added. The samples were allowed to temperature and light adapt for 1 h. Plasma samples were prepared by addition of 100 μL of the plasma sample

to 10 mL of the liquid scintillation fluid. All samples were counted on a Beckman LS 6500 Scintillation Counter.

MTX in tissue and plasma was analyzed using a previously reported UV-HPLC method (Cairnes and Evans, 1982) with minor modifications. Briefly, brain tissue samples were homogenized with equal volumes of 0.1 M Tris (pH 8) containing the internal standard aminopterin (10 μg/mL), 20 μl of TCA was added to 200 μl of the homogenate and centrifuged at 20,000 × *g* for 20 min. An amount of 50 μL of the supernatant was then injected on to an HPLC under the chromatographic conditions stated below. Plasma samples were treated with 10 μl of 50% TCA, centrifuged at 20,000 × *g* for 20 min and 50 μL supernatant analyzed for MTX utilizing UV detection at λ_{max} of 280 nm. MTX was eluted using a reverse phase C_{18} 10 μm Bondapak (Phenomenex, 150 mm × 3.9 mm) column with 15% ACN in 0.01 M ammonium formate buffer.

2.3. Distribution and statistical data analysis

2.3.1. Brain to plasma ratios for studies with sucrose

Brain (*B*) to plasma (*P*) ratio (*B*/*P*) of [^{14}C]-sucrose at 5 min was determined as follows:

$$B/P = \frac{(\text{dpm/g}) \text{ in brain}}{(\text{dpm/mL}) \text{ in plasma}} \tag{1}$$

B/*P*-fold enhancement ratio to indicate the amount of enhancement produced by Δ*G* treatment was calculated as

$$\text{fold enhancement ratio } (R) = \frac{(B/P)_{\Delta G/\text{PI}}}{(B/P)_{\text{control}}} \tag{2}$$

where, $(B/P)_{\Delta G/\text{PI}}$ is the brain to plasma ratio of [^{14}C]-sucrose in the Δ*G* treated group (with or without the PIs) and $(B/P)_{\text{control}}$ is the brain to plasma ratio of [^{14}C]-sucrose in the control group. The *B*/*P* ratios were compared for statistical significance using a Student's *t*-test or ANOVA with Dunnett's post-hoc where appropriate at $p < 0.05$.

2.3.2. Brain distribution studies with paclitaxel and MTX

Area under the concentration–time curve (AUC) and the variance associated with the AUC was calculated for the treatment and control groups using Bailer's method (Bailer, 1988). At each time point, the mean sample concentration was estimated following the expression given below:

$$\bar{Y}_q = \frac{1}{n_q}\left[\sum_{1}^{n_q} y_{q,\text{r}}\right] \tag{3}$$

where $\bar{Y}_q$ is the average brain concentration of the drug q^{th} time point, n_q the number of sample points at *q*th point, and $y_{q,\text{r}}$ is the concentration of the *r*th animal at the *q*th time point. The trapezoidal rule was then applied to the average concentrations to estimate the AUC:

$$\widehat{\text{AUC}} = \sum_{q=0}^{Q} w_q \bar{Y}_q \tag{4}$$

where

$$w_q = \frac{(t_1 - 0)}{2}, \quad q = 0 \tag{5}$$

$$w_q = \frac{(t_{q+1} - t_{q-1})}{2}, \quad q = 1, \ldots, Q-1 \tag{6}$$

$$w_q = \frac{(T_Q - t_{Q-1})}{2}, \quad q = Q \tag{7}$$

The variance of the estimated AUC at each time interval was estimated using the following expression:

$$\text{var}(\widehat{\text{AUC}}) = \frac{\sum_{q=0}^{Q} w_q^2 s_q^2}{n_q} \tag{8}$$

where s_q is the variance of the concentration at the *q*th time point. Total variance was calculated as a summation of variance at each time interval. Ninety-five percent confidence intervals were calculated for the groups and used to determine statistical significance. The ratio of AUC brain to AUC plasma was used to calculate the plasma to tissue partitioning. Further, fold enhancement of the Δ*G* treated group was calculated as

$$R = \frac{\widehat{\text{AUC}}_{\text{Brain-}\Delta G}/\text{AUC}_{\text{Plasma-}\Delta G}}{\widehat{\text{AUC}}_{\text{Brain-Control}}/\text{AUC}_{\text{Plasma-Control}}} \tag{9}$$

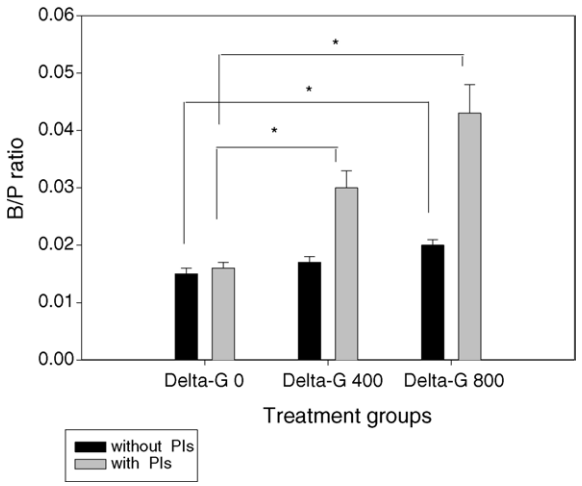

Fig. 1. Mean (±S.D.) of B/P ratio of sucrose in Sprague–Dawley rats for the control (ΔG 0) and treatment groups (ΔG 400 and 800) on concomitant dosing with 400 and 800 mg/kg ΔG with and without PIs via the intracarotid catheter. $^{*}p < 0.05$ vs.control.

3. Results

3.1. Brain distribution studies with sucrose and ΔG

The objective of the sucrose arm of this study was to assess a dose range for ΔG that should be administered in rats to observe an appreciable brain penetration enhancement effect. Sucrose was administered with 400 and 800 μg/kg ΔG with and without PIs. Fig. 1 illustrates the results. The B/P [^{14}C]-sucrose ratio for the control groups with and without the administration of PIs was 0.016 ± 0.001 and 0.015 ± 0.001, respectively, with no significant difference. The B/P ratios for the 0, 400, and 800 μg/kg ΔG treatment in the absence of PI treatment was 0.015 ± 0.001, 0.017 ± 0.001, and 0.020 ± 0.001, respectively. The B/P of the 800 μg/kg treatment group was significantly different ($p < 0.05$) as that of the control, whereas that of 400 μg/kg treatment did not shown any significant difference. When the PI cocktail was administered along with ΔG at 0, 400, and 800 μg/kg, the B/P ratios were 0.016 ± 0.001, 0.03 ± 0.003, and 0.043 ± 0.005, respectively. In this case, both the 400 and the 800 μg/kg ΔG groups had significantly higher ratios ($p < 0.05$) as compared to the control group.

In the group where sucrose was administered with ΔG alone, a 1.1 and 1.33-fold enhancement in the mean B/P ratio was observed with the 400 and 800 μg/kg doses, respectively. As seen from the plot, on administration of ΔG along with the PIs there was a further increase in B/P ratio (1.88 and 2.68 with the 400 and 800 μg/kg doses) caused by ΔG.

3.1.1. Brain distribution studies with paclitaxel, MTX and ΔG

MTX and paclitaxel, two anticancer agents with poor permeability across the BBB, were selected to evaluate the effect of ΔG on the brain distribution of compounds that show limited transport into the CNS. The brain distribution of MTX after a 50 mg/kg intra-arterial dose was determined over a 10 min period in the absence and presence of ΔG (0, 200, 400 and 600 μg/kg) when administered along with PIs. The experiment employed a destructive sampling strategy and therefore application of Bailer's method to estimate the AUC would allow statistical analysis of the data. The brain and plasma $AUC_{0-10\,min}$ for MTX as determined by Bailer's method for each dose of ΔG are presented in Table 1. In addition, the B/P ratios for MTX in the presence and absence of ΔG are also presented in Table 1. As seen from the table, there is a significant reduction ($p < 0.05$) in the systemic availability of MTX when comparing

Table 1
Brain and plasma mean area under the concentration–time curve ($AUC_{0-10\,min}$) and B/P ratio of MTX for the control and treatment groups in male Sprague–Dawley rats on concomitant dosing with 200, 400 and 600 μg/kg of ΔG with PIs via the intracarotid catheter

Group	Mean AUC brain$_{0-10\,min}$ (lower 95% CI, upper 95% CI) (μg min/g)	Mean AUC plasma$_{0-10\,min}$ (lower 95% CI, upper 95% CI) (μg min/mL)	B/P
Control	108 (70.0, 146)	14623 (12624, 16623)	0.00737
200	317[a] (261, 373)	5393 (4923, 5864)	0.059
400	607[a] (422, 792)	3359 (3096, 3622)	0.181
600	822[a] (684, 960)	4691 (4071, 5309)	0.175

[a] Significantly different from control based on 95% CI.

Table 2
Brain and plasma mean area under the concentration–time curve ($AUC_{0–60\,min}$) and *B*/*P* ratio of paclitaxel for the control and treatment group in male Sprague–Dawley rats on concomitant dosing with 800 μg/kg of Δ*G* with PIs via the intracarotid catheter

Group	Mean AUC brain$_{0–60\,min}$ (lower 95% CI, upper 95% CI) (ng min/g)	Mean AUC plasma$_{0–60\,min}$ (lower 95% CI, upper 95% CI) (ng min/g)	*B*/*P*
Control	1.17 (1.00, 1.35)	41.15 (31.30, 51.01)	0.028
800	3.04[a] (2.73, 3.35)	27.82 (15.97, 39.71)	0.109

[a] Significantly different from control based on 95% CI.

the control group ($AUC_{0–10\,min}$ = 14,623 μg min/mL) versus the 200 ($AUC_{0–10\,min}$ = 5393 μg min/mL), 400 ($AUC_{0–10\,min}$ = 3359 μg min/mL) and 600 ($AUC_{0–10\,min}$ = 4691 μg min/mL) treatment groups. The distribution of MTX as indicated by $AUC_{0–10\,min}$ (317, 607, 822 μg min/g) into the brain was significantly increased in each of treatment group (200, 400, and 600 μg/kg) when compared to the control ($AUC_{0–10\,min}$ = 108 μg min/g). As such, an increase of over three- to seven-fold in the exposure of MTX in the brain was observed in the three treatment groups. A corresponding 8–24-fold increase was seen in the *B*/*P* ratio of MTX, both due to the enhanced distribution of MTX into the brain as well as the reduced systemic exposure of this agent in plasma.

The total amount of [^{3}H]-paclitaxel present in the brain was estimated by calculating the $AUC_{0–60\,min}$ by Bailer's method and comparing the AUC values in the two treatment groups. The data is presented in Table 2. A significant increase ($p < 0.05$) in the brain distribution of [^{3}H]-paclitaxel was observed on dosing with 800 μg/kg of Δ*G*. As such, the $AUC_{0–60\,min}$ of the control group was 1.17 ± 0.08 and 3.04 ± 0.26 ng min/g for the Δ*G* treated group. The Δ*G* treated animals displayed approximately a two-fold increase in the brain exposure to [^{3}H]-paclitaxel as compared to the control group. There was no significant difference in the plasma distribution of [^{3}H]-paclitaxel. The *B*/*P* ratio was 0.028 and 0.109 in the control and treatment groups, respectively, translating into a fold enhancement of 3.9.

4. Discussion

Effective delivery of compounds to the brain has been a challenging problem to the pharmaceutical scientist for many years. In spite of this, more than 99% of central nervous system (CNS) drug development research is focused on drug discovery and only 1% is allocated to CNS drug delivery (Pardridge, 1997). Although several factors contribute to the low brain levels of CNS active drugs, the presence of the blood–brain barrier (BBB) plays a key role in preventing their transport to the brain (Bart et al., 2000). The presence of highly resistant complex tight junctions is an important component of the physical barrier. The tight junctions or zonula occludens are specialized structures between the capillary endothelial cells that securely separate the blood compartment from the brain and almost completely eliminate the paracellular pathway for the passage of compounds (Lutz and Siahaan, 1997).

Methods involving modulation of the BBB have proven to be effective in increasing the brain distribution of anticancer agents (Neuwelt, 1984; Louis et al., 2000; Siegal and Zylber-Katz, 2002; Pardridge, 2003b). Osmotic BBB disruption has been one of the first approaches to this end. Intracarotid infusion with hypertonic mannitol has been used to open the BBB in experimental animals and in humans (Kroll and Neuwelt, 1998). Other methods involving localized delivery of small hydrophilic anticancer agents to brain tumors using intra arterial and intravenous infusions of bradykinin and its analogue RMP-7 have been shown to be effective in animal models and in humans (Inamura et al., 1994; Prados et al., 2003). However both these methods are fraught with severe side effects like morbidity and are still under evaluation.

Fasano et al. have identified zonula occludens toxin (ZOT), a 45 kDa protein elaborated by *V. cholerae*, which activates a protein kinase C (PKC) dependent complex intracellular cascade of events that regulate tight junction competency in the small intestine (Fasano et al., 1995). ZOT exerts its action on tight junctions by mimicking Zonulin, a ubiquitous molecule that regulates tight junction permeability. ZOT is capable of binding to the Zonulin receptor on the luminal

surface of the intestine and reversibly opening the tight junctions between intestinal epithelial cells (Fasano et al., 1991, 1995; Fasano and Uzzau, 1997a; Fasano et al., 1997). Both in vitro and in vivo studies have shown that ZOT significantly enhances the intestinal absorption of a variety of *structurally dissimilar* co-administered therapeutic agents, including insulin, mannitol, inulin, paclitiaxel, acylcovir, cyclopsorin A, and PEG4000. Of significant importance, is that Zonulin, the putative endogenous ligand for the ZOT receptor has been found in the brain (Lu et al., 2000).

ΔG is the 12 kDa active fragment of ZOT that has been shown to reversibly and transiently open tight junctions in epithelial and endothelial cells (Cox et al., 2002; Karyekar et al., 2003). In previously reported work from this lab, it has been shown that the paracellular transport of a number of molecular weight markers (PEG 4000, dextran and inulin) and therapeutic agents (acyclovir, cyclosporin, doxorubicin, paclitaxel) was significantly enhanced with the concomitant use of ΔG (Cox et al., 2002; Karyekar et al., 2003). In addition, ΔG has been shown to enhance the systemic availability of both hydrophilic markers and therapeutic agents (Salama et al., 2003, 2004, 2005).

The first objective of the study reported herein was to determine an effective dose range for ΔG required to enhance the brain distribution of sucrose. Sucrose is an non-metabolized commonly used low molecular weight (MW 342.3) paracellular marker. A number of proteases, like aminopeptidases and angiotensin converting enzymes, are expressed at the BBB and in the systemic circulation. The PIs bestatin, leupeptin and captopril have been shown to inhibit the activity of the aminopeptidases, serine and thiol proteases and angiotensin converting enzymes, respectively. PIs have been used in in vitro and in vivo studies with proteins to effectively prevent degradation of the protein (Augustijins and Borchardt, 1995; Salama et al., 2003). ΔG being a protein, is susceptible to metabolic degradation by the proteases in the systemic circulation and at the BBB. In studies evaluating the effect of ΔG on the intestinal absorption of therapeutic agents, Salama et al. (2003, 2004, 2005) observed a significant enhancement in the biological effect of the protein when dosed along with the PIs. Therefore in order to examine the need for protection of ΔG from degradation by proteases, when doesed via the intracarotid route, the biological effect of ΔG on the brain distribution of sucrose was evaluated with and without the use of PIs (captopril, bestatin and leupeptin).

The sampling times in the various arms of this study was selected considering the stability of the protein ΔG, its proposed mode of action and the disposition of the compounds being evaluated. For instance, the distribution of sucrose into the brain is predominantly determined by its concentrations in plasma. It is rapidly cleared from plasma. Therefore sampling at an early time point, such as 5 min, will be more effective in delineating any possible differences due to treatment with ΔG. Also, since the effect of degradation by proteases on the biological activity of the protein was being evaluated, early sampling times were selected. MTX and paclitaxel, have a longer half life in plasma as compared to sucrose and therefore the distribution of MTX and paclitaxel were evaluated over a longer duration of time (Scheufler et al., 1981; Beijnen et al., 1994; Lesser et al., 1995).

We observed a significant difference in the B/P ratio at both the doses of ΔG (400 and 800 μg/kg) when administered with the protease inhibitors (Fig. 1). In the ΔG treatment groups without the protease inhibitors, a significant difference was observed only in the higher dose group (800 μg/kg). Also, there was no significant difference in the B/P values of the control groups with and without protease inhibitors. From these observations we may infer: (1) that the protease inhibitors are essential in preventing the degradation of the protein and thereby serve to enhance its activity as seen from the B/P values, and (2) that the protease inhibitors are themselves not responsible for the increase in the distribution of the compound. Based on these results the dose of ΔG effective when co-administered with the PIs (400 μg/kg) and two doses bracketing this effective dose (200 and 600 μg/kg) were selected for the distribution study with MTX and the higher dose (800 μg/kg) was selected for distribution studies with paclitaxel.

The second objective of this study was to determine the brain distribution of two anticancer agents, MTX and paclitaxel, in the absence and presence of ΔG. Methorexate (MW 454.4) is a weak organic acid, is negatively charged at neutral pH and therefore diffuses very slowly across biological membranes. This results in very poor permeability across the blood–brain barrier (Cosolo and Christophidis, 1987; Neuwelt et al., 1991). Also, MTX is a substrate for the efflux pumps MRP1 and BCRP expressed at the BBB (Borst et al.,

2000; Breedveld et al., 2004). This further reduces the intracellular concentrations of MTX. As seen from Table 1, treatment with ΔG resulted in a significantly higher distribution of MTX into the brain in all the dose groups. In our study, we observed a seven-fold increase in the brain distribution of MTX at the highest dose evaluated (600 μg/kg). This may appear to be modest when compared to the 10–100-fold increase in the brain distribution of MTX reported with the use of intraarterial infusion of mannitol (Kroll et al., 1998). However, differences in the method of administration (bolus dose versus infusion) and the use of increased doses of ΔG may result in a further increase in the brain distribution.

Paclitaxel (MW 854.9) is a high molecular weight compound, is extremely lipophilic and is a substrate of the efflux pump *p*-glycoprotein (van Asperen et al., 1997). Therefore, paclitaxel exhibits very poor permeability across the BBB and into the brain (Fellner et al., 2002; Kemper et al., 2003; Rice et al., 2003). Paclitaxel is a large molecule and therefore the highest dose of ΔG was used in this experiment. As seen from the data discussed above, the PIs did not themselves enhance the distribution of sucrose or MTX. Therefore, the PIs were not included in the control group and were included in the treatment group to protect ΔG from degradation. A point to be noted is that paclitaxel is predominantly metabolized by the CYP 450 isozymes (Cresteil et al., 1994; Harris et al., 1994) and the PIs used in this study are not known to be CYP inhibitors. As seen from Table 2, the brain distribution of paclitaxel was significantly higher in the treatment group compared to the control. On treatment with 800 μg/kg of ΔG, over a three-fold increase in the B/P ratio was observed.

For a drug delivery technology to the brain to be viable, it should not only be effective in enhancing the brain distribution of therapeutic agents but should also be non-toxic to the endothelial and brain cells. PET studies in humans have demonstrated that the BBB opening following osmotic disruption, leaves the BBB wide open for 40 min. The BBB returns to baseline levels after about 6 h (Siegal et al., 2000). This relatively long period over which the BBB function is incompletely restored would have a significant bearing on the safety of treatment regimens. The tj opening effect of ΔG is characterized by a rapid return to baseline values in vitro (Karyekar et al., 2003). The dose dependent decrease in TEERs observed by Karyekar et al. in BBMECs on treatment with ΔG returned to baseline values (same as controls) within 10 min of removal of the protein. This would prove be significant in vivo. Further studies in brain tumor models examining the efficacy and safety of concomitant dosing of anticancer agents with ΔG are required to completely characterize its role in drug therapy.

To summarize, we conclude that ΔG is effective in increasing the brain distribution of both hydrophilic and lipophilic compounds. Compared to ZOT, ΔG is susceptible to enzymatic degradation and requires the use of PIs for activity. The protein appears to significantly enhance the distribution of poorly permeable compounds into the brain. This ΔG technology may have the potential to be comprehensive because it has been shown to reversibly modulate tight junctions in the small intestine, and BBB and can potentially enhance the drug delivery of structurally dissimilar macromolecules. Drug delivery approaches, by their nature, need to be developed on a molecule-by-molecule basis. This research has taken the opposite approach, and has evaluated the influence of modifying the anatomical structures that control molecule transport. Furthermore, this approach may produce an effective, safe, broadly enabling and encompassing solution for the challenges that exist in drug delivery.

References

Artursson, P., 1990. Epithelial transport of drugs in cell culture. I. A model for studying the passive diffusion of drugs over intestinal absorptive (Caco-2) cells. J. Pharm. Sci. 79, 476–482.

Augustijins, P.F., Borchardt, R.T., 1995. Transport and matabolism of sleep inducing peptide in cultured human intestinal epithelial cell monolayers. Drug Metab. Dispos. 23, 1372–1378.

Bailer, J.A., 1988. Testing for the equality of areas under the curves when using destructive measurement techniques. J. Pharmacokinet. Biopharm. 16, 303–309.

Bart, J., Groen, H.J., Hendrikse, N.H., van der Graaf, W.T., Vaalburg, W., de Vries, E.G., 2000. The blood–brain barrier and oncology: new insights into function and modulation. Cancer Treat. Rev. 26, 449–462.

Behin, A., Hoang-Xuan, K., Carpentier, A.F., Delattre, J.Y., 2003. Primary brain tumours in adults. Lancet 361, 323–331.

Beijnen, J.H., Huizing, M.T., ten Bokkel Huinink, W.W., Veenhof, C.H., Vermorken, J.B., Giaccone, G., Pinedo, H.M., 1994. Bioanalysis, pharmacokinetics, and pharmacodynamics of the novel anticancer drug paclitaxel (Taxol). Semin. Oncol. 21, 53–62.

Borst, P., Evers, R., Kool, M., Wijnholds, J., 2000. A family of drug transporters: the multidrug resistance-associated proteins. J. Natl. Cancer Inst. 92, 1295–1302.

Bradbury, M.W., 1984. The structure and function of the blood–brain barrier. Fed. Proc. 43, 186–190.

Breedveld, P., Zelcer, N., Pluim, D., Sonmezer, O., Tibben, M.M., Beijnen, J.H., Schinkel, A.H., van Tellingen, O., Borst, P., Schellens, J.H., 2004. Mechanism of the pharmacokinetic interaction between methotrexate and benzimidazoles: potential role for breast cancer resistance protein in clinical drug–drug interactions. Cancer Res. 64, 5804–5811.

Cairnes, D.A., Evans, W.E., 1982. High performance liquid chromatographic assay of methotrexate, 7-hydroxymethotrxate, 4-deoxy-4-amino-N10-methylpteroic acid and sulfamethoxazole in serum, urine and cerebrospinal fluid. J. Chromatogr.: Biomed. Appl. 231, 103–110.

Corn, B.W., Marcus, S.M., Topham, A., Hauck, W., Curran Jr., W.J., 1997. Will primary central nervous system lymphoma be the most frequent brain tumor diagnosed in the year 2000? Cancer 79, 2409–2413.

Cosolo, W., Christophidis, N., 1987. Blood–brain barrier disruption and methotrexate in the treatment of a readily transplantable intracerebral osteogenic sarcoma of rats. Cancer Res. 47, 6225–6228.

Cote, T.R., et al., 1996. Epidemiology of brain lymphoma among people with or wihtout accquired immunodeficiency syndrome. J. Natl. Cancer Inst. 88, 675–679.

Cox, D.S., Gao, H., Raje, S., Scott, K.R., Eddington, N.D., 2001. Enhancing the permeation of marker compounds and enaminone anticonvulsants across Caco-2 monolayers by modulating tight junctions using zonula occludens toxin. Eur. J. Pharm. Biopharm. 52, 145–150.

Cox, D.S., Raje, S., Gao, H., Salami, N.N., Eddington, N.D., 2002. Enhanced permeability of molecular weight markers and poorly bioavailable compounds across Caco-2 cell monolayers using the absorption enhancer, zonula occludens toxin. Pharm. Res. 19, 1680–1688.

Cresteil, T., Monsarrat, B., Alvinerie, P., Treluyer, J.M., Vieira, I., Wright, M., 1994. Taxol metabolism by human liver microsomes: identification of cytochrome P450 isozymes involved in its biotransformation. Cancer Res. 54, 386–392.

Di Pierro, M., Lu, R., Uzzau, S., Wang, W., Margaretten, K., Pazzani, C., Maimone, F., Fasano, A., 2001. Zonula occludens toxin structure-function analysis. Identification of the fragment biologically active on tight junctions and of the zonulin receptor binding domain. J. Biol. Chem. 276, 19160–19165.

Fasano, A., Baudry, B., Pumplin, D.W., Wasserman, S.S., Tall, B.D., Ketley, J.M., Kaper, J.B., 1991. *Vibrio cholerae* produces a second enterotoxin, which affects intestinal tight junctions. Proc. Natl. Acad. Sci. U.S.A. 88, 5242–5246.

Fasano, A., Fiorentini, C., Donelli, G., Uzzau, S., Kaper, J.B., Margaretten, K., Ding, X., Guandalini, S., Comstock, L., Goldblum, S.E., 1995. Zonula occludens toxin modulates tight junctions through protein kinase C-dependent actin reorganization, in vitro. J. Clin. Invest. 96, 710–720.

Fasano, A., Uzzau, S., 1997a. J. Clin. Invest. 99, 1158–1164.

Fasano, A., Uzzau, S., 1997b. Modulation of intestinal tight junctions by Zonula occludens toxin permits enteral administration of insulin and other macromolecules in an animal model. J. Clin. Invest. 99, 1158–1164.

Fasano, A., Uzzau, S., Fiore, C., Margaretten, K., 1997. The enterotoxic effect of zonula occludens toxin on rabbit small intestine involves the paracellular pathway. Gastroenterology 112, 839–846.

Fellner, S., Bauer, B., Miller, D.S., Schaffrik, M., Fankhanel, M., Spruss, T., Bernhardt, G., Graeff, C., Farber, L., Gschaidmeier, H., Buschauer, A., Fricker, G., 2002. Transport of paclitaxel (Taxol) across the blood–brain barrier in vitro and in vivo. J. Clin. Invest. 110, 1309–1318.

Fischer, H., Gottschlich, R., Seelig, A., 1998. Blood–brain barrier permeation: molecular parameters governing passive diffusion. J. Membr. Biol. 165, 201–211.

Golden, P.L., Pollack, G.M., 2003. Blood–brain barrier efflux transport. J. Pharm. Sci. 92, 1739–1753.

Harris, J.W., Rahman, A., Kim, B.R., Guengerich, F.P., Collins, J.M., 1994. Metabolism of taxol by human hepatic microsomes and liver slices: participation of cytochrome P450 3A4 and an unknown P450 enzyme. Cancer Res. 54, 4026–4035.

Huennekens, F.M., 1994. The methotrexate story: a paradigm for development of cancer chemotherapeutic agents. Adv. Enzyme Regul. 34, 397–419.

Inamura, T., Nomura, T., Bartus, R.T., Black, K.L., 1994. Intracarotid infusion of RMP-7, a bradykinin analog: a method for selective drug delivery to brain tumors. J. Neurosurg. 81, 752–758.

Karyekar, C.S., Eddington, N.D., 2002a. Proceedings of the 17th National Meeting on Annual American Association of Pharmaceutical Scientists, Toronto, Canada.

Karyekar, C.S., Eddington, N.D., 2002b. Proceedings of the 17th National Meeting on Annual American Association of Pharmaceutical Scientists, Toronto, Canada.

Karyekar, C.S., Fasano, A., Raje, S., Lu, R., Dowling, T.C., Eddington, N.D., 2003. Zonula occludens toxin increases the permeability of molecular weight markers and chemotherapeutic agents across the bovine brain microvessel endothelial cells. J. Pharm. Sci. 92, 414–423.

Kemper, E.M., van Zandbergen, A.E., Cleypool, C., Mos, H.A., Boogerd, W., Beijnen, J.H., van Tellingen, O., 2003. Increased penetration of paclitaxel into the brain by inhibition of *P*-Glycoprotein. Clin. Cancer Res. 9, 2849–2855.

Kroll, R.A., Neuwelt, E.A., 1998. Outwitting the blood–brain barrier for therapeutic purposes: osmotic opening and other means. Neurosurgery 42, 1083–1099 (discussion 1099–1100).

Kroll, R.A., Pagel, M.A., Muldoon, L.L., Roman-Goldstein, S., Fiamengo, S.A., Neuwelt, E.A., 1998. Improving drug delivery to intracerebral tumor and surrounding brain in a rodent model: a comparison of osmotic versus bradykinin modification of the blood–brain and/or blood–tumor barriers. Neurosurgery 43, 879–886 (discussion 886–889).

Legler, J.M., Ries, L.A., Smith, M.A., Warren, J.L., Heineman, E.F., Kaplan, R.S., Linet, M.S., 1999. Cancer surveillance series [corrected]: brain and other central nervous system cancers: recent trends in incidence and mortality. J. Natl. Cancer Inst. 91, 1382–1390.

Lesser, G.J., Grossman, S.A., Eller, S., Rowinsky, E.K., 1995. The distribution of systemically administered [^{3}H]-paclitaxel in rats: a quantitative autoradiographic study. Cancer Chemother. Pharmacol. 37, 173–178.

Levin, V.A., 1980. Relationship of octanol/water partition coefficient and molecular weight to rat brain capillary permeability. J. Med. Chem. 23, 682–684.

Louis, D.N., Posner, J., Jacobs, T., Kaplan, R., 2000. Report of the Brain Tumor Progress Review Group. National Cancer Institute, National Institute of Neurological Disorders and Stroke, pp. 19–20.

Lu, R., Wang, W., Uzzau, S., Vigorito, R., Zielke, H.R., Fasano, A., 2000. Affinity purification and partial characterization of the zonulin/zonula occludens toxin (Zot) receptor from human brain. J. Neurochem. 74, 320–326.

Lutz, K.L., Siahaan, T.J., 1997. Molecular structure of the apical junction complex and its contribution to the paracellular barrier. J. Pharm. Sci. 86, 977–984.

Neuwelt, E.A., 1984. Therapeutic potential for blood–brain barrier modification in malignant brain tumor. Prog. Exp. Tumor Res. 28, 51–66.

Neuwelt, E.A., Goldman, D.L., Dahlborg, S.A., Crossen, J., Ramsey, F., Roman-Goldstein, S., Braziel, R., Dana, B., 1991. Primary CNS lymphoma treated with osmotic blood–brain barrier disruption: prolonged survival and preservation of cognitive function. J. Clin. Oncol. 9, 1580–1590.

Pardridge, W.M., 1997. Drug delivery to the brain. J. Cereb. Blood Flow Metab. 17, 713–731.

Pardridge, W.M., 2001. Brain drug targeting and gene technologies. Jpn. J. Pharmacol. 87, 97–103.

Pardridge, W.M., 2003b. Blood–brain barrier drug targeting: the future of brain drug development. Mol. Interv. 3, pp. 90–105, 51.

Pardridge, W.M., Triguero, D., Yang, J., Cancilla, P.A., 1990. Comparison of in vitro and in vivo models of drug transcytosis through the blood–brain barrier. J. Pharmacol. Exp. Ther. 253, 884–891.

Prados, M.D., Schold, S.J.S., Fine, H.A., Jaeckle, K., Hochberg, F., Mechtler, L., Fetell, M.R., Phuphanich, S., Feun, L., Janus, T.J., Ford, K., Graney, W., 2003. A randomized, double-blind, placebo-controlled, phase 2 study of RMP-7 in combination with carboplatin administered intravenously for the treatment of recurrent malignant glioma. Neuro-oncol 5, 96–103.

Reggev, A., Djerassi, I., 1988. The safety of administration of massive doses of methotrexate (50 g) with equimolar citrovorum factor rescue in adult patients. Cancer 61, 2423–2428.

Rice, A., Michaelis, M.L., Georg, G., Liu, Y., Turunen, B., Audus, K.L., 2003. Overcoming the blood–brain barrier to taxane delivery for neurodegenerative diseases and brain tumors. J. Mol. Neurosci. 20, 339–343.

Rubin, L.L., Staddon, J.M., 1999. The cell biology of the blood–brain barrier. Annu. Rev. Neurosci. 22, 11–28.

Salama, N.N., Fasano, A., Lu, R., Eddington, N.D., 2003. Effect of the biologically active fragment of zonula occludens toxin, ΔG, on the intestinal paracellular transport and oral absorption of mannitol. Int. J. Pharm. 251, 113–121.

Salama, N.N., Fasano, A., Thakar, M., Eddington, N.D., 2004. The effect of ΔG on the transport and oral absorption of macromolecules. J. Pharm. Sci. 93, 1310–1319.

Salama, N.N., Fasano, A., Thakar, M., Eddington, N.D., 2005. The impact of ΔG on the oral bioavailability of low bioavailable therapeutic agents. J. Pharmacol. Exp. Ther. 312, 199–205.

Scheufler, E., Zetler, G., Iven, H., 1981. Pharmacokinetics and organ distribution of methotrexate in the rat. Pharmacology 23, 75–81.

Siegal, T., Rubinstein, R., Bokstein, F., Schwartz, A., Lossos, A., Shalom, E., Chisin, R., Gomori, J.M., 2000. In vivo assessment of the window of barrier opening after osmotic blood–brain barrier disruption in humans. J. Neurosurg. 92, 599–605.

Siegal, T., Zylber-Katz, E., 2002. Strategies for increasing drug delivery to the brain: focus on brain lymphoma. Clin. Pharmacokinet. 41, 171–186.

Unger, C., Eibl, H., von Heyden, H.W., Krisch, B., Nagel, G.A., 1985. Blood–brain barrier and the penetration of cytostatic drugs. Klin. Wochenschr. 63, 565–571.

van Asperen, J., van Tellingen, O., Sparreboom, A., Schinkel, A.H., Borst, P., Nooijen, W.J., Beijnen, J.H., 1997. Enhanced oral bioavailability of paclitaxel in mice treated with the P-glycoprotein blocker SDZ PSC 833. Br. J. Cancer 76, 1181–1183.

Yazdanian, M., 1999. Blood–brain properties of human immunodeficiency virus antiretrovirals. J. Pharm. Sci. 88, 950–954.