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Research Paper

Etoposide modulates the effects of oral morphine analgesia by targeting the intestinal P-glycoprotein

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Abstract

Objectives Opioids and anticancer compounds such as etoposide (ETP) are substrates of P-glycoprotein (P-gp), an ATP-dependent efflux pump. Chemotherapy compounds may impact on the analgesic effect of opioids such as morphine when the two drugs are co-administered. In this study, we used a mouse model to determine if there is a pharmacological interaction between ETP and morphine, focusing on the involvement of intestinal P-gp.

Methods P-gp drug efflux activity was measured by an in-situ closed loop method with Rhodamine 123, a P-gp substrate. The analgesic effect of morphine was determined by the tail-flick test. Intestinal P-gp expression levels were determined by Western blot.

Key findings ETP and morphine significantly decreased the intestinal Rhodamine 123 efflux activity of P-gp. Oral morphine analgesia was significantly enhanced when co-administered with ETP. However, repeated pretreatment (7 days) with oral ETP significantly decreased the oral morphine-induced analgesia, in a cyclosporine A (a P-gp inhibitor) reversible manner. Furthermore, repeated ETP significantly up-regulated intestinal P-gp expression.

Conclusions It may be important to consider aspects of therapeutic design such as the administration route or scheduling of drugs in patients receiving concurrent chemotherapy and opioid therapy to avoid pharmacokinetic interactions between the two agents.

Introduction

Opioids, the gold standard analgesics for the control of pain in cancer patients, are now often used for the palliative care of cancer patients.[1] The World Health Organization (WHO) guidelines for palliative care encourage the initiation of palliative care early in a patient's course of treatment,[1] and it has been suggested that an increasing number of patients undergoing chemotherapy are also receiving opioids to treat cancer-related pain. It is well established, that the analgesic effects of opioids may differ from one patient to another, owing to the different characteristics of pain and pharmacokinetics between individuals.[2] When considering the outcomes of palliative care conducted in parallel with chemotherapy, the role of drug–drug interactions between opioids and anticancer chemotherapy agents are often neglected as a point of study.

The pharmacokinetic factors affecting the analgesic effects of opioids include drug metabolising enzymes (e.g. cytochrome P-450), drug-transporters (e.g. P-glycoprotein; P-gp) and/or opioid receptors (μ, δ and κ-opioid).[2,3] To date, most research has focused on the interaction between P-gp and opioids.[4,5] P-gp is an ATP-dependent drug efflux transporter that recognizes a number of drugs, including opioids, calcium-channel blockers and anticancer chemotherapy agents.[6] Research generated in this laboratory and elsewhere has shown that the analgesic effect of morphine was significantly elevated in mice lacking the P-gp encoding gene *mdr1*.[4,7] In these *mdr1* knockout mice, subcutaneous administration of morphine significantly enhanced brain morphine concentrations, and it is believed that the marked increase in morphine-related analgesia is owing to a functional loss of

P-gp operating at the blood–brain barrier.[4] However, P-gp is also expressed in the kidney, liver and intestine, and the involvement of P-gp in these tissues has not as yet been discussed at length. Current WHO guidelines recommend that patients be treated with orally administered analgesics wherever possible.[1] Given that intestinal P-gp is an important regulator of the mechanisms underlying orally administered drug absorption, P-gp may also affect the pharmacokinetics or pharmacodynamics of orally administered opioids.

In addition to opioids, the bioavailability of orally administered drugs that act on P-gp, such as human immunodeficiency virus protease inhibitors or tyrosine kinase inhibitors, have also been reported to be increased in *mdr1* knockout mice.[8,9] Furthermore, competitive inhibition of P-gp or the induction of P-gp by substrate drugs has been reported to affect the pharmacokinetics and pharmacodynamics of opioids.[7,10] Thus, putative drug–drug interactions, acting via intestinal P-gp, which occur between orally administered opioids and anticancer compounds, have been implicated in cancer patients receiving opioids to treat cancer pain in addition to receiving chemotherapy. However, the specific interactions are yet to be elucidated.

In this study, we sought to analyse whether P-gp acts as an underlying mechanism modulating the pharmacological interaction between morphine and etoposide (ETP), an orally administered anticancer drug.

Materials and Methods

Animals

Male ddY mice (Japan SLC Inc., Shizuoka, Japan) (4–5 weeks old) were provided with food and water *ad libitum*, and were housed in an animal room maintained at 24°C and 55 ± 5% humidity according to a 12 h light/dark cycle (light phase 0800–2000 hours). All procedures were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, adopted by the Japanese Pharmacological Society. Additionally, all experiments were approved by the animal ethics committee at Kobe Gakuin University (approval no. A 090130-1).

Drug administration

Morphine was obtained from Takeda Co., Ltd (Osaka, Japan). The mice were divided into two groups, those receiving oral morphine (30 and 50 mg/kg) and those receiving subcutaneous morphine (1, 5, 7 and 10 mg/kg). Each group was compared with a vehicle (water) control group. The mice were also treated orally with 10 mg/kg ETP (Sigma, St Louis, MO, USA) dissolved in water, and cyclosporine A (CsA; Wako, Osaka, Japan) (100 and 300 mg/kg, p.o.) dissolved in Cremophor EL and ethanol (6.71 : 3.29). ETP was administered concomitantly with morphine administration or pre-administered (once a day for 7 days) before morphine administration. CsA was administered at the same time as morphine.

Tail-flick test

The morphine analgesia against thermal stimuli was assessed with the tail-flick test.[11,12] The mice were gently held with the tail positioned in a tail-flick apparatus (MK-330B; Muromachi Kikai Co., Ltd, Tokyo, Japan) for radiant thermal stimulation of the dorsal surface of the tail. The intensity of the thermal stimulus was adjusted to cause the animal to flick its tail within 3 to 4 s as the baseline of the tail-flick latency. The tail-flick latency was measured before oral morphine administration and then every 30 min for 120 min thereafter. A 10-s cut-off time was set to minimize tissue damage. The area under the curve (AUC) value for morphine analgesia in each mouse was then calculated.

In-situ closed loop methods

Ileal P-gp activity was evaluated as described previously.[13] Briefly, the mice were fasted for at least 16 h and then deeply anesthetized with isoflurane (2%). The upper and lower ends of the ileum (14 cm) were then ligated. Next, 1.2 ml of Krebs Henseleit bicarbonate buffer solution containing 52 μM Rhodamine 123 (Rho123; Sigma) was administrated into the ileum. At 0 and 20 min following Rho123 administration, the fluorescent intensity of the Rho123 that remained in the ileum was measured with a fluorescence microplate reader (excitation wavelength: 485 nm; absorption wavelength: 535 nm; Perkin Elmer, Kanagawa, Japan). The percentage Rho123 concentration in the loop at 20 min was calculated compared with values obtained at 0 min.

Preparation of membrane fractions from intestinal mucosa

Experiments were performed as previously described with some modifications.[13,14] Briefly, the ileal mucosa membrane was obtained from mice. After homogenization (400 rev/min, 20 strokes) in homogenizing buffer, the homogenate was centrifuged at 3000*g* for 10 min at 4°C. The supernatant was then further centrifuged at 15 000*g* for 15 min at 4°C. The residual membrane fractions were resuspended in lysis buffer. The protein concentrations were then measured using the Lowry method (DC Protein Assay kit II; Bio-Rad, Hercules, CA, USA).

Western blot analysis for intestinal P-gp expression

Western blot analysis was carried out as previously described.[13] Briefly, the proteins extracted from the ileal mucosal membrane fraction and were separated by

electrophoresis (50 μg/lane) on a 7.5% SDS-polyacrylamide gel and then transferred onto a nitrocellulose membrane. After blocking in blocking buffer consisting of Tris-buffered saline (pH 7.6), 0.1% Tween 20 and 5% blocking agent (GE Healthcare UK Ltd, Bucks, England), the membranes were incubated with primary antibodies directed against P-gp (mAb C219, 1 : 200 dilution; Calbiochem, San Diego, CA, USA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (clone 6C5, 1 : 20 000; Chemicon, Temecula, CA, USA). The membrane was then incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody (1 : 2000; Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). The immunoreactive bands were visualized using a Light Capture system (ATTO, Tokyo, Japan) with an enhanced chemiluminescent substrate for horseradish peroxidase detection (ECL Western Blotting system; GE Healthcare UK Ltd). Signal intensity of the immunoreactive bands was determined by using specialized software (CS-Analyzer version 3.0; ATTO, Tokyo, Japan).

Measurement of morphine concentrations

Experiments were preformed as previously described.[15,16] Briefly, blood and brain samples were collected 15, 30 and 60 min after morphine administration. Serum was separated by centrifugation (880g, 3000 rev/min for 10 min at 4°C). The brain was homogenized in 1 ml of pure water by sonication (20 s). The mixture of sample (100 μl of serum or brain homogenate) and 40% K_2HPO_4 solution (1 ml) was shaken with 5 ml of ethylacetate for 20 min and then centrifuged at 2000*g* for 5 min at 4°C. The organic layer was collected, and the aqueous layer was re-extracted with 5 ml of ethylacetate. Then, the morphine in the organic layer was extracted with 1 ml of 1 M acetic acid, and a 0.9-ml portion of the aqueous layer was lyophilized. The samples were dissolved in 200 μl of 0.01 M HCl, and 20 μl was analysed by high-performance liquid chromatography with electrochemical detection: column, Eicompak MA-ODS (Eicom, Kyoto, Japan); mobile phase, 0.1 M citric acetate buffer (pH 3.9)/methanol (82 : 18) containing 3 mg/l EDTA and 150 mg/l sodium octane sulfonate; flow rate, 1 ml/min; detector, ECD-100 (Eicom) 750 mV Ag/AgCl; temperature, 25°C. The data were analysed by use of the AUC of the time-dependent changes in serum morphine concentration or in brain morphine content.

Statistical analysis

Data were expressed as means ± SEM. Statistical significance was assessed with an unpaired Student's *t*-test or one-way analysis of variance followed by Scheffe's test or Dunnett's test. Differences were regarded as statistically significant when the *P* value was less than 0.05.

Results

Effect of ETP and morphine on the Rho123 efflux activity of ileal P-gp

As shown in Figure 1, with the in-situ loop method, the ileal concentration of Rho123 decreased by 40% after Rho123 injection into the ileal loop (i.e. Rho123 was absorbed). Co-administration of morphine or ETP with Rho123 significantly decreased the ileal Rho123 concentration, which indicates enhanced Rho123 absorption, or a decrease in P-gp-dependent Rho123 efflux activity (Figure 1).

Effect of co-administration of oral ETP on the analgesic effect of oral morphine

The tail-flick test clearly demonstrated that co-administration of oral ETP with morphine increases the latent tail withdrawal time during thermal stimulation. Sig-

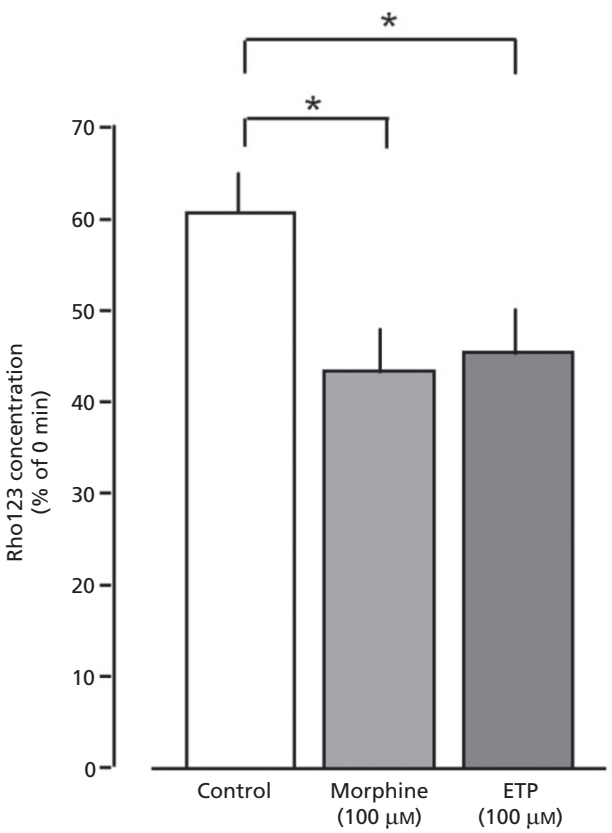

Figure 1 Effect of etoposide (ETP) and morphine on the Rhodamine 123 (Rho123) efflux activity of ileal P-glycoprotein analysed by the in-situ closed loop method. The concentration of Rho123 in the ileal loop was measured 20 min after Rho123 injection with or without the indicated drugs. The control group was treated with water. *$P < 0.05$, significantly different compared with the control group (Dunnett's test); $n = 5$, control; $n = 5$, Morphine; $n = 5$, ETP.

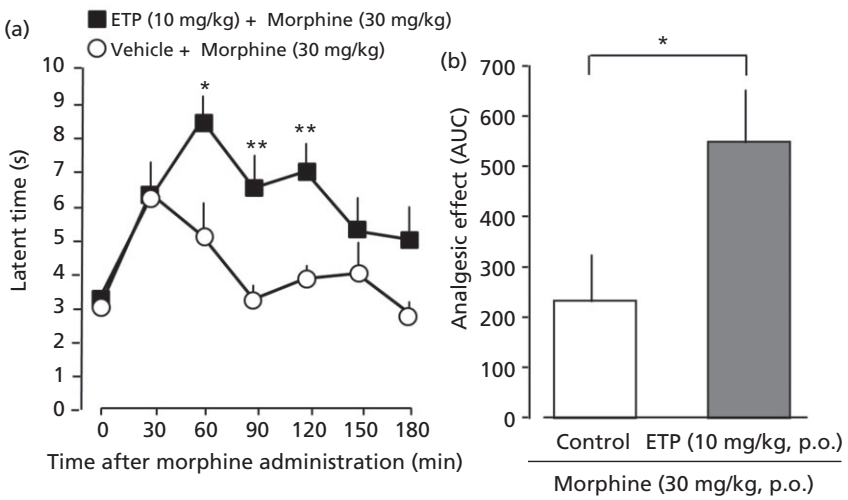

Figure 2 Effect of a single oral administration of etoposide (ETP) on the analgesic effect of oral morphine. The analgesic effect of morphine (30 mg/kg, p.o.) was evaluated by the tail-flick test. The control group was treated with water (0.1 ml/10 g). (a) Time course of the analgesic effect of morphine. (b) Area under the curve calculated from Figure 2a. $*P < 0.05$, significantly different compared with the control group (Student's *t*-test); $n = 10$, control; $n = 10$, ETP.

nificant differences were observed at 60–120 min following morphine administration when compared with the vehicle treated control group (Figure 2a). The AUC of the analgesic effect of morphine clearly indicated that orally produced morphine analgesia was significantly enhanced following co-administration of oral ETP (Figure 2b).

Effect of repeated oral ETP on the analgesic effect of oral morphine

At 7 days after repeated oral ETP treatment, the time-course analysis revealed that the analgesic effect of oral morphine (30 and 50 mg/kg) was lower than that in the vehicle treated control group, whereas there were no changes in the nociceptive response in the absence of morphine between the two groups (Figure 3a and b). Furthermore, the AUC analysis of the analgesic effect of oral morphine was significantly decreased in the ETP treated group compared with the control group (Figure 3c).

Effect of repeated oral ETP on the serum concentration and brain content of morphine

At 7 days after repeated oral ETP treatment, both the serum concentration and brain content of oral morphine (30 and 50 mg/kg) was lower than that in the vehicle treated control group (Figure 4a and b). The significant decrease was observed at a morphine dose of 50 mg/kg. On the other hand, the brain-to-blood ratio was not different between the ETP treated group and the control group (Figure 4c).

Effect of P-gp inhibitor on the repeated oral ETP-induced attenuation of the analgesic effect of oral morphine

We confirmed that the analgesic effect of oral morphine (30 mg/kg) was dose-dependently and significantly enhanced by CsA (100 and 300 mg/kg) co-administration (Figure 5a). Furthermore, the attenuated analgesic effect of oral morphine by repeated oral ETP treatment was significantly suppressed by CsA co-administration (Figure 5b).

Effect of repeated oral ETP on the analgesic effect of subcutaneous morphine

There was no difference between the control group and the repeated oral ETP treated group when the dose-dependent subcutaneous morphine analgesia was analysed. Thus, the analgesic effect of subcutaneous morphine was not altered by repeated oral ETP administration (Figure 6).

Changes in the analgesic effect of oral morphine and intestinal P-gp expression after ceasing repeated oral ETP treatment

The analgesic effect of oral morphine was significantly decreased on Day 1 following repeated oral ETP treatment. This effect was still observed on Day 3 after the cessation of repeated oral ETP treatment. However, 7 days after stopping the oral ETP treatment, the analgesic effect of oral morphine was comparable with baseline (control) levels. Furthermore, there were significant differences between Day 3 and Day 7

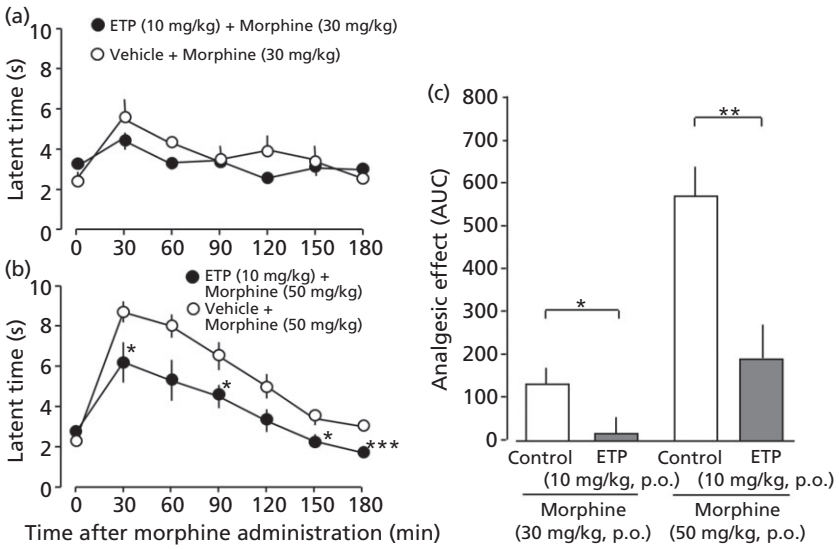

Figure 3 Effect of repeated oral administration of etoposide (ETP) on the analgesic effect of oral morphine. After mice were treated with ETP (10 mg/kg, p.o.) once a day for 7 days, the analgesic effect of morphine (30 and 50 mg/kg, p.o.) was evaluated by the tail-flick test. (a, b) Time courses of the analgesic effect of morphine. (c) Area under the curve calculated from Figure 3a, 3b. The control group was treated with water (0.1 ml/10 g). $*P < 0.05$, $**P \leq 0.01$, significantly different compared with the control group (Student's *t*-test); control: $n = 9$, 30 mg/kg morphine; $n = 8$, 50 mg/kg morphine; ETP: $n = 7$, 30 mg/kg morphine; $n = 8$, 50 mg/kg morphine.

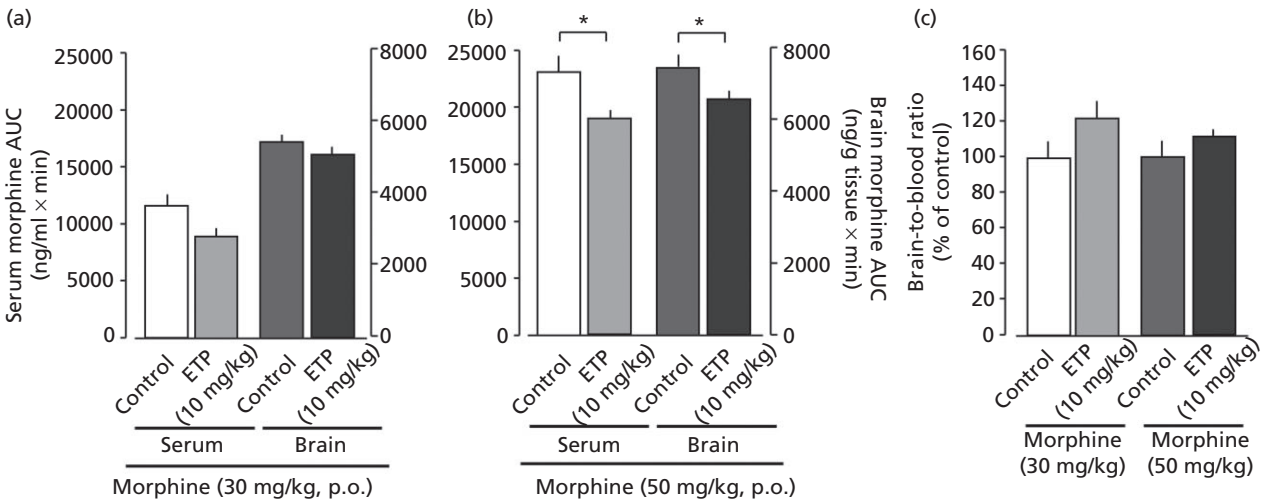

Figure 4 Effect of repeated oral administration of etoposide (ETP) on the serum concentration and brain content of morphine. After mice were treated with ETP (10 mg/kg, p.o.) once a day for 7 days, the serum concentration and brain content of morphine was evaluated by HPLC-ECD at 0, 15, 30 and 60 min after administration of morphine (30 and 50 mg/kg, p.o.). (a, b) Area under the curve of serum concentration and brain content of morphine. (c) Brain-to-blood ratio calculated from Figure 4a, 4b. The control group was treated with water (0.1 ml/10 g). $*P \leq 0.05$, significantly different compared with the control group (Student's *t*-test); control: $n = 5$, 30 mg/kg morphine; $n = 8$, 50 mg/kg morphine; ETP: $n = 5$, 30 mg/kg morphine; $n = 9$, 50 mg/kg morphine.

after the cessation of repeated ETP (Figure 7a). On the other hand, ileal P-gp expression was significantly increased on Day 1 after repeated ETP treatment, but had disappeared by Day 7 after the cessation of ETP treatment (Figure 7b).

Discussion

Most important pharmacokinetic drug interactions are reported to occur at the level of drug metabolism or protein

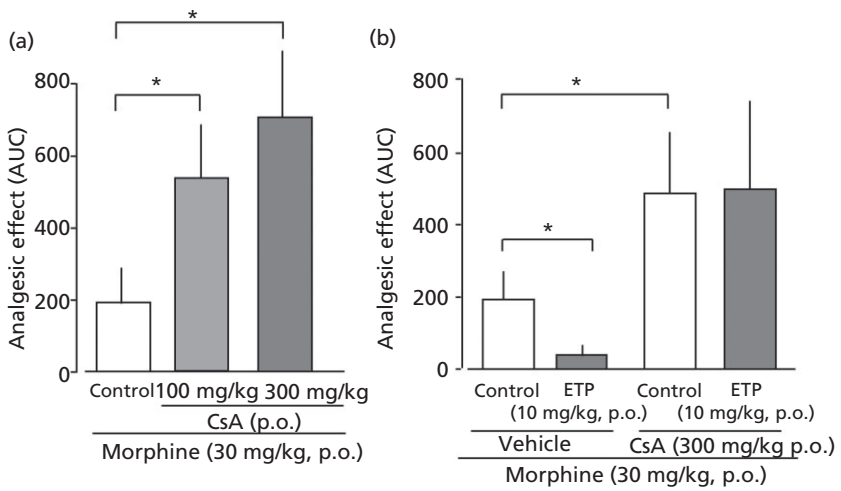

Figure 5 Effect of oral administration of cyclosporine A (CsA) on repeated etoposide (ETP) induced increase in the analgesic effect of oral morphine. (a) Dose-dependent effect of oral CsA on the analgesic effect of morphine. The analgesic effect of morphine (30 mg/kg, p.o.) was evaluated by the tail-flick test. CsA was orally co-administered with morphine. The control group was treated with ethanol/Cremophor EL (0.1 ml/10 g). *$P < 0.05$ significantly different compared with the control group (Dunnett's test); control: $n = 4$; CsA 100 mg/kg: $n = 5$; CsA 300 mg/kg: $n = 4$. (b) After mice were treated with ETP (10 mg/kg, p.o.) once a day for 7 days, the analgesic effect of morphine (30 mg/kg, p.o.) was evaluated by the tail-flick test. CsA was orally co-administered with morphine. Water and ethanol/Cremophor EL were administered instead of morphine and CsA, respectively, in the control group. *$P < 0.05$, significantly different compared with the control group (Scheffe's test); vehicle: $n = 5$, control; $n = 7$, ETP; CsA: $n = 3$, control; $n = 4$, ETP.

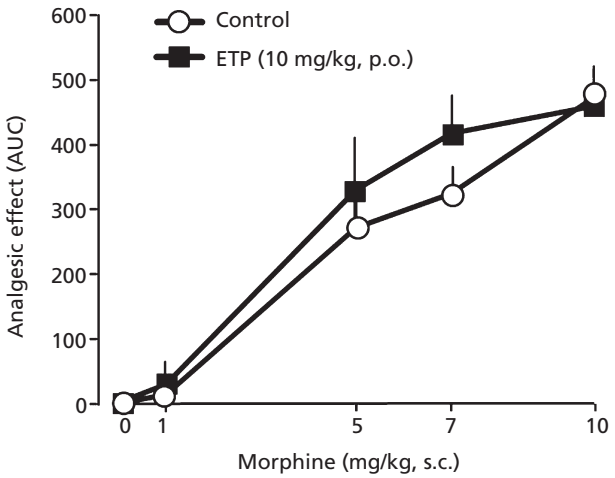

Figure 6 Effect of repeated oral administration of etoposide (ETP) on the analgesic effect of subcutaneous morphine. After mice were treated with ETP (10 mg/kg, p.o.) once a day for 7 days, the analgesic effect of morphine (10 mg/kg, s.c.) was evaluated by the tail-flick test. The control group was treated with saline (0.1 ml/10 g) instead of ETP. Morphine 1 mg/kg: $n = 4$, control; $n = 4$, ETP; morphine 5 mg/kg: $n = 5$, control; $n = 6$, ETP; morphine 7 mg/kg: $n = 4$, control; $n = 5$, ETP; morphine 10 mg/kg: $n = 4$, control; $n = 4$, ETP.

binding,[17,18] however, the drug interactions occurring at the level of transmembrane drug transport are also important.[3] As we have previously reported, the analgesic effect of morphine is regulated by P-gp, a drug-efflux transporter, found at the blood–brain barrier.[4,5] Since P-gp is expressed throughout the body, the contribution of P-gp in tissues other than the blood–brain barrier, such as the liver, kidney and intestine, may be important in the regulation of morphine-based analgesia. In this study, we focused on the role of intestinal P-gp, based on the idea that P-gp acts as the first barrier for the absorption of oral drugs in intestine. Despite the current WHO guidelines recommending that opioids should be administered orally in a palliative care setting,[1] few studies have focused on the interaction between intestinal P-gp and opioids. In this study, we found that orally administered CsA, an inhibitor of P-gp, significantly increased the analgesic effect of morphine, supporting the possibility that the analgesic effect of orally administered morphine may be regulated by intestinal P-gp.

Since some P-gp substrates could potentially act as competitive substrate inhibitors of P-gp,[19] it could be hypothesized that anticancer drugs may have the ability to modulate the analgesic effect of morphine. Interactions between anticancer drugs and opioids have been reported *in vitro* and *in vivo*.[20] In particular, pretreatment for 8 days with oxycodone, a P-gp opioid substrate, increased P-gp expression in association with a decrease of tissue distribution of paclitaxel, an anticancer drug that also acts on P-gp, [20]. However, the pharmacological interaction between these drugs has not been examined. Herein, we focused on the effect of the pharmacological interaction with respect to morphine analgesia.

In this study we analysed the drug–drug interaction between ETP and morphine, specifically focusing on the role

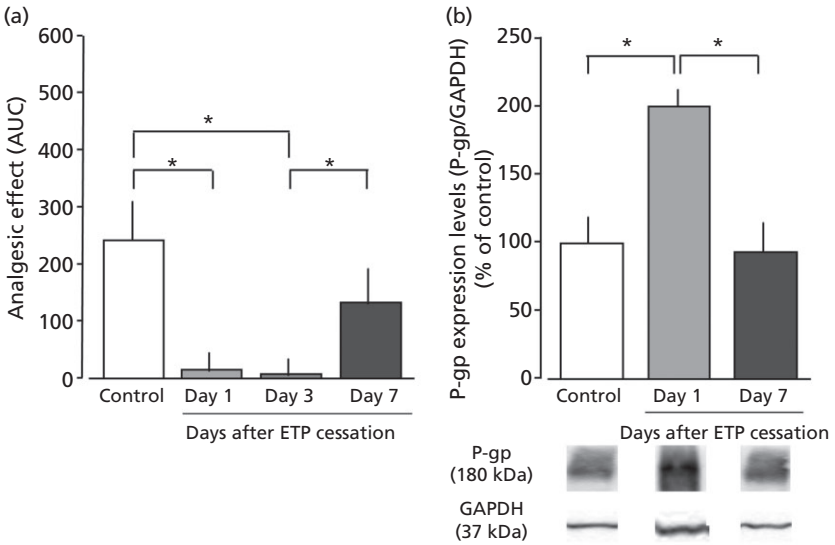

Figure 7 Changes in the analgesic effect of oral morphine and intestinal P-glycoprotein (P-gp) expression levels after repeated etoposide (ETP) pretreatment and the effect of cessation of ETP administration. At the indicated periods after cessation of repeated ETP (10 mg/kg, p.o.), the analgesic effect of morphine (30 mg/kg, p.o.) (a) and ileal P-gp expression levels (b) were evaluated by the tail-flick test and Western blot analysis, respectively. The control group was treated with water (0.1 ml/10 g) instead of ETP. $*P < 0.05$, significantly different compared with the control group (Scheffe's test); control: $n = 4$, Day 1; $n = 14$, Day 3; $n = 5$, Day 7.

of P-gp. We clearly showed that oral ETP, a compound frequently used for the treatment of small cell lung carcinomas and other high-grade neuroendocrine tumours,[21] modulates the analgesic effect of oral morphine. In this study we found that ETP works differently depending on whether it was used in conjunction with, or prior to, morphine administration. Specifically, morphine-related analgesia was significantly increased when morphine was concomitant with orally administered ETP. In contrast, the analgesic effect was decreased when mice were pretreated with ETP for 7 days before morphine administration. There may not be a sole effect of ETP on the baseline nociceptive response against thermal stimulation used in this study. Since the results of the in-situ closed loop experiments clearly showed that co-administration of morphine and ETP with Rho123, a typical P-gp substrate, significantly increased the absorption of Rho123, suggesting an inhibition of P-gp mediated drug efflux, we hypothesized that the enhancement of morphine analgesia by ETP co-administration may be due to the competitive inhibition of morphine efflux activity of P-gp by ETP in intestine.

The negative effect of repeated oral pretreatment with ETP on oral morphine analgesia and serum concentration and brain content of morphine may be due to an up-regulation of P-gp leading to enhanced morphine-efflux by P-gp, possibly by inhibiting morphine absorption. Although it has already been established that longterm exposure to P-gp substrates induces up-regulation of P-gp via the transcriptional activation of pregnane X receptors,[22] the precise mechanism of up-regulation of P-gp by ETP in this study is still unknown. Importantly, repeated oral ETP did not have an impact on subcutaneous morphine analgesia, suggesting that repeated oral ETP may influence the intestinal absorption of morphine. In the clinic, it is usual to treat cancer by intermittent repeated courses of anticancer chemotherapy (e.g. 1–3 weeks of therapy followed by cessation of treatment).[23,24] The results of this study have important implications on the use of morphine in patients for palliative care, especially following repeated longterm treatment with compounds that also act on P-gp.

Another important finding of this study was that the effect of repeated oral ETP pretreatment on the analgesic effect of oral morphine and intestinal P-gp expression completely disappeared when ETP treatment ceased. Given that changes in the analgesic effect of oral morphine 7 days after repeated oral ETP treatment, and 7 days after cessation of ETP, appear to be coupled to intestinal P-gp expression, it is possible that changes in intestinal absorptive processes may underscore the effects on morphine analgesia. It may also be important to consider the effect of repeated oral ETP on factors other than P-gp, such as UDP-glucuronosyltransferase or cytochrome P-450, which are hepatic metabolic enzymes known to act on morphine. Alternatively, changes in the sensitivity of the μ-opioid receptors may also contribute to the regulation of morphine pharmacodynamics and/or pharmacokinetics.[3] However, the finding that there was no difference in the

analgesic effect of subcutaneously delivered morphine supports our theory that changes in intestinal P-gp underlie the ETP-induced attenuation of oral morphine analgesia.

Recent evidence has shown that introducing early palliative care leads to significant improvements in the quality of life and survival outcome of cancer patients.[25] When cancer treatment is coupled with palliative care, it is important to consider the pharmacological interactions between chemotherapeutic drugs and the opioids used for pain relief. The results of the present study suggest that timing of drug delivery may be important to avoid potential pharmacokinetic interactions between the anticancer drugs and opioids. In the future, it may be of interest to investigate whether there are any interactions between drugs used for chemotherapy and palliative care other than those tested in this study.

Conclusions

We clearly demonstrated that the analgesic effect of orally administered morphine was significantly enhanced by oral ETP co-administration. In contrast, repeated pretreatment with oral ETP significantly attenuated the intestinal absorption and analgesic effect of orally administered morphine. Interestingly, ETP had no influence on the analgesic effect of subcutaneous morphine. Furthermore, changes in the analgesic effect of oral morphine after repeated oral ETP administration were coupled to changes in intestinal P-gp expression, suggesting that intestinal P-gp is an important determinant underlying the interaction between ETP and morphine. Thus, the findings of this study show that the route and timing of chemotherapeutic drug and opioid analgesic administration are important clinical considerations from a drug–drug interaction perspective.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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