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Research paper

Fluorescein transport properties across artificial lipid membranes, Caco-2 cell monolayers and rat jejunum

Katja Berginc *, Simon Žakelj, Lea Levstik, Darko Uršič, Albin Kristl

Faculty of Pharmacy, University of Ljubljana, Ljubljana, Slovenia

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Abstract

Membrane transport characteristics of a paracellular permeability marker fluorescein were evaluated using artificial membrane, Caco-2 cell monolayers and rat jejunum, all mounted in side-by-side diffusion cells. Modified Ringer buffers with varied pH values were applied as incubation salines on both sides of artificial membrane, cell culture monolayers or rat jejunum. Passive transport according to pH partition theory was determined using all three permeability models. In addition to that, active transport of fluorescein in the M–S (mucosal-to-serosal) direction through rat jejunum was observed. The highest M–S P_{app} values regarding the active transport through the rat jejunum were observed in incubation saline with pH 6.5. Fluorescein transport through the rat jejunum was inhibited by DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid) and α-CHC (α-cyano-4-hydroxycinnamic acid). Thus, we assume that two pH-dependent influx transporters could be involved in the fluorescein membrane transport through the intestinal (jejunal) epithelium.

One is very likely an MCT (monocarboxylic acid cotransporter) isoform, inhibited by specific MCT inhibitor α-CHC, while the involvement of the second one with overlapping substrate/inhibitor specificities (most probably a member of the organic anion-transporting polypeptide family, inhibited at least partially by DIDS) could not be excluded.

Keywords: Fluorescein; Fluorescein uptake transporter; MCT (monocarboxylic acid cotransporter); DIDS; α-CHC; Mucolytic agents; pH partition theory

1. Introduction

Fluorescein (FLU), a fluorescent dye, is widely used as a paracellular marker for monitoring the tight junction integrity during *in vitro* experiments performed on various permeability models, i.e. animal tissue and Caco-2 cell monolayers. However, recent scientific data have revealed that beside simple transcellular and paracellular passive diffusion according to pH partition theory there are also other mechanisms of permeation involved in the FLU membrane transport. FLU has been shown to be a substrate for efflux MRP-2 transporters [1] and more importantly, a substrate for an apically located absorptive transport protein [2–4], named ''intestinal fluorescein transporter'' [3]. Itagaki et al. [3], Konishi et al. [4] and Kuwayama et al. [2] studied the nature and characteristics of the fluorescein transporter on Transwell® grown Caco-2 cells. The authors of all three papers agree that fluorescein transporter is a pH-dependent protein, insensitive to DIDS or α-CHC inhibition but sensitive to competitive inhibition by MCT substrates (salicylic and benzoic acids but not L-lactate). The influence of high concentration (10–20 mM) of MCT substrates (salicylic, benzoic and lactic acids), various inhibitors (DIDS, α-CHC) and acidic incubation salines (pH 5.5) on FLU transport was investigated without monitoring the cell monolayer viability in the above-mentioned studies. Furthermore, the FLU membrane transport was monitored only for 15 min after a 10-min long preincubation, while our experience with the permeability studies on Caco-2 monolayers shows that the lag

* Corresponding author. Faculty of Pharmacy, Aškerčeva 7, 1000 Ljubljana, Slovenia. Tel.: +386 1 476 95 00; fax: +386 1 425 80 31.
E-mail address: katja.sturm@ffa.uni-lj.si (K. Berginc).

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time (calculated as the intercept on the *X*-axis in the plot of the permeated amount of fluorescein versus time) is between 5 and 15 min in spite of a 20-min long preincubation period. Thus the equilibrium between the donor solution and the Caco-2 cell monolayers might not have been achieved in the experiments of Kuwayama et al. [2]. It must also be stressed that the fluorescein cell accumulation and not permeability was measured in the study by Itagaki et al. [3].

Regarding all this, we have decided to reevaluate the above-mentioned observations. Therefore in the present work FLU transport properties were characterized not only through the Caco-2 cell monolayers, but also through artificial lipid membranes and rat jejunum *in vitro*. Additional information was gained by comparing the influence of incubation salines with various pH values on both sides of the artificial lipid membranes and on both sides (apical/mucosal and basolateral/serosal) of Caco-2 cell monolayers and rat jejunum, all mounted in side-by-side diffusion chambers. In some experiments mucolytic agents, cystein and DTT (dithiothreitol), were applied on the mucosal side of the rat jejunum *in vitro* to destruct mucus and therefore abolish microclimate pH influence on the "intestinal fluorescein transporter" activity. The influence of two inhibitors, DIDS (inhibitor of anion transport) previously shown to inhibit active uptake of both, MCT and OATP-B (organic anion-transporting polypeptide-B) substrates [5–7], and α-CHC (specific MCT 1–4 inhibitor) [11] was also evaluated. During the experiments with the intestinal tissue *in vitro* and cell monolayers the epithelium viability and integrity were controlled by measuring the electrophysiological parameters. Only tissue segments and cell monolayers, which were not deteriorated by the presence of inhibitors or extreme pH values, were used for the final evaluation of the results.

2. Materials and methods

2.1. Materials

Fluorescein sodium, DIDS, α-CHC, cystein hydrochloride anhydrous and DTT were from Sigma–Aldrich Chemie (Deisenhofen, Germany). All these and other chemicals (i.e. incubation saline salts) used in this study were of analytical grade. All experiments were performed in side-by-side diffusion chambers using modified Ringer solutions with various pH values (5.6, 6.0, 6.5, 7.0, 7.5 and 8.0) on apical/mucosal and basolateral/serosal sides as the incubation media. The salts were also purchased from Sigma Chemie (Deisenhofen, Germany).

2.2. Transport studies across the artificial membrane

Permeability across the artificial lipid membrane was studied in Sweetana–Grass type diffusion chambers custom made at the University of Utrecht; The Netherlands (the volume of donor and acceptor solution is 2.5 mL, exposed surface area is 1.22 cm^2) as previously described [8]. Briefly, a cellulose nitrate membrane filter with 0.1 μm pore size (Sartorius, Göttingen, Germany) previously impregnated with 1-octanol for 1 day was used as the artificial lipid membrane. After removing the excess of 1-octanol by pressing the membrane between two pieces of blotting paper, the membrane was cut into six pieces and mounted in the diffusion chambers where it was exposed to the appropriate Ringer buffer, for 90 min to be saturated with the aqueous phase. During the experiments, samples of 250 μL were removed from the acceptor compartment at 10-min intervals up to 60 min and replaced with the equal volume of the appropriate Ringer buffer. FLU was detected by fluorescence detector Tecan GENious ($\lambda_{ex} = 485$ nm, $\lambda_{em} = 535$ nm).

2.3. In vitro transport studies across Caco-2 cells

Caco-2 cells obtained from American Tissue Culture Collection (ATCC) HTB.37, lot 2463681 were used in the experiments. Cell monolayers were grown on Snapwell Costar culture inserts with a polycarbonate membrane (diameter of 12 mm and pore size of 0.4 μm). 100,000 cells/filter membrane were used for seeding. Cells were grown 21 days and the medium was changed every 2 days. At day 15, the measured TEER (transepithelial electrical resistance) was in the range of 450–650 Ω cm^2 for Caco-2 cells grown on filter membranes, used for the subsequent testing of permeability.

The inserts with Caco-2 cells were carefully rinsed with Ringer buffer (pH 7.4) and then placed between two compartments of EasyMount side-by-side diffusion chambers (Physiologic Instruments, San Diego, USA). The bathing solution was maintained at 37 °C and continuously oxygenated and circulated by bubbling carbogen. The 2.5 mL of incubation salines on the apical side (if studying the apical-to-basolateral (AP–BL) transport) or serosal side (if studying the basolateral-to-apical (BL–AP) transport) contained 10 μM of FLU and 10 mM glucose (BL–AP) or 10 mM mannitol (AP–BL) in Ringer buffer. After 25 min of preincubation the samples of 250 μL were withdrawn from the acceptor side every 20 min up to 120 min and analyzed for FLU as previously described. The samples were replaced by fresh Ringer buffer containing all necessary ingredients at appropriate concentrations.

Salicylic and benzoic acids were analyzed by HPLC system (Series 1100, Hewlett-Packard, Waldbrom, Germany). A Gemini 3u C6-Phenyl 110 column (5 μm, 150 × 4.6 mm, Phenomenex) was used at 35 °C. The mobile phase was composed of phosphate buffer (pH 3.0) and acetonitrile (65:35). For detection UV diode array detector was used at 297 and 230 nm wavelength. FLU was detected by fluorescence detector Tecan GENious ($\lambda_{ex} = 485$ nm, $\lambda_{em} = 535$ nm).

2.4. In vitro transport studies across rat jejunum

The experiments conform to the Law for the protection of animals (Republic of Slovenia) and are registered at the

Veterinary Administration of the Republic of Slovenia. They were performed in the manner as described previously [1]. Rat jejunum, located 25–60 cm distally from the pyloric sphincter, was obtained from male Wistar rats (250–320 g), fasted 18 h prior to the experiments, after euthanasia and laparotomy and rinsed with ice-cold 10 mM glucose Ringer solution. Tissue was cut into 3 cm long segments, excluding visible Peyer's patches. The intestinal segments were opened along the mesenteric border, stretched onto special inserts with exposed tissue area of 1 cm^2 and then placed between two compartments of Easy-Mount side-by-side diffusion chambers (Physiologic Instruments, San Diego, USA). The experimental procedure continued as it is already described in Section 2.3 for Caco-2 cell monolayers.

2.5. Electrical measurements

The diffusion chambers were equipped with two pairs of Ag/AgCl electrodes for measuring transepithelial potential difference (PD) and short circuit current (I_{sc}) with multichannel voltage–current clamp (model VCC MC6, Physiologic Instruments). The tissue viability and integrity were checked by monitoring PD, I_{sc} and TEER every 20 min. In viable rat tissues and Caco-2 cells, PD, I_{sc} and TEER did not change significantly over 120 min. The average TEER from 20 to 120 min was calculated and also used for assessing tissue/cell integrity and viability. The average PD, I_{sc} and TEER values for Caco-2 cells were -1.5 ± 0.3 mV, 4.3 ± 0.8 μA and $396 \pm 8\ \Omega\,cm^2$, respectively. The viability of rat jejunum was additionally checked by recording the increase of I_{sc} and PD after the addition of stock glucose solution to the mucosal compartment at the end of experiment (final glucose concentration was 25 mM). Tissue segments were considered viable if the absolute value of PD after the addition of glucose was lower than −1.0 mV and if the average of TEER values recorded during the experiment was between 20 and 40 $\Omega\,cm^2$. The Caco-2 cell culture monolayers were considered viable if the average TEER values between 20 and 120 min were in the range of 450 and 750 $\Omega\,cm^2$ and if they exhibited a PD greater than −0.5 mV during the experiment.

2.6. Data analysis and statistics

The apparent permeability coefficient (P_{app}) was calculated as follows

$$P_{app} = \frac{dc}{dt}\frac{V}{c_0 A}, \quad (1)$$

where dc/dt is the change in concentration per unit time in the acceptor compartment under steady state conditions, V is the volume of acceptor compartment (2.5 mL), A is the exposed surface area (1 cm^2) and c_0 is the initial concentration of the substance in donor solution.

Results are presented as means ± SD of at least three measurements. Data were statistically evaluated using F-test for testing the equality of variances. Afterwards the appropriate 2-tailed Student's t-test ($p < 0.05$ for significant (S) differences and $p > 0.05$ for not significant (NS) differences) was used to test the equality of means. Paired Student's t-test was used where appropriate.

3. Results and discussion

Apparent permeability coefficients (P_{app}) of FLU through the rat jejunum/Caco-2 cell monolayers were determined in M–S/AP–BL (mucosal-to-serosal/apical-to-basolateral) and S–M/BL–AP (serosal-to-mucosal/basolateral-to-apical) directions. Artificial lipid membranes are not polarized. Thus the P_{app} values of FLU through this model were evaluated only in one direction. Ringer incubation solutions with different pH values were used on mucosal/serosal, apical/basolateral or "donor"/"acceptor" sides for rat jejunum, Caco-2 cell monolayers or artificial lipid membranes, respectively.

3.1. Transport of fluorescein through the artificial lipid membrane

FLU possesses two ionization groups (carboxyl moiety with pK_{a1} 4.36 and phenol moiety with pK_{a2} 6.38) [2]. Permeability of FLU decreased if the pH of donor solution increased from 5.6 ($P_{app} = 2.80 \pm 0.48 \times 10^{-4}$ cm/s) to 6.5 ($P_{app} = 1.01 \pm 0.50 \times 10^{-4}$ cm/s) and to 7.5 ($P_{app} = 3.99 \pm 0.49 \times 10^{-6}$ cm/s), while the pH on the acceptor side was kept at 6.5. On the other hand, if the donor solution was kept at constant pH 6.5, P_{app} values obtained at pH 5.6 and 7.5 of the acceptor solution were $3.87 \pm 1.21 \times 10^{-5}$ cm/s and $1.61 \pm 0.25 \times 10^{-4}$, respectively. These results demonstrate that the permeation of FLU through the lipid membrane is influenced by the FLU ionization in the donor and in the acceptor solutions, indicating that it permeates through the artificial membrane according to pH partition theory.

3.2. Transport of fluorescein through the Caco-2 cell monolayers

According to the results, presented in Table 1, AP–BL and BL–AP P_{app} values of FLU decreased with the increasing pH of the donor solution. FLU is ionized to a much smaller extent in more acidic solutions. Thus, less ionized form of a compound can, according to the pH partition theory [9] and similar to our results obtained with artificial lipid membranes, permeate through the Caco-2 monolayer more readily. Our results (Table 1), indicating that permeability of FLU depends on the pH of incubation media, are in accordance with the findings of Itagaki et al. [3], Konishi et al. [4] and Kuwayama et al. [2]. However, these authors believe that the higher AP–BL P_{app} values observed in more acidic conditions are due to the increased activity of the "intestinal fluorescein transporter". We thus performed further experiments to establish whether the

Table 1
AP–BL and BL–AP P_{app} values of FLU through Caco-2 cell monolayers, incubated in modified Ringer buffers with different pH values on both sides of the cell monolayers

Incubation pH		P_{app} ($\times 10^{-6}$ cm/s)		R	S/NS
AP	BL	M–S	S–M		
6.5	6.5	5.38 ± 0.34	5.67 ± 0.14	0.95	NS
7.4	7.4	0.55 ± 0.23	0.63 ± 0.11	0.87	NS
6.5	7.4	5.30 ± 0.09	0.33 ± 0.06	16.06	S
7.0	7.0	0.94 ± 0.06	1.28 ± 0.07	0.73	S
7.4	6.5	0.26 ± 0.15	5.23 ± 0.43	0.05	S
5.6	6.5	21.2 ± 3.12	–	–	–
6.5	5.6	3.33 ± 0.07	–	–	–

In the last two experiments only AP–BL FLU permeability was monitored, therefore no data for BL–AP P_{app} values are presented (–). Data are presented as means ± SD of three or more cell monolayers. The ratio (R) between average AP–BL and average BL–AP P_{app} values for each experiment and the statistical comparison (S/NS) between AP–BL and BL–AP P_{app} values are also included.

Table 2
M–S and S–M P_{app} values of FLU through the rat jejunum at different pH of incubation buffer on mucosal (M) and serosal (S) side.

pH		P_{app} FLU ($\times 10^{-6}$ cm/s)		R	S/NS
M	S	M–S	S–M		
6.0	6.0	7.44 ± 4.54	2.54 ± 0.51	2.93	S
6.5	6.5	17.8 ± 2.43	3.68 ± 0.25	4.84	S
7.0	7.0	6.9 ± 2.35	4.02 ± 0.53	1.72	NS
7.4	7.4	3.21 ± 0.89	4.09 ± 0.80	0.78	NS
8.0	8.0	3.28 ± 0.74	2.54 ± 0.40	1.29	NS
5.6	7.4	16.4 ± 0.5	4.17 ± 1.37	3.93	NS
6.5	7.4	8.59 ± 1.31	5.64 ± 0.49	1.52	S
7.4	6.5	3.02 ± 0.65	5.73 ± 1.39	0.53	NS
5.6	6.5	11.4 ± 2.54	2.1 ± 0.34	5.43	S
7.4	5.6	2.33 ± 0.33	3.98 ± 1.50	0.59	NS
6.5	5.6	9.96 ± 3.68	2.94 ± 0.74	3.39	S

The ratio (R) between average M–S and average S–M P_{app} values and the statistical comparison (S/NS) between M–S and S–M P_{app} values are also included in the table.

pH-dependent FLU transport observed on Caco-2 cell monolayers is a consequence of the "intestinal fluorescein transporter" activity.

AP–BL and BL–AP P_{app} values of MCT substrates, salicylic and benzoic acids, were determined. When the incubation salines of the same pH (7.5) were used on both sides of the cell monolayers, no asymmetrical permeability characteristics could be observed (the measured P_{app} values for salicylic and benzoic acid were $1.16 \pm 0.22 \times 10^{-5}$ and $2.51 \pm 0.26 \times 10^{-5}$ cm/s in the AP–BL direction, respectively, while they were $1.12 \pm 0.05 \times 10^{-5}$ and $2.49 \pm 0.49 \times 10^{-5}$ cm/s in the BL–AP direction, respectively). Lowering the apical pH value to 6.5 (with the pH 7.5 of BL solution) caused only the AP–BL P_{app} values of both acids to increase ($7.33 \pm 0.22 \times 10^{-5}$ cm/s for salicylic and $2.07 \pm 0.04 \times 10^{-4}$ cm/s for benzoic acid) but the P_{app} values in BL–AP direction remained the same. There was no competitive inhibition between salicylic and benzoic acid under the tested pH gradient conditions. These results indicate that there is most probably none or very low expression of MCTs in the studied Caco-2 cell monolayers.

Furthermore no influence of salicylic or benzoic acid could be detected regarding the FLU permeability when it was applied to the donor solution on the apical side under the same pH gradient (pH 6.5/pH 7.5) together with benzoic acid or both, salicylic and benzoic acids, in 1 or 5 mM concentrations. These data strongly suggest that the "intestinal fluorescein transporter" is not present in the Caco-2 cells used in this study.

3.3. Transport of fluorescein through the rat jejunum in vitro

We also examined the influence of incubation saline pH on FLU permeability through the rat jejunum *in vitro* (Table 2). Fig. 1 shows the M–S and S–M P_{app} values of FLU, measured when the pH value of both incubation salines (mucosal and serosal) was the same. The S–M P_{app} values (–) did not change significantly with increasing pH of the incubation saline and are all in the range of $3-6 \times 10^{-6}$ cm/s. On the other hand, the M–S P_{app} values (♦) strongly depend on the pH value of the incubation saline. Moreover, the highest M–S FLU permeability was observed at pH 6.5 and not at the lowest pH of incubation medium tested (6.0). At pH 6.5 only 43% of FLU is in its monoanion form and the percentage increases with lowering pH. These results are also not in accordance with our previously obtained permeability results through artificial membrane and Caco-2 cell monolayers and cannot be explained by simple passive diffusion according to the pH partition theory [9]. Active uptake of FLU through the rat jejunal epithelium is obviously triggered by the slightly acidic conditions (pH 6.5) in the incubation saline. The results in Table 2 characterize the pH dependency of the "intestinal fluorescein transporters" in more details. Incubation salines with different pH values on each side of the tissue were used to evaluate the effect of the transtissue pH gradient on the FLU transport. Determined P_{app} values, presented in Table 2, clearly show that the FLU permeability in the M–S direction was the highest in the case when the pH on both sides of the intestinal tissue was 6.5. It was therefore concluded that the "intestinal fluores-

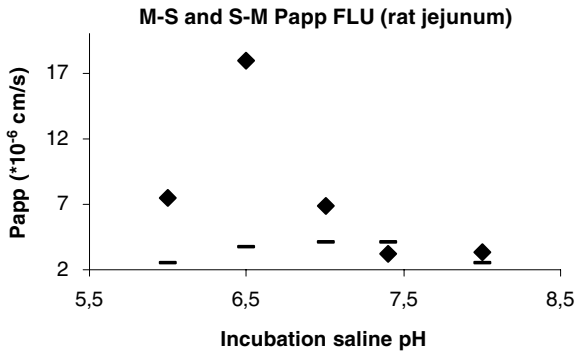

Fig. 1. The M–S (♦) and S–M (–) P_{app} values of FLU through the rat jejunum, when the pH value of Ringer incubation saline is the same on both sides.

Table 3
The average M–S and S–M P_{app} values and their standard deviations for FLU, determined in the presence of mucolytic substances (Cys and DTT) or two inhibitors (α-CHC and DIDS) on mucosal side at pH 6.5 of salines on both sides of the tissue

	P_{app} ($\times 10^{-6}$ cm/s)		*t*-test
	M–S	S–M	S/NS
Control [a]	17.8 ± 4.5	3.7 ± 0.3	–
1 mM Cys	14.8 ± 1.7	3.6 ± 0.8	NS
5 mM Cys	13.7 ± 2.2	5.7 ± 0.9	NS
1 mM DTT	18.0 ± 4.3	4.1 ± 0.2	NS
1 mM α-CHC	12.0 ± 3.7	5.9 ± 2.9	S
1 mM DIDS	10.1 ± 2.6	4.6 ± 0.7	S

The comparison between M–S P_{app} values of FLU, determined in the presence of these compounds, and M–S P_{app} values of FLU alone (control) are given as insignificant (NS, $p > 0.05$) or significant (S, $p < 0.05$).
[a] The reference taken from Table 2.

cein transporter'' activity is the highest at this pH value (pH 6.5) of the incubation saline, because even the highest pH gradient used in these experiments (mucosal pH 5.6 and serosal pH 7.4) could not induce higher FLU transport than that observed at pH 6.5.

Previous studies [2–4] as well as our results (Table 1) regarding pH characteristics of FLU transport through Caco-2 cells are in contradiction with the results obtained on the rat jejunum *in vitro*. The importance of mucus for the FLU transport was thus tested. For this purpose, mucolytic agents [1 and 5 mM cystein, 1 mM DTT (dithiothreitol)] were applied to the mucosal side of the rat jejunal tissue mounted in the diffusion chambers and incubated in Ringer buffer pH 6.5 on both sides. In the presence of mucolytic agents, the microclimate pH and pH of the incubation saline are the same [8,10]. The results in Table 3 show no significant change in M–S P_{app} values of FLU obtained in the presence of mucolytic agents compared to control (M–S P_{app} values of FLU alone at pH 6.5 of incubation media on both sides of the tissue). Therefore one can conclude that the activity of the ''intestinal fluorescein transporters'' does not depend on the maintenance of the acidic microclimate at the mucosal surface. Furthermore, the lack of mucus (and acidic microclimate) in the Caco-2 model is thus not responsible for the absence of active fluorescein transport.

Additionally, the influence of two inhibitors (α-CHC, a MCT 1–4 inhibitor, and DIDS, an inhibitor of anion exchange) on FLU transport through the rat jejunum was also studied at pH 6.5. Both inhibitors were previously shown not to influence the uptake of FLU into Caco-2 cells [2–4], in our experiments however, the M–S P_{app} values of FLU significantly decreased after the addition of both inhibitors to mucosal side of the tissue, compared to M–S P_{app} values for FLU alone (Table 3). In both experiments inhibitors were added to mucosal side of the tissue 1 h after the start of the experiment. The M–S P_{app} values of FLU have decreased by 33% and by 30% after the addition of DIDS and α-CHC, respectively. The partial inhibition of FLU transport by α-CHC most probably indicates the involvement of the MCT 1–4 transporter/isoform. Additionally, the FLU active transport is also diminished in the presence of DIDS – an agent, which was previously shown to inhibit the active uptake not only of MCT substrates, but also of estrone-3-sulphate and pravastatin, both known OATP-B (organic anion-transporting polypeptide B) substrates [5–7]. Thus, OATPs could also be involved in the FLU active transport.

4. Conclusions

Fluorescein permeates through the artificial lipid membranes and Caco-2 cell monolayers by passive diffusion according to modified pH partition theory. In experiments performed with Caco-2 cell monolayers, no competitive inhibition could be observed between FLU and salicylic/benzoic acids.

On the other hand, active transport of FLU through the rat jejunum in M–S direction was observed with the most pronounced effect when the pH of incubation salines was 6.5 on both sides of the tissue. The activity of ''intestinal fluorescein transporter'' could be inhibited by DIDS and α-CHC.

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