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RESEARCH ARTICLES

Absorption of Etoposide (VP-16-213) from the Small Intestine of the Rat. The Potential Role of Mucus as an Absorption Rate Limiting Barrier

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Received: September 3, 1984; accepted: February 19, 1985.

Abstract: The absorption of etoposide (VP-16-213) was investigated in a perfused intestinal loop. The absorbed amount of drug was determined by collecting the blood draining the loop via cannulation of the efferent jejunal vein. The absorption rate of VP-16-213 strongly depended on the composition of the perfusion medium. The addition of taurocholate to an aqueous etoposide solution enhanced the absorption rate. When *N*-acetylcysteine was added to an aqueous solution, the absorption rate dropped significantly.

The absorption of etoposide in man after oral administration is erratic (1). In rats relatively high concentrations of etoposide were found in the intestine. This finding may indicate binding of etoposide to intestinal mucus. In the literature, mucus binding has been described for various drugs (e. g. 2–7). In most cases, the transport rate of the drug from the intestinal lumen to the blood was decreased if a drug-mucus interaction occurred. Only 10–20 % of benzomethamine, a monoquaternary compound, was taken up over 3–4 hours; absorption mainly occurred during the first 45 minutes. It was shown, that binding to mucus was responsible for the slow absorption rate (8, 9). Meli et al. (10) observed that ethynylestradiol cyclopentyl ether disappeared from the intestinal lumen by 85 % within 7.5 minutes. Almost the entire amount that disappeared was bound to the intestinal tissue from where it was released very slowly. The absorption of some water soluble dyes was linearly related to the binding of these substances to the intestinal mucus (11–14). The binding to intestinal mucus was essential for the absorption of these dyes, because digestion of the mucus glycoproteins, and even the glycocalyx, caused a severe decrease in the absorption of these dyes.

If the intestinal mucus is a significant barrier to the absorption of a drug, attempts can be made to promote absorption by reducing the influence of the mucus. A possible way to increase the diffusion rate through the mucus might be to decrease the apparent viscosity of the gel. Organic compounds, containing thiol groups such as dithiothreitol, 2-mercaptoethane-sulfonate, glutathione and cysteine derivatives can reduce the disulfide bridges in the core of the glycoprotein molecule, thereby, breaking up the native structure of the glycoproteins (15–17). This results in a decreased elasticity and an increased ciliary transport rate (18–20). The addition of 2 % *N*-acetylcysteine (N-ac) causes a 40 % reduction in consistency of the mucus after a contact time of 60 minutes (17). The optimal pH for the action of *N*-acetylcysteine (N-ac) lies around 7 (21, 22). *N*-acetylcysteine also enhances the binding of some antibiotics to mucus (2).

The resistance of the mucus layer in the absorption process can also be changed by bile salts. It was shown that bile salts affect mucus viscosity and elasticity (23); therefore, these compounds might influence the absorption kinetics of mucus interacting drugs. The benificial effect of bile salts on the absorption process of a number of drugs has been reported (24–26). In general, the mechanism behind the observed phenomena is not clear. An increase in the "apparent" solubility, or a change in membrane or mucus characteristics might play a role.

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In this study an attempt was made to enhance the absorption rate of the antineoplastic agent etoposide. *N*-Acetylcysteine was added to the aqueous solution as it was expected that this compound would decrease the viscosity of the mucus. A concentration of 4 % was used, since it was reported by Schiller et al. (27) that this concentration did not damage the intestinal mucosa of the rat. Taurocholate (TC) micelles were used to increase the "apparent" solubility of the drug and thereby the concentration gradient over the intestinal barrier. Besides, these micellar dispersions might affect the resistance of the absorption barrier. The concentrations of taurocholate were 10 and 40 mM. These concentrations are in the physiological range (28). The experimental technique enabled the determination of the drug immediately after absorption. The blood draining the perfused loop was collected by cannulating the efferent jejunal vein. With this technique whole body distribution and metabolism was avoided.

Materials and Methods

Animals

Male Wistar rats, 285 ± 26 g (mean ± S.D.) were used. The animals were not fasted prior to the experiments.

Absorption Experiments

The preparation and perfusion technique is described elsewhere (29). Briefly, after intraperitoneal injection of urethane (1 g/kg body weight) the abdomen was opened and a suitable segment of the jejunum was exposed. Loops of 6.7 ± 1.3 cm (mean ± S.D.) were cleared from food material and rinsed with an isotonic saline solution until the effluent was clear. The amount of etoposide absorbed was measured in the blood draining the perfused segment via cannulation of the efferent jejunal vein. The total blood volume of the animal was kept constant by an infusion of heparinized donor blood via a jugular vein. All perfusion solutions were prepared one hour prior to the experiment. A volume of 1.0 ml perfusate was oscillated at a flow rate of 0.88 ml/min. The standard perfusion solution contained (g/l): NaCl 5.2, KCl 0.35, $MgSO_4 \cdot 7\ H_2O$ 0.29, $CaCl_2 \cdot 2\ H_2O$ 0.27, $NaHCO_3$ 1.50, KH_2PO_4 0.06, mannitol 13.0. All ingredients were of pharmacopoeial grade. Etoposide concentrations were 0.1 and 2.0 mg/ml. The pH was 6.5. *N*-Acetylcysteine (OPG, Utrecht) solutions were prepared by dissolving 4 g *N*-acetylcysteine in 100 ml demineralized water; the pH was adjusted to 6.5 with a sodium hydroxide solution. Micellar solutions were prepared by dissolving sodium taurocholate (98 % pure, Sigma Chemicals, St. Louis, MO) and etoposide (Bristol Myers, New York, NY) in methanol. The solvent was evaporated with a Rotavapor RE 120 (Büchi, Flawil, Switserland) and was maintained under reduced pressure for two hours to completely remove the solvent. Then the standard perfusion solution was added to obtain the desired concentration of micelles and the film was dispersed using glass beads.

Determination of Etoposide

Plasma was obtained by centrifugation of the blood samples at 2000 rpm for 10 minutes. The plasma samples were either extracted immediately or were frozen until extraction. They were analyzed according to the method of Holthuis et al. (30) – a reversed phase HPLC analysis with electrochemical detection after extraction from plasma – with two modifications. First, the structural analogue tenoposide was used as an internal standard and second, all organic extracts were filtered through a phase separating filter (Whatman PS-1) prior to evaporation.

The mucus layer was separated from the intestinal tissue using a method recommended by Marriott (personal communication). A piece of Kleenex tissue was gently pressed on the opened loop and removed from the tissue after a few seconds. The amount of etoposide in the intestinal tissue was measured by extracting the tissue after grinding with 5 g of dried sodium sulphate. 4.0 ml Dichloroethane (DCE) was added, and the mixture was vortexed for 1 minute. After standing overnight at 4°C, the mixture was filtered through a swab of cotton wool, and the extract was filtered through a phase separating filter. Another 3.0 ml DCE was poured over the powder and filtered in the same way. The organic solvent was evaporated at room temperature under nitrogen and the residue was dissolved in 1.0 ml eluent for the HPLC determination. The Kleenex tissue was extracted twice with 3.0 ml DCE. After evaporation of the organic solvent at room temperature under nitrogen, the residue was dissolved in the HPLC eluent. The presence of taurocholate or *N*-acetylcysteine did not affect the assay.

Histological Investigations

In separate experiments, intestinal loops were perfused for 1 hour with the test solutions. After removing the loop, a thin ring was immersed in Bouin's solution (saturated picric acid solution (150) + 40 % formaldehyde solution (50) + glacial acetic acid (10). After 24 hours, the Bouin solution was replaced by 70 % alcohol. After drying with pure alcohol, the tissue was imbedded in glycolomethacrylate and 5 μm coupes were cut. The coupes were stained with P.A.S. (Periodic Acid Schiff) – Haemaluin (Mayer). After drying, they were imbedded in malinol and examined at a magnification of 40 and 100x.

Data Analysis

The flux of etoposide (ng/min·cm) was calculated by multiplication of the plasma concentration (ng/ml) and the bloodflow (ml/min·cm). The length of the intestinal segment was determined at the end of an experiment. The segment was excised and stressed with a 10 gram weight for 60 seconds. No correction was made for the decreasing concentration of etoposide in the lumen because the fluxes were rather low (see Results and Discussion). The total amount of etoposide in the intestinal tissue and in the mucus layer was expressed as μg/cm intestinal length. No correction for bloodflow variation was applied in the different experiments, because the flow was kept constant during the experiment, and the inter-experimental variation was small. For statistical analysis of the etoposide fluxes Student's t-test was used. Differences were considered significant when $P < 0.05$.

Results and Discussion

Flux of Etoposide

The penetration of drugs through the epithelium might lead to an impaired absorption because of a damaging effect on the epithelial cells (31). Figure 1 shows a typical plot of the cumulative amount of etoposide in the blood draining the perfused jejunal segment as a function of time. A standard perfusion solution with a concentration of 0.1 mg/ml etoposide

was used. The flux of etoposide from the lumen to the blood – measured at the blood site – was constant with time. Besides, macroscopically and microscopically no changes in epithelial structure could be observed after the experiment. From these results it is concluded that the integrity of the intestinal barrier was retained.

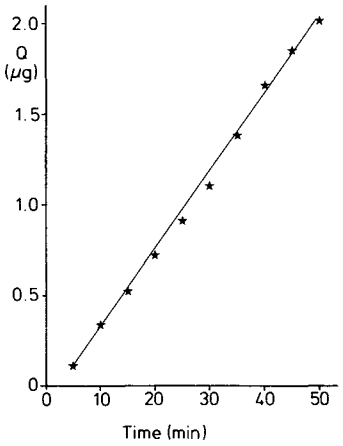

Fig. 1 Plot of the cumulative amount of etoposide in the blood (μg) vs time. The concentration is 0.1 mg/ml etoposide in standard perfusion solution.

Influence of Taurocholate Micelles on the Flux of Etoposide

Concentration of etoposide: 0.1 mg/ml. The effect of solubilization of the drug was investigated. At 37°C the solubility of etoposide was slightly higher than 0.1 mg/ml. Both with the pure standard solution and with the solution containing the micelles a drug concentration of 0.1 mg/ml was taken. The results are presented in Table I. This table shows that the flux of etoposide was enhanced in the presence of micelles by a factor of 3.3. There was no significant difference between the amounts recovered from the intestinal wall with or without TC. The same applied to the etoposide content of the mucus. Because of the high lipophilicity of etoposide, the drug is mainly contained in the micelles. This means that the free concentration of the drug in solution will be decreased. Three hypotheses might be proposed to explain the enhancement of the flux. A) An increased diffusion rate of the drug containing TC micelles through the mucus barrier results in an enhanced mass transport rate of etoposide towards the membrane compared to the free drug in the standard solution. The gel filtration concept (2) provides the mechanistic background to explain this increased diffusion rate. B) The presence of TC reduces the consistency of the mucus and therefore increases the transport rate of the free and the micellar bound fraction of the drug. C) An effect of taurocholate on the epithelial membrane. However, it was demonstrated by Muranishi et al. (26) in exsorption experiments that taurocholate in this concentration and with a comparable perfusion rate had no effect on the membrane of the large intestine. Therefore, hypothesis C is not very likely.

Concentration of etoposide: 2 mg/ml. It was possible to dissolve up to 2.5 mg of etoposide per ml in the presence of 10 mM TC. In a further series of experiments the flux of etoposide from micellar solutions (10 and 40 mM TC) containing 2 mg/ml of etoposide was measured. The results are presented in Table I. The data shown in this table indicate that for both the 10 and the 40 mM TC solutions the flux of etoposide (2 mg/ml) was enhanced by a factor of 18 compared to the standard perfusion solution containing 0.1 mg/ml etoposide. Apparently, in this concentration range no TC concentration dependent effect on the diffusion rate occurred (cf. hypotheses A and B). The enhancement of the etoposide flux in 10 mM TC solutions is not linearly related to the drug concentration (11.7 vs 61.2 ng/min·cm). This deviation from linearity cannot be ascribed to an increased free drug concentration for the 2 mg/ml etoposide dispersion. The free drug concentration does not surpass 0.1 mg/ml, and from this concentration only a flux of about 3.5 ng/min·cm can be expected. To explain this non-linearity in the absorption of etoposide in 10 mM TC solutions, two ways of reasoning can be followed. 1) It is likely that the micelles contain etoposide and TC in very widely varying ratios: a ratio of 1 molecule of etoposide to 50 molecules of TC for the 0.1 mg/ml, 10 mM TC dispersion and a ratio of 1 molecule of etoposide to 2.5 molecules of TC in the 2 mg/ml, 10 mM TC dispersion. This difference in molar ratio will have an impact on the characteristics (e. g. particle size, shape, charge) of the micelles. 2) The transport process through the intestinal membrane might be concentration dependent. Then, for dispersions containing 2 mg etoposide/ml and 10 mM TC the transport rate is limited because of saturation effects. However, for lipophilic compounds like etoposide saturable transport processes through the intestinal membrane are rather rare.

Table I. Flux and distribution of etoposide under various conditions.

	Flux[1]	Intestine[2]	Mucus[2]
0.1 mg/ml Etoposide			
Standard solution	3.5±1.7 (6)	0.5±0.4 (6)	1.2±0.8 (6)
10 mM TC	11.7±2.2 (4)	0.3±0.1 (4)	0.7±0.3 (4)
4 % N-ac	n.d. (5)	0.4±0.1 (5)	3.4±1.7 (5)
2 mg/ml Etoposide			
10 mM TC	61.2±1.4 (5)	24.2±7.7 (5)	16.6±10.3 (5)
40 mM TC	68.6±20.8 (3)	31.4 (2)	16.4 (2)

1=ng/min·cm intestinal wall, mean ± SD, number of experiments in parentheses; 2=μg/cm intestine, mean ± SD, number of experiments in parentheses; n.d. = not detectable
TC = taurocholate
N-ac = *N*-acetylcysteine, pH 6.5

The recovery of etoposide from the intestinal wall and mucus (expressed as a fraction of the initial amount of drug in the perfusion medium) was similar for the 0.1 and 2 mg/ml etoposide solutions. This points to a non specific binding of etoposide to intestinal and mucus components.

Meli et al. (10) suggested that the intestine together with the mucus layer could behave as a separate compartment. A drug diffuses rapidly into it, but is only slowly released into the blood. An experiment was carried out to test whether this situation occurred with etoposide. A solution of 10 mM TC containing 2 mg/ml etoposide was instilled in a jejunal loop. After 15 minutes (see arrow, Fig. 2) the solution was removed and the loop was rinsed with 1.0 ml standard perfusion solution without the drug. The course of the plasma level of etoposide was followed over 45 minutes. The results are shown in Fig. 2.

After 45 minutes the flux of etoposide was still 50 % of the value after 15 minutes. This means that the intestinal tissue

together with the mucus layer indeed can behave as a reservoir for the release of certain drugs. The total amount of etoposide in the intestinal tissue in these experiments was 12.8±3.6 μg/cm (mean ± SD, n = 6). The total amount in the mucus was 14.7±6.8 μg/cm (mean ± SD, n = 6).

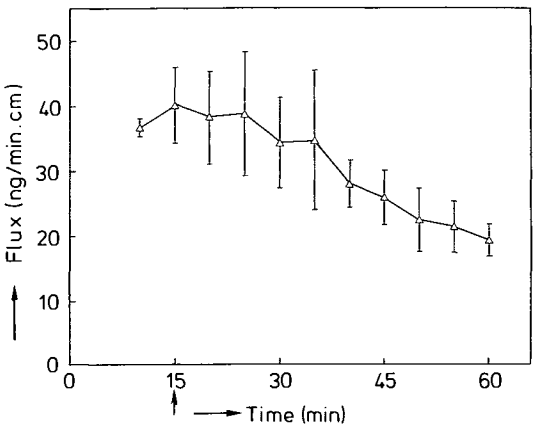

Fig. 2 Plot of the decrease of the flux of etoposide with time after instillation of a solution containing 2 mg/ml etoposide for 15 minutes. Results are expressed as mean ± SD (n=6). For details: see text.

Influence of N-acetylcysteine on the Flux of Etoposide

The results using a solution of 4 % *N*-acetylcysteine are shown in Table I. At a pH value where the activity of *N*-acetylcysteine is maximum (22) the flux of etoposide dropped significantly. The three dimensional structure of the glycoproteins might be destroyed by the action of *N*-acetylcysteine (i. e. breaking up sulfide bonds in the native glycoprotein molecule), leading to a closer packing of the subunits and resulting in a significant decrease in the flux of etoposide. Simultaneously, more binding sites would become available by breaking the native glycoprotein, and therefore, the amount of etoposide bound to the glycoprotein might increase. The results indeed indicate a tendency to recover a larger amount of etoposide from mucus after perfusion with the *N*-acetylcysteine solution than with a standard perfusion solution. This finding shows that a reduction of the consistency of intestinal mucus does not necessarily imply that the flux of a drug increases.

Histological Examination

The light microscopic examination showed no change compared to the control preparation in the appearance of the villi after perfusion with either the micellar solutions or the *N*-acetylcysteine solution.

It can be concluded, that the intestinal mucus is most probably the rate limiting barrier in the absorption process of etoposide, because: A) by using bile salt micellar systems the flux of etoposide increased. This was probably caused by a reduction of the resistance of the mucus barrier. Work is in progress to elucidate the underlying mechanism. B) Increasing the resistance of the mucus layer with *N*-acetylcysteine resulted in a significant decrease of the flux of etoposide and an increase of the amount of etoposide recovered from the mucus layer.

Acknowledgement

The authors are grateful to Mrs. I. Groothuis-Pielage for excellent technical assistance.

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