The Effect of Clodronate on the Integrity and Viability of Rat Small Intestine in Vitro—A Comparison with EDTA

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Although substances, which can increase the paracellular permeability of intestinal mucosa, could be very helpful for increasing the bioavailability of hydrophilic drugs, they are not used therapeutically due to the possibilities of acute or long-term toxicity (intestinal inflammations due to penetration of bacterial fragments into subepithelial spaces). In this paper the abilities of a calcium chelator EDTA and clodronate (a first generation bisphosphonate) to increase the paracellular permeability were assessed using rat jejunum in side-by-side diffusion chambers while the viability of the tissue was monitored by transepithelial potential difference. Although clodronate is less potent than EDTA in depleting calcium from the intestinal tissue, it significantly increased the paracellular permeability of viable rat jejunum “in vitro” when tested at 15 mM and higher concentrations (the highest therapeutic dose dissolved in 250 ml gives a 22 mM solution of clodronate). This effect was reversible under “high-calcium” conditions. Since clodronate therapy does not have any long-term consequences it was concluded that a safe, transient increase of small intestinal permeability is possible. However, the acute gastrointestinal undesired effects, which can develop during the therapy with high doses of clodronate, might also occur after oral applications of paracellular permeability enhancers. Namely, 30 mM and higher concentrations of clodronate caused a loss of the tissue viability in all rat jejunal segments tested in “in vitro” conditions. A similar effect was observed with much lower concentrations of EDTA.

Key words paracellular permeability enhancer; clodronate; rat jejunum; side-by-side diffusion chamber; calcium depletion; EDTA

Hydrophilic drugs cannot cross the cell membrane and are absorbed in the intestine mainly through the paracellular pathway. Their oral bioavailability is usually low since this pathway is severely restricted by the presence of the tight junctions. One of the possible strategies for solving this problem would be a controlled and reversible opening of the tight junctions, which could be achieved by compounds called paracellular permeability enhancers (PPEs). However, presently none of the known PPEs (Ca$^{2+}$ chelators, bile salts, anionic surfactants etc.) is used pharmaceutically since they cause, or might cause, toxicity (mucosal damage) “in vivo”. The main “tools” used in the research of PPEs are “in vitro” models of the intestinal epithelium, such as Caco-2 monolayers while experimental animals can be used for “in vivo” studies.1)

We can only speculate on the possible side effects of substances, which are known to increase the “in vitro” paracellular permeability, while the side effects of registered drugs are well documented. Therefore, instead of clinically testing a substance, which has been proven to increase the paracellular permeability “in vitro”, a therapeutic agent, already used in clinical practice could be tested for its “in vitro” ability to increase the paracellular permeability—an activity similar to that of PPEs. From the undesired gastrointestinal effects of such therapeutic agent or from its gastrointestinal tolerability the possibility for safe use of PPEs could be assessed. Thus, we have evaluated the “in vitro” ability of clodronate (a bisphosphonate) to increase the paracellular permeability of rat jejunum.

Bisphosphonates are a group of drugs prescribed for the treatment of osteoporosis, Paget’s disease, tumor-induced hypercalcemia, bone metastases and other diseases characterized by elevated bone resorption.2,3) They are capable of chelating divalent metal ions such as Ca$^{2+}$, Mg$^{2+}$ and Fe$^{2+}$.4) Some have been shown to increase the paracellular permeability of Caco-2 monolayers; PEG$_{400}$ flux was increased by tiludronate at concentrations above 20 mM added to both sides (apical and basolateral) of the Caco-2 monolayers. Pamidronate and zoledronate (also bisphosphonates) altered the mannitol flux for 30% at 10.3 and 40.7 mM respectively, when added only to the apical side of the Caco-2 monolayers. Pamidronate and zoledronate at concentrations of 30 mM, but not at 3 and 10 mM also caused a leakage of the plasma marker raffinose into the lumenal perfusate in the perfused rat ileal loop “in vivo”.2,5) A sugar absorption test has also shown that pamidronate can increase the intestinal permeability in humans.6) The presumed mechanism by which these substances increase the “in vitro” epithelial permeability is similar to that of Ca$^{2+}$ chelators—a group of PPEs (EDTA (ethylene diamine tetraacetic acid), EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N’-tetraacetic acid) etc.). However, bisphosphonates are much less potent for binding Ca$^{2+}$.3) Therefore, their “in vitro” effects on the integrity of epithelia can be expected at higher concentrations.

Clodronate is a first generation bisphosphonate. Its bioavailability is only 1—2%4) and it is freely soluble in water.9) The most commonly used oral dose of clodronate is 1600 mg/d given in a single dose.10,11) Therefore its expected gastrointestinal concentration can be estimated to 22 mM if 250 ml is the estimated volume for the dissolution of the given dose.12,13) We have decided to compare the ability of clodronate to modulate the paracellular permeability by opening the tight junctions of rat small intestine “in vitro” with that of EDTA. Similar to other Ca$^{2+}$ chelators, EDTA can deplete the extracellular Ca$^{2+}$, which is necessary for the maintenance of the paracellular barrier.14,15) Experiments were performed on isolated segments of rat jejunum mounted in side-by-side diffusion chambers. The tissue viability after the...
treatment with clodronate or EDTA was also evaluated.

MATERIALS AND METHODS

Materials Clodronic acid (disodium salt) was purchased from Sigma Aldrich Chemie (Steinheim, Germany), disodium salt of EDTA from Kemika (Zagreb, Croatia) and fluorescein sodium (FLU) from Fluka (Deisenhofen, Germany).

“In Vitro” Intestinal Permeability Studies Rat jejunum from male Wistar rats (250—320 g) was obtained, prepared and mounted in Easy Mount side-by-side diffusion chambers (Physiologic Instruments, San Diego, CA, U.S.A.) as described in a previous work.15 Both compartments were filled with 2.5 ml of Ringer buffer supplemented with 10 mM d-glucose or 10 mM mannitol on serosal and mucosal side of the tissue, respectively. The composition of Ringer buffer in mM was: 139.0 Na+, 5.0 K+, 1.2 Ca2+, 1.2 Mg2+, 121.8 Cl−, 25.0 HCO3−, 0.4 H2PO4 and 1.6 HPO42−. The tissue was kept at 37 °C throughout the experiments and the pH of the incubation saline was 7.5. Incubation salines were oxygenated and circulated by bubbling with carbogen (95% O2 and 5% CO2).

A saline without Ca2+ and Mg2+15 was necessary for the preparation of donor (mucosal) solutions containing EDTA or clodronate to ensure that the nominal concentrations of these substances were indeed the concentrations of their free form (not complex). In some experiments a saline with an increased concentration of Ca2+ (10 mM) was also used. To avoid the precipitation with calcium, phosphates had to be omitted from this saline. The osmolarity of all modified incubation salines was adjusted by NaCl.

After the tissue segments were placed in the diffusion chambers, 25 min was allowed for equilibration. During the experiments samples of 250 μl were withdrawn from the acceptor compartment every 20 min and replaced by the same volume of Ringer buffer containing 10 mM d-glucose. Each experiment was performed in three 80 min long “phases”.

Control of the Tissue Viability and Integrity Transepithelial potential difference (PD) and short circuit current (Isc) were measured by a multi channel voltage-current clamp (models VCC MC6 or VCC MC8, Physiologic Instruments). The transepithelial electrical resistance (TEER) was calculated from PD and Isc according to the Ohm’s law. Isc and TEER were corrected for fluid resistance prior to mounting the tissue in the diffusion chamber system. PD correlates well with the tissue viability in this type of experiments.16

The donor and acceptor solution (on mucosal and on serosal side, respectively) for the first phase of the experiments were prepared with standard Ringer buffer. The donor (mucosal) solution contained 10 mM mannitol and 5 μM hydrophilic paracellular permeability marker (FLU). In this phase the permeability of FLU under standard conditions (Pdiff) was measured. The tissue viability was controlled by checking PD and Isc every 20 min during the experiments. Tissue segments, which did not exhibit satisfactory PD and TEER values during the first phase of the experiment, were excluded from the study. PD (indicator of the tissue viability) had to be above 1.0 mV and stable. TEER was used to detect the tissue segments with poor integrity prior to the treatment with EDTA or clodronate (i.e., the intestinal mucosa can be damaged while excising the intestine from the euthanized animal or later while mounting the tissue segment in the diffusion chambers). Tissue segments with TEER values below 20Ω×cm² were excluded.

For the second phase the donor solution on the mucosal side of the tissue was replaced by a modified (Ca2+ and Mg2+-free) Ringer buffer containing the appropriate concentration of EDTA or clodronate. The absolute value of PD during this phase was altered because of the different ion concentrations on mucosal and serosal sides of the tissue caused by the addition of EDTA or clodronate in the form of sodium salts. Consequently, the tissue viability could not be reliably controlled during the second phase. For the control experiments, the donor solution on the mucosal side of the tissue was replaced by a modified (Ca2+ and Mg2+-free) Ringer buffer without EDTA or clodronate. Changes in the integrity of rat jejunal mucosa (i.e., changes in its barrier function) due to the opening of tight junctions were detected by measuring the permeability of the hydrophilic paracellular permeability marker FLU.

The third phase of the experiment was performed under the same conditions as the first phase. In this phase the PD again reliably reflected the tissue viability. Based on the experience with our system, we can say with confidence that the absolute values of PDGLU (PD after the addition of d-glucose to the mucosal compartment (final concentration was 25 mM) at the end of the experiment) higher than 1.0 mV indicate reliably, that the viability of rat jejunal enterocytes has not been affected to the point where it could influence the permeability properties of the isolated tissue. This includes normal paracellular permeability and normal function of intestinal active transport systems. Therefore the viability of tissue segments with PDGLU higher than 1.0 mV was evaluated as “good”. Poor tissue viability (indicated by lower PDGLU values) was considered to be a consequence of the treatment with EDTA or clodronate in the previous phase of the experiment. FLU Pdiff was measured in this phase.

Analytical Procedures and Data Analysis The concentrations of FLU were measured by fluorescence (λEX=485 nm, λEM=535 nm) on 96-well plates by a microtiter reader (Tecan, Salzburg, Austria).

The Pdiff values of the investigated substances were calculated by the following equation:

$$P_{diff} = \frac{dQ}{dt} \times \frac{1}{A C_0} \text{[cm/s]}$$  \hspace{1cm} (1)

where dQ/dt is the steady-state appearance rate of FLU on the acceptor side of the tissue, A is the exposed tissue area and C0 is the initial concentration of the investigated substance in the donor compartment.

For the statistical comparison the differences between the permeabilities of FLU in the second and in the first phase (Pdiff1−Pdiff2) were calculated for each tissue segment. These differences were then compared to those obtained from the control experiments where no EDTA or clodronate were present during the second phase. At least 3 measurements were obtained for each concentration of the examined substances tested. Dunnett t-tests were used for this comparison.

RESULTS The influences of EDTA and clodronate on the permeability coefficient of the paracellular marker FLU are shown on
Figs. 1a and b, respectively.

2 mM EDTA and higher concentrations were sufficient to provoke a statistically significant ($p < 0.05$) increase of the FLU permeability. At 9 mM a plateau was reached. Up to the highest concentration of EDTA tested (35 mM) the $P_{\text{eff}}$ of FLU remained 3 to 4 times higher relative to its $P_{\text{eff}}$ value. Already when 2 mM EDTA was tested, only 2 out of 4 tissue segments met the criterion for "good viability" at the end of the experiment. None of tissue segments treated with 3 mM or higher concentrations of EDTA had the $PD_{\text{GLU}}$ value above 1 mV.

The effect of clodronate on the permeability of FLU is evident (Fig. 1b), although it is not as strong as the effect of EDTA. It caused a noticeable increase of the FLU permeability at 10 mM concentration (Fig. 1b). The effect (compared to the control values—no clodronate) was statistically significant ($p < 0.05$) at 15 mM and higher concentrations. The tissue viability was not affected at this concentration. The viability of 3 out of 4 tissue segments treated with 20 mM clodronate and 2 out of 4 treated with 25 mM clodronate was good, while none of the tissue segments treated with 30 or 35 mM clodronate exhibited a $PD_{\text{GLU}}$ value above 1.0 mV.

The different effects of 15 mM and 35 mM clodronate on the FLU permeability through rat jejunal segments can also be seen on Fig. 2. In the first phase of the experiment both lines (circles and triangles) are parallel. In the second phase the permeability of FLU through the intestine treated with 35 mM clodronate in the mucosal solution is slightly higher. The tissue was no longer viable after the treatment with 35 mM clodronate and its permeability to FLU even increased in the third phase in spite of removing clodronate from the mucosal side. When the tissue was treated with 15 mM clodronate, its viability remained normal and the permeability of FLU did not increase in the third phase. The third series of data presented on Fig. 2 (squares) was obtained when 10 mM $Ca^{2+}$ was present on both sides of the tissue throughout the experiment and 15 mM clodronate was present on the mucosal Side of the Tissue during the second phase of the Experiment.

Results are shown as means±S.D. Statistically significant increases of permeability of FLU are indicated by asterisks above the columns. The numbers of tissue segments, which showed good viability after the treatment with clodronate and the numbers of all tissue segments tested at each concentration of EDTA are given in italic/bold, respectively.
DISCUSSION

Some authors consider the loosening of the tight junctions “in vitro” to be “damage to the intestinal mucosa” and relate it to the expected gastrointestinal toxicity “in vivo” of the drug tested. On the contrary, in our present work the evaluation of the tissue viability was based only on the PD and PDGLU values, while the increase of the paracellular permeability alone was not considered to be a “toxic effect”. We have seen that clodronate can induce a small but obvious increase of the permeability of the rat jejunal epithelium while it does not affect its viability. Furthermore, the loss of the epithelial integrity can be reversed if the tissue is viable and if calcium is present in a sufficiently high concentration (Fig. 2, squares).

A partial loss of the tissue viability “in vitro” was noticed in the concentration range, which is presumably reached in the small intestine after the administration of the highest single 1600 mg dose of clodronate (22 mM). On the other hand, the concentrations, which cause poor viability in all the tissue segments tested “in vitro”, are even higher (30, 35 mM). Therefore one could expect that the “in vitro” impairment of the tissue viability would be a better predictor of “in vivo” gastrointestinal adverse effects than the “in vitro” loss of the rat jejunal tissue integrity.

Some authors speculate that bisphosphonates can enhance their own transport through the gastrointestinal epithelium. Moreover, the bioavailability of some bisphosphonates like pamidronate and tiludronate is dose dependent, being slightly better at higher doses. This is in accordance with our findings, that the “in vitro” exposure of the mucosal side of the jejunal tissue to clodronate increases the paracellular permeability. Additionally, such an increase in the paracellular permeability might also affect the pharmacokinetics of other hydrophilic drugs with very low bioavailability if they were taken with clodronate.

As expected, EDTA induces an opening of the tight junctions in the rat jejunal mucosa and influences its viability at much lower concentrations than clodronate. However, both substances cause a similar change in the rat jejunal permeability, if they are applied at the concentrations, which cause a partial loss of the tissue viability. There are several possible mechanisms for the increase of the paracellular permeability. The assumption that clodronate increases the paracellular permeability through the same mechanism as EDTA—a depletion of extracellular Ca++, was confirmed by the fact that the tissue integrity could be restored after the treatment with clodronate when “high-Ca++” conditions were used.

The lowest concentration of EDTA, which caused a statistically significant increase in the FLU permeability, also affected the rat jejunal viability in 2 out of 4 segments tested. This confirms, that the implementation of the existing Ca++ depleting PPEs like EDTA in any kind of a clinical study on humans would be inappropriate.

As explained by Ward et al. a useful PPE would have to exhibit a controlled, transient and reversible opening of intestinal tight junctions. Furthermore its use should not result in increased exposure of the subepithelial spaces to intestinal bacteria or their byproducts. Namely, a major concern limiting the development and use of PPEs is that the opening of the tight junctions might cause local intestinal inflammation similar to that observed in intestinal inflammatory diseases like the inflammatory bowel syndrome—IBS. A hypothesis is that IBS patients have an abnormally leaky colonic epithelium that allows luminal bacterial fragments to penetrate into the subepithelial spaces, resulting in an inflammatory response. No reports could be found on clodronate therapy resulting in IBS-like complications or in any other permanent damage of the gastrointestinal tract. A possible explanation is that clodronate is at least partially saturated with bivalent cations by the time it reaches the colon where the risk for an inflammatory response is the highest. This is due to the presence of a much higher population of bacteria in the lower parts of the gastrointestinal tract. A similar reasoning could be applied for a Ca++ depleting PPE—meaning that it should not cause any long-term damage to the lower gastrointestinal tract even if it would increase the paracellular permeability in the upper small intestine.

A therapeutic use of Ca++ depleting PPEs like EDTA remains hindered by their cytotoxicity “in vitro”, which can be observed in our experiments as poor viability of the rat jejunum. However, the ability of many PPEs to increase paracellular permeability is not a direct result of their toxic actions.

CONCLUSIONS

By the chelation of the extracellular Ca++ clodronate significantly increases the paracellular permeability when tested in concentrations, which are probably reached in the small intestine following the administration of a single 1600 mg dose. At higher concentrations it also affects the viability of the rat jejunum “in vitro”.

EDTA has a much stronger effect on the paracellular permeability, however it coincides with a loss of the tissue viability.

The “in vitro” impairment of the tissue viability as shown by PD is a much better predictor of the “in vivo” gastrointestinal tolerability of drugs or PPEs than the “in vitro” increase of the paracellular permeability. It was shown that clodronate has similar characteristics as PPEs in “in vitro” conditions. The clinical experience with clodronate shows that it is not likely that the use of PPEs would have “IBS-like” long-term consequences.

REFERENCES AND NOTES