Aged Garlic Extract Stimulates P-Glycoprotein and Multidrug Resistance Associated Protein 2 Mediated Effluxes

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The growing concomitant consumption of drugs and herbal preparations such as garlic, and the numerous reports about the influence of herbal preparations on intestinal transport, led us to evaluate the influence of aged garlic extract on the transport function and electrophysiological parameters of the small intestinal mucosa. Aged garlic extract induced increase of the absolute value of transepithelial potential difference and of the short-circuit current in both permeability models tested (rat jejunum, Caco-2 cell monolayers) without affecting transepithelial electrical resistance. It also caused a significant increase of the P-glycoprotein and multidrug resistance associated protein 2 mediated effluxes through rat jejunum of marker substrates Rhodamine 123 and 2,4-dinitrophenyl-5-glutathione, respectively. Rhodamine 123 efflux through the Caco-2 cell monolayers was not altered by aged garlic extract, whereas the efflux of 2,4-dinitrophenyl-5-glutathione increased significantly. So altered activity of the important transport proteins could significantly change the pharmacokinetic properties of conventional medicines taken concomitantly with aged garlic extract.

Key words P-glycoprotein; multidrug-resistance associated protein 2; aged garlic extract; Rhodamine 123; 2,4-dinitrophenyl-5-glutathione; fluorescein

Bioactive natural products are consumed as food, dietary supplements and to a lesser extent as pharmaceutical products. A vast number of phytochemicals present in plant-based functional foods and dietary supplements exert not only desired biological effects but also complex interactions, which demonstrate themselves as pharmacological effects. Phytochemicals can alter the pharmacokinetic properties of conventional pharmaceuticals by enzyme inhibition/activation (cytochrome P450, glutathione S-transferase, glucuronyl transferase), P-glycoprotein (Pgp) activation or inhibition, altered Pgp expression and membrane fluidity, modulated drug solubility and uptake, and others.1,2) The most common users of herbal medicines are elderly patients prone to conditions treated by chronic therapy with conventional medicines. Therefore, the interactions of herbal medicines with prescribed drugs are very likely in this subpopulation and could result in cases of clinically adverse reactions.3,4) It is also important since the consumption of herbal medicines (such as the best selling representatives St. John’s worth, ginseng, garlic, Echinacea, saw palmetto and kava) is rising continuously.3,5)

P-glycoprotein and multidrug resistance associated protein 2 (MRP-2) are ATP-dependent multidrug transporter proteins, expressed in several cancer cell lines and in normal tissues of liver, kidney, blood–brain barrier, placenta and small intestine.2,6,7) The intestinal secretion of drugs and/or their metabolites from enterocytes into the intestinal lumen by apically located efflux proteins constitutes a potential biochemical barrier that can limit the rate and extent of drug absorption.2,7) The modulatory effect of several food components has been established, especially on Pgp, while data on MRP-2 are sparse.2,8) High-throughput screening essays, utilizing Pgp, human liver microsomes and cDNA expressed cytochromes, have shown the influence of garlic, garlic preparations and other phytochemicals on Pgp activity and on cytochrome P (Cyp) 450 2C9, 2C19, 2D, and 3A4 enzyme function.2,5,9,10) Garlic also significantly increased the activity of lipase and amylase, and decreased the activity of chymotrypsin, lactase and maltase.11) An interaction of garlic with Cyp3A4 metabolism and Pgp transport was even suspected to cause the case of extreme gastrointestinal toxicity of ritonavir.12)

Garlic and its extracts are used in particular because of their cholesterol and blood pressure lowering effects and their ability to prevent infections, atherosclerotic processes and to improve circulation.3) Garlic supplements are available as powder, garlic essential oil, macerated garlic oil and as aged garlic extract (AGE).2,5,13) AGE is formulated by soaking sliced raw garlic (Allium sativum) in 5—20% aqueous ethanol for up to 20 months at room temperature. The extract is then filtered and concentrated under reduced pressure at low temperature. The content of water soluble compounds is relatively high, while that of oil-soluble compounds is low.14) In the main, AGE comprises water-soluble sulphur containing compounds such as S-allyl-β-cysteine (SAC), S-allylmethylcysteine (SAMC), saponins, etc. The purpose of the 20 month long aging process is to eliminate the majority of the irritating, acidic and oxidizing oil-soluble sulphur substances (diallyl sulphide (DAS), diallyl disulphide (DADS), diallyl trisulfide, methyl allyl disulphide, etc.) that are responsible for the characteristic odour and taste of raw garlic.3,13)

The aim of the present study was to characterize the influence of AGE on the transporter function and electrophysiological parameters of intestinal mucosa “in vitro.” More specifically, the influence of AGE on the transport of marker substances Rhodamine 123 (Rho123) and 2,4-dinitrophenyl-5-glutathione (DNP-SG) for Pgp and MRP-2 transporters, respectively, was evaluated. The chosen “in vitro” permeability models were rat jejunum and Caco-2 cell monolayers.

MATERIALS AND METHODS

Materials Fluorescein sodium (FLU), Rho123, verapamil, quercetin, acivicin and salts for incubation salines were from Sigma Aldrich Chemie (Deisenhofen, Germany). SAC standard was from TCI Europe NV (Zwijndrecht, Bel-
gium). All chemicals used in this study were of analytical grade. 2,4-Dinitrophenyl-S-glutathione (DNP-SG) was synthesized according to Hinchman et al. Its purity and identity were confirmed by HPLC and NMR analysis.

Kyolic® liquid aged garlic extract was produced by Wakanuga of America Co., Ltd. (Mission Viejo, CA, U.S.A.), lot number 5H04A. The AGE used in this study contained 1.27 g/l SAC, the compound normally used for the standardization of AGE. AGE composition is described in more detail by Amagase. In the range of 450—750 μm2, the Caco-2 cell monolayers were grown on Snapwell Costar culture inserts with a polycarbonate membrane (diameter 12 mm and pore size 0.4 μm). 100000 cells/filter membrane were used for seeding and the medium was changed every 2 d. At day 15, transepithelial electrical resistance (TEER) was measured for each filter with Caco-2 cell monolayers. If the TEER values were in the range of 450—750 Ω cm2, the Caco-2 cell monolayers were used for the subsequent testing of permeability at day 21.

The inserts with Caco-2 cells were carefully rinsed with Ringer buffer and then placed between two compartments of EasyMount side-by-side diffusion chambers (Physiologic Instruments, San Diego, U.S.A.). Two point five milliliters of bathing solution (Ringer buffer) on each side of the Caco-2 cell monolayer was maintained at 37 °C and continuously oxygenated and circulated by bubbling with carbogen (95% O2, 5% CO2). Ten millimolar glucose and 10 mM mannitol were always added to the basolateral (BL) and apical (AP) sides, respectively. After 25 min of preincubation, 0.5 ml of the concentrated solution containing one or two investigated substances was added to the AP side (if studying apical-to-basolateral (AP-BL) transport) or the BL side (if studying basolateral-to-apical (BL-AP) transport). Two hundred fifty microliters samples were withdrawn from the acceptor side every 15 min up to 75 min, and replaced each time by fresh Ringer buffer containing all necessary ingredients at appropriate concentrations. In the second phase of the experiment, AGE concentration was added to the AP side of Caco-2 cell monolayers to give 1% (v/v) final concentration of AGE. The sampling continued every 15 min up to 150 min. The withdrawn samples were replaced by fresh Ringer buffer with all necessary ingredients. In the third phase of the experiment, the appropriate specific transporter inhibitor was added to the AP side and the experiment continued for an additional 75 min with 15 min sampling periods. Only those Caco-2 cell monolayers with TEER values that remained constant during the whole experiment were used.

The specific inhibitors of Pgp used in the experiments were quercetin or verapamil. Quercetin is a specific substrate—competitive inhibitor for the H-site of Pgp, while verapamil is a less site-specific Pgp inhibitor. As expected, preliminary experiments performed in our laboratory have shown the efflux of Rho123 could be significantly inhibited by 200 μM apical verapamil concentrations. The Rho123 efflux ratio decreased from 27.9 to 3.9. The preliminary experiments with rat small intestine have also confirmed, that the efflux ratio of rat123 (24.7) decreases significantly in the presence of mucosal verapamil (200 μM) to 4.5. Higher verapamil concentrations (tested up to 1 mM) can further decrease the Rho123 efflux ratio to 2.9 but they also affect the tissue viability and integrity. Therefore a uniform 200 μM verapamil is used for all Pgp inhibition studies.

Acivicin, an irreversible inhibitor of γ-glutamyl transpeptidase, was used in 1 mM concentration during experiments with DNP-SG to prevent its enzymatic degradation in the apical/mucosal incubation medium.

In Vitro Transport Studies across Rat Small Intestine

The experiments conformed to the Law for the Protection of Animals (Republic of Slovenia) and were registered at the Veterinary Administration of the Republic of Slovenia. They were performed in the manner described previously. Rat small intestine was obtained from male Wistar rats (250—320 g) fasted 18 h prior to the experiments. After euthanasia and laparotomy, the intestine was rinsed with ice-cold 10 mM glucose Ringer solution. Jejunum, located 25—60 cm distally from the pyloric sphincter, and ileum, 20 cm proximally from the ileo-caecal junction, were used for the experiments. The intestinal tissue was cut into 3 cm long segments, excluding visible Peyer’s patches. These intestinal segments were opened along the mesenteric border, stretched onto inserts with an exposed tissue area of 1 cm2, then placed between two compartments of EasyMount side-by-side diffusion chambers (Physiologic Instruments, San Diego, U.S.A.). The experimental procedure was continued in the same manner as described above for Caco-2 cell monolayers.

Electrical Measurements

The diffusion chambers were equipped with two pairs of Ag/AgCl electrodes for measuring transepithelial potential difference (PD) and short circuit current (Isc) with a multi channel voltage-current clamp (model VCC MC6, Physiologic Instruments). The viability and integrity of tissue and Caco-2 cells were checked by monitoring PD, Isc and TEER every 15 min during the experiments. In viable rat tissues and Caco-2 cells, TEER did not change significantly over 150 or 225 min periods. The average TEER from 15 min to 150 or 225 min was calculated and also used for evaluating the tissue/cell integrity and viability. The viability of rat small intestine was additionally checked by recording the increase of Isc 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 PD value after the addition of glucose was lower than −0.1 mV and if the average TEER values recorded during the experiment were between 20 and 400 Ω cm2. The Caco-2 cell culture monolayers were considered viable if the average TEER values between 15 and 150 or 225 min were in the range of 500 and 800 Ω cm2, and if they exhibited a PD lower than −0.5 mV during the experiment.

HPLC Parameters

Concentrations of FLU and Rho123 were measured with a fluorospectrometer detector Tecan GENious (λex=485 nm, λem=535 nm). One hundred microliters of a sample was diluted with 100 μl of 0.025 M NaOH to measure the concentration of FLU and with 100 μl of 0.01 M HCl for Rho123.

DNP-SG was analyzed by HPLC (Series 1100, Hewlett-Packard, Waldbronn, Germany). A Chromolith SpeedRod C-18 (50×4.6 mm) column, with a mobile phase composed of ammonium acetate buffer and acetonitrile (84:16) at 35 °C, was used. The flow rate was 2.5 ml/min, the injection volume...
100 μl, and the retention time of DNP-SG 0.8 min. UV detection was at 335 nm.

DADS and DAS in AGE were also quantified by HPLC, with a Syngene Fusion-RP 80 (250×4.6 mm; 4 micron) column at 50 °C and a 100% phosphate buffer (pH 4.7) as mobile phase. The flow rate was 1.5 ml/min, the injection volume 5 μl, and the retention times of DADS and DAS were 6.3 and 4.7 min, respectively. UV detection was at 220 nm.

**Data Analysis** The apparent permeability coefficient (P_{app}) was calculated according to Eq. 1:

\[
P_{\text{app}} = \frac{dc}{dt} \frac{V}{c_0 A}
\]

where dc/dt is the change in concentration of the examined substance in the acceptor compartment per unit time under steady state conditions, V is the volume of the acceptor compartment, A the exposed surface area (1 cm² for rat jejunum and 1.13 cm² for Caco-2 cell monolayers) and c₀ the initial concentration of the examined substance in the donor solution.

Results in Tables and Figures are presented as means±S.D. of at least 3 measurements. Data were evaluated statistically using SPSS 16.0 for Windows. Where appropriate, F-test for testing the equality of variances and, afterwards, 2-tailed Student’s t-test (α=0.05), were used. Otherwise, one way ANOVA, followed by Bonferroni post-hoc test were applied.

**RESULTS**

The Influence of AGE on the Electrophysiological Parameters of Rat Jejunum and Caco-2 Cell Monolayers After the addition of AGE at 1% (v/v) concentration to the mucosal or apical side of rat jejunum or Caco-2 cell monolayers, changes of electrophysiological parameters (PD, I_{sc}, TEER) were recorded (Table 1). While the absolute values of PD and I_{sc} increased, TEER values were practically unaltered. This indicated that tight junctions were most probably not affected by AGE, which was confirmed by the permeability of the paracellular marker FLU, measured concomitantly. The permeability of FLU was 3.2±0.7×10^{-6} cm/s in the M-S and 5.0±1.5×10^{-6} cm/s in the S-M direction through rat jejunum, which does not differ significantly from the values obtained during control experiments (3—6×10^{-6} cm/s for both directions) performed in the absence of AGE.\(^{17}\) Furthermore, the FLU permeability through Caco-2 cell monolayers also remained unaltered in the presence of apical AGE (1% (v/v)) (2-tailed Student test; p=0.502 for AP-BL and p=0.089 for BL-AP FLU permeabilities). The AP-BL and BL-AP FLU permeabilities were 1.4±0.4×10^{-7} cm/s and 3.9±0.6×10^{-7} cm/s in the first, reference phase (before AGE addition), and 1.6±0.4×10^{-7} cm/s and 5.8±1.4×10^{-7} cm/s in the presence of AGE.

Transport Properties of Rho123 in the Presence of AGE Rho123 was the chosen marker substrate for evaluating the influence of AGE on the activity of Pgp transporters. Caco-2 cell monolayers and rat jejunum were used for this purpose.

It is known that Pgp activity is higher in the distal small intestine than in the jejunum.\(^{18}\) Our results showed that the S-M/M-S permeability ratio of Rho123 for ileum (16.2) was not significantly greater (one-way ANOVA followed by post-hoc Bonferroni test) than for jejunum (12.2), justifying the use of the much shorter rat ileum in the experiments instead of the very long jejunum. All further experiments with rat intestinal tissue were thus performed with jejunum.

The influence of different mucosal AGE concentrations (2, 1 and 0.5% (v/v)) on Pgp function was then evaluated by measuring S-M P_{app} values of Rho123 before and after the addition of AGE to the mucosal side of rat jejunum (Table 2). The average S-M P_{app} values of Rho123 increased in the presence of AGE at all concentrations tested, although the effect was statistically significant only at 1% (v/v) of AGE (with a 4.5-fold increase; 2-tailed Student test p=0.017). Therefore this concentration was used in further experiments.

The three-phase experiments, described in Materials and Methods were further performed with Caco-2 cell monolayers and rat jejunum. Thus, the second phase—the addition of 1% (v/v) AGE to the mucosal/apical side—was followed by the third phase with Pgp inhibitor, verapamil or quercetin, added to the same side (Table 3).

In the reference phase, S-M/BL-AP Rho123 permeability values were significantly greater than M-S/AP-BL values. The efflux of Rho123, which was triggered by AGE through rat jejunum (but not through Caco-2 cells), was inhibited by verapamil (one-way ANOVA followed by post-hoc Bonferroni test; p=0.006). The same inhibitory effect of verapamil on Rho123 efflux was observed through Caco-2 cell monolayers. Quercetin, a specific marker for the H-site of Pgp, had no impact on Rho123 efflux through rat jejunum and Caco-2 cells.

**The Influence of AGE on DNP-SG Transport** 2,4-
Table 3. The Average M-S/AP-BL and S-M/BL-AP Permeability Values and Their Standard Deviations for Rho123 and FLU (Reference), in the Presence of AGE and after the Addition of Inhibitor (Verapamil 100 μM, Quercetin 40 μM) Determined through Rat Jejunum and Caco-2 Cell Monolayers

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<thead>
<tr>
<th>Rat jejenum</th>
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<td></td>
<td>M-S</td>
<td>S-M</td>
<td>M-S</td>
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<tr>
<td>Phase 1 Reference</td>
<td>2.8±1.6</td>
<td>4.0±1.0</td>
<td>0.3±0.1</td>
<td>6.6±2.3</td>
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<td>3.8±2.0</td>
<td>13.4±3.0</td>
<td>0.7±0.4</td>
<td>16.2±4.5</td>
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<td>Phase 3 Verapamil</td>
<td>5.4±3.8</td>
<td>7.8±1.1</td>
<td>0.9±0.7</td>
<td>6.7±1.8</td>
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<tr>
<td>Phase 1 Reference</td>
<td>2.5±0.7</td>
<td>3.4±0.7</td>
<td>0.3±0.1</td>
<td>4.1±1.0</td>
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<tr>
<td>Phase 2 AGE</td>
<td>3.3±0.6</td>
<td>7.6±1.2</td>
<td>0.3±0.1</td>
<td>8.3±1.0</td>
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<tr>
<td>Phase 3 Quercetin</td>
<td>4.6±0.9</td>
<td>7.7±0.5</td>
<td>0.5±0.1</td>
<td>8.0±0.9</td>
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<tr>
<th>Caco-2 cell monolayers</th>
<th>AP-BL</th>
<th>BL-AP</th>
<th>AP-BL</th>
<th>BL-AP</th>
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<tr>
<td>Phase 1 Reference</td>
<td>0.5±0.1</td>
<td>4.8±0.6</td>
<td>0.5±0.1</td>
<td>9.3±0.6</td>
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<tr>
<td>Phase 2 AGE</td>
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<td>10.8±1.1</td>
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<td>Phase 3 Verapamil</td>
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<tr>
<td>Phase 1 Reference</td>
<td>0.5±0.1</td>
<td>4.8±0.6</td>
<td>0.6±0.1</td>
<td>9.5±1.5</td>
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<tr>
<td>Phase 2 AGE</td>
<td>0.7±0.1</td>
<td>6.0±0.7</td>
<td>0.7±0.0</td>
<td>10.3±1.0</td>
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<tr>
<td>Phase 3 Quercetin</td>
<td>0.7±0.1</td>
<td>4.5±0.6</td>
<td>0.7±0.2</td>
<td>8.4±0.9</td>
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Fig. 1. M-S/AP-BL and S-M/BL-AP DNP-SG $P_{\text{app}}$ Values for Rat Jejunum (Reference (ref) Conditions, Hypo-Osmotic (hypo) Conditions (205 mOsm Ringer buffer, No AGE) and the Experiment with 1% (v/v) AGE (AGE) on the Mucosal Side with Iso-Osmotic Ringer Buffer) and Caco-2 Cell Monolayers (Iso-Osmotic Ringer Buffer (iso) and with 1% (v/v) AGE on the Apical Side (+AGE) in the Second Phase of the Experiment)

Dinitrophenyl-S-glutathione (DNP-SG) is a substrate for MRP-2 transporters. MRP-2 proteins are expressed in the villus tips in the jejunum and, to a much lesser extent, in the distal ileum. All experiments performed with DNP-SG were therefore conducted with rat jejunum as described previously. The reference experiment with DNP-SG and without AGE on the mucosal side (reference (ref)) indicated higher M-S than S-M permeability of the marker (Fig. 1). Therefore we assumed that the MRP-2 apical expression or activity was too low for the efflux of DNP-SG to be detected. Gerk and Vore showed that hypo-osmotic buffer induced endocytic membrane retrieval of MRP-2 proteins to the apical side of hepatic cell cultures. Assuming that a similar effect could take place in rat jejunal enterocytes, 205 mOsm Ringer buffer was used during another experiment. Under hypo-osmotic conditions the measured S-M $P_{\text{app}}$ values of DNP-SG were significantly higher than the M-S values (2-tailed Student test, $p=0.025$), and than the S-M $P_{\text{app}}$ values determined under iso-osmotic (reference) conditions ($p=0.033$). Similar results, i.e., higher S-M than M-S $P_{\text{app}}$ values of DNP-SG, were obtained under iso-osmotic conditions with AGE in the medium on the mucosal side of the intestinal tissue. Furthermore, S-M $P_{\text{app}}$ values determined in iso-osmotic condition with AGE were also significantly higher than S-M $P_{\text{app}}$ values determined under hypo-osmotic conditions (2-tailed student test, $p=0.034$). This means that, in this case, MRP-2 transport of DNP-SG was activated by 1% (v/v) AGE.

This experiment, with DNP-SG in iso-osmotic Ringer buffer and AGE in the second phase, was also performed with Caco-2 cell monolayers. After the addition of AGE to the apical side of the cell monolayers, the BL-AP DNP-SG permeability values significantly increased (2-tailed Student test, $p=0.037$) while the AP-BL permeabilities were not affected by the AGE (Fig. 1).

**Active Secretion of FLU in the Presence of Pgp Substrate**

The tight junction integrity marker FLU diffuses across the membrane mainly through the paracellular route. However, in the presence of slightly acidic mucosal pH or 10 mM D-glucose on the mucosal side of rat jejunum, FLU is a substrate for the influx MCT (monocarboxylic acid cotransporter) and OATP (organic anion-transporting polypeptide) or efflux MRP-2 and Pgp transporters, respectively.

Although FLU M-S and S-M permeabilities were not affected by AGE alone (see above), the addition of AGE at final 1% (v/v) concentration in the presence of Rho123 caused a significant increase in S-M $P_{\text{app}}$ values of FLU through rat jejunum, while M-S $P_{\text{app}}$ remained unaltered (Table 3, one-way ANOVA followed by Bonferroni). The Pgp inhibitor verapamil induced a significant decrease of FLU efflux, while quercetin had no effect.

The same experiments were performed with Caco-2 cell monolayers. In the first, reference phase, there was a significant difference between BL-AP and AP-BL permeabilities of FLU (2-tailed Student test, $p=0.002$, Table 3), although the TEER values were all between 521 Ω cm² and 913 Ω cm² throughout each experiment. Therefore the integrity of tight junctions during these experiments was not compromised and could not be the cause of the higher BL-AP permeabilities. However, in previous studies we observed that, in the presence of any Pgp substrate and/or inhibitor, FLU BL-AP permeabilities exceed AP-BL permeabilities by at least two fold (Table 4). The reason for FLU efflux in the reference phase is therefore most probably due to higher Pgp activity, triggered by Pgp substrate Rho123 (Table 3).

In the second phase, the presence of AGE did not affect FLU permeabilities (2-tailed Student test), as in the case of rat jejunum (Table 3). However, the addition of verapamil caused a significant decrease of FLU efflux, while quercetin (a Pgp inhibitor that binds only to the H-site of Pgp) had no influence on FLU BL-AP permeability.

**DISCUSSION**

AGE contains mainly water-soluble phytochemicals, since the oil-soluble compounds are unstable and decompose dur-
ing the aging process. The oil-soluble phytochemicals (DADS, DAS) activate the transient receptor potential family of nonselective cation channels20) and cause changes of electrophysiological parameters of cardiac21) and intestinal22) cells by modulating intracellular Ca\textsuperscript{2+} and/or K\textsuperscript{+} concentrations. The electrophysiological changes were also observed in this study when the whole garlic extract was used. Our HPLC analysis of AGE confirmed that, in spite of the aging process, during which the content of these oil-soluble, irritant, oxidizing and acidic compounds in AGE should degrade,13) they were still present in 1% (v/v) AGE apical solution (10.5 μM diallyl sulphide and 38.3 μM diallyl disulphide). The changes of PD and I\textsubscript{sc} of rat jejunal mucosa and Caco-2 cell monolayers following the addition of AGE to the mucosal/apical compartment showed that AGE modulates the electrogenic activity of one or more membrane channels or other electrogenic transporters expressed in the apical membrane of enterocytes and Caco-2 cells. We assumed therefore that the concentration of oil-soluble compounds in the AGE, in spite of the aging process, remains sufficient to induce electrophysiological effects in the intestinal mucosa. The presence of DADS and DAS were confirmed by HPLC.

The complexity of interactions between garlic components and efflux transporters (Pgp, MRP-2) could be explained by several regulatory mechanisms of Pgp and MRP-2. Although the regulation of efflux proteins is still not well understood, we tried to elucidate the most likely mechanisms involved in AGE induction of Pgp and MRP-2 transporters activity “in vitro.”

The observed increase of Rho123 efflux through the rat jejunum in the presence of AGE on the mucosal side (Tables 2, 3) could be the consequence of the direct influence of garlic phytochemicals from AGE binding to Pgp transporters. Pgp has four binding sites, I, II, III and IV, which are involved in the transport of substrates (transport sites I, II and III) and/or in the regulation of this transport (regulatory sites II and IV). Each transport/regulatory site can switch between high and low affinity conformations as a consequence of complex allosteric interactions.23) The transport sites that preferentially bind Rho123 and Hoechst 33342 (a marker substance, transported by the H-site of Pgp), termed the R- and H- sites, have been shown to interact in a positive cooperative manner. Rho123 stimulates Hoechst 33342 transport and vice versa.5) Therefore substances bound to one site competitively inhibit the transport of other substrates to that site and stimulate the transport of substrates to the other site. This stimulatory effect is concentration dependent—above the optimal concentration, the stimulatory effect diminishes.6) It is possible that such an effect was observed when AGE was added in 2% (v/v) concentration, because the effect was less pronounced than at 1% (v/v) AGE in mucosal bathing solution (Table 2).

According to the above-described theory of the positive cooperative effect between different binding sites of Pgp, the stimulation of Rho123 efflux by the R-site could be triggered by the binding of AGE compounds to one of the three remaining binding/regulatory sites of Pgp. However, if a component or components of AGE would increase the Rho123 efflux through the rat jejunum, the same effect of AGE should also be observed through Caco-2 cell monolayers, but this was not the case (Table 3). An increase of a transmembrane protein (like Pgp) activity could also be induced by the changes of electrophysiological membrane characteristics that were observed in the presence of AGE. But, like allosteric regulation, direct activation of Pgp by increased potential difference is not likely, because the changes of PD and I\textsubscript{sc} were observed also on Caco-2 cells where no additional Pgp activity was detected after AGE addition. The fact that AGE does not induce Pgp mediated transport through Caco-2 cell monolayers above its normal activity indicates that it probably interferes with another cellular mechanism for the regulation of Pgp activity in rat intestinal mucosa. Such a control mechanism might be absent in cancerous cell lines like Caco-2 in our study because of an adaptation that provides them with stronger multi-drug resistance by keeping their efflux transporters continuously maximally active. The difference between the Pgp regulatory mechanisms in rat jejunum and in the Caco-2 cell line is also evident when observing the paracellular permeability of FLU. FLU, whose permeability was not affected by AGE alone, became a Pgp substrate in rat jejunum in the presence of AGE and Rho123 together. It was most probably transported by a site other than the H-site of Pgp, because its efflux could be inhibited by verapamil while there was no competitive inhibition after quercetin addition. However, in the Caco-2 cell line, the efflux of FLU is already high enough in the presence of Pgp substrate/inhibitor, and no additional influence can be observed after the addition of AGE. FLU is not transported by the H-site of Pgp in the cancerous cell line either, because quercetin did not affect FLU efflux.

The results obtained with DNP-SG (Fig. 1) show that, without activation of MRP-2 apical expression or activity in rat jejunum, these effects are too small for detecting efflux of the specific marker DNP-SG. This is in accordance with the previous observations of Kubitz et al. made on rat hepatocytes.24) The regulation of MRP-2 activity is a complex process. Allosteric regulation is possible in this case too; similar to Pgp, MRP-2 transporters have two binding/regulatory sites, which act in a positive cooperative manner. The retrieval of MRP-2 proteins to the apical membrane of hepatocytes has been observed after exposure of a hepatic cell line to hypo-osmotic conditions.3) Our results (“hypo” and AGE in Fig. 1, and Caco-2 measurement of DNP-SG permeability in iso-osmotic conditions with AGE) reveal that this regulatory mechanism is also present in the enterocytes of the rat jejunal mucosa and Caco-2 cell monolayers. The ob-

<table>
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<th>Experiment with:</th>
<th>FLU $P_{app} (\times 10^{-7} \text{cm/s})$</th>
<th>R</th>
<th>Pfp</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>Verapamil</td>
<td>1.8 ± 2.6</td>
<td>S + 1</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Quinidine</td>
<td>2.3 ± 1.5</td>
<td>S + 1</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Ranitidine</td>
<td>2.1 ± 1.2</td>
<td>S</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Captopril</td>
<td>2.2 ± 0.2</td>
<td>S</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Metoprolol</td>
<td>5.5 ± 2.3</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Naproxen</td>
<td>4.9 ± 2.4</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Atenolol</td>
<td>7.7 ± 1.0</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

Factor R represents the ratio between the average BL-AP and AP-BL FLU permeabilities.
erved activation of MRP-2 in the presence of AGE in iso-osmotic conditions could thus be caused either by allosteric regulation or by membrane retrieval of transporter proteins. Further experiments are necessary to clarify this phenomenon.

In conclusion, garlic phytochemicals from AGE induced a significant alteration of electrophysiological parameters, PD and Isc, of rat jejunum and Caco-2 cell monolayers, while TEER remained unchanged. AGE also increased the activity of Pgp and MRP-2 in rat jejunum, but only MRP-2 in Caco-2 cells “in vitro.” These results strongly indicate that concomitant consumption of AGE and a drug, subjected to intestinal Pgp and/or MRP-2 efflux could lead to decreased, or perhaps even insufficient, absorption of the drug. Extensive further research on “in vitro” and “in vivo” models is necessary to better understand the function and regulation of efflux transporters, while only a clinical study with human volunteers could unequivocally determine the exact effect of garlic and garlic preparations on the bioavailability of drugs that are Pgp and/or MRP-2 substrates.

REFERENCES