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# Effect of nonylphenol ethoxylates (NPEs) on barrier functions of epithelial cell membranes: Opening of tight junctions and competitive inhibition of P-gp-mediated efflux

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**Abstract**

The effect of nonylphenol ethoxylates (NPEs) on selected barrier functions of biological membranes, such as tight junction and P-gp efflux pump of epithelial membranes, against the transport of xenobiotics was examined. The Caco-2 cell line was used to evaluate the transport of mannitol and daunomycin across the cell monolayer as well as the cellular uptake of daunomycin. In the presence of NPEs, the transport of mannitol was increased, with NP-9 showing a maximal effect, and the transepithelial electrical resistance (TEER) was reduced. The onset of this effect of NP-9 was fairly rapid and reversible for a short term (e.g., 2 h) treatment, while irreversible for a long term (e.g., 72 h) treatment. In the presence of NP-9, the apical uptake of daunomycin was increased, suggesting competitive inhibition between NP-9 and daunomycin in the efflux via the P-gp system. However, a 72 h pretreatment of the cells with NP-9 (up to 1000 nM) did not affect the apparent cellular uptake of daunomycin, suggesting no significant effect of NPEs on the expression of P-gp. In conclusion, NPEs appear to rapidly open the tight junction of epithelial cell membranes and to competitively inhibit the efflux of P-gp substrates, thereby reducing the self-protection ability of the organism against xenobiotics or hazardous environmental compounds that are transported via the paracellular pathway (i.e., uptake) or the P-gp system (i.e., efflux).


*Keywords:* Nonylphenol ethoxylates; Barrier functions; Caco-2; Tight junction; P-glycoprotein

## 1. Introduction

Nonylphenol ethoxylates (NPEs, Fig. 1) are synthetic surfactants that are inexpensive enough to be commonly used in a variety of household products, and, as the result, are quite common in surface water

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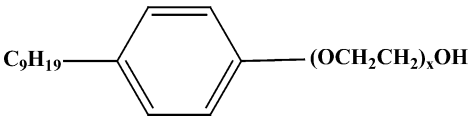


Fig. 1. Chemical structures of nonylphenol ethoxylates (NPEs) for NP-4, NP-7, NP-9, NP-10 and NP-35 which contain 4, 7, 9, 10 and 35 ethoxylates, respectively. The number following NP- indicates the average number of ethylene oxide units per molecule at position *x*.

and other aquatic environments that receive sewage discharges (Maguire, 1999). NPEs have been used as wetting agents and as intestinal permeability enhancers to improve oral drug delivery (Swenson et al., 1994a). They are rapidly absorbed orally and topically and are actively excreted into the urine of healthy subjects (Monteiro-Riviere et al., 2000, 2003; Charuk et al., 1998). It has been suggested that long term exposure to NPEs might have an impact on the reproductive systems in various animal species including humans (Kang et al., 2003), in that it exhibits a potent spermicidal effect in the testis (Kang et al., 2003). The inhibition of $Ca^{2+}$ pumps on the testis endoplasmic reticulum has been suggested as a possible mechanism for this spermicidal effect (Monteiro-Riviere et al., 2003; Charuk et al., 1998; Minami et al., 2000). Recent reports suggest that NPEs may function as hormone disrupters (Monteiro-Riviere et al., 2003; Minami et al., 2000). Most interestingly, NPEs have been reported to be potential *Plasmodium falciparum* P-gp substrates and drug efflux inhibitors, and are under examination as reversal agents for malarial quinoline resistance (Crandall et al., 2000; Ciach et al., 2003).

Biological membranes serve as barriers against the permeation of xenobiotics into the systemic circulation or specific organs. Membrane junction complexes between the epithelial cells of the membrane, such as desmosomes, adherens junctions and tight junctions, appear to constitute the barrier function, with the tight junction making the major contribution (Erwin et al., 1999; Knipp et al., 1997). In general, surfactants are known to lower the barrier function of biological membranes through affecting the integrity (Eva et al., 1992) of the junction complexes. Thus, the effect of NPEs, a new category of surfactants, on the barrier function of tight junctions was studied. This is of special interest in that the tight junction contributes to the blood–testis barrier (Erwin et al., 1999; Holash et al., 1993; Janecki et al., 1992). In addition to the tight junction, the P-glycoprotein (P-gp), an ATP-dependent efflux pump, often also functions as a barrier against the permeation of xenobiotics across biological membranes (Monteiro-Riviere et al., 2003). NPEs are known to be substrates of P-gp (Charuk et al., 1998; Loo and Clarke, 1998). Thus, it seems reasonable to assume the existence of an interaction between NPEs and P-gp, which may influence the barrier function of the cell membranes. In this regard, the effect of NPEs on the function of P-gp was also investigated in the present study.

Information on this matter, as well as on the toxicity (Swenson et al., 1994a; Harrison et al., 1997), will aid in developing a better understanding, for example, of the effect of NPEs on the reproductive systems (Kang et al., 2003) and of the mechanism for malarial quinoline resistance reversal (Crandall et al., 2000; Ciach et al., 2003). The Caco-2 cell monolayer was utilized as a model biological membrane that contains tight junctions and the P-gp system.

## 2. Materials and methods

### 2.1. Materials

Unradiolabeled daunomycin was a kind gift from Dong-A Pharm. Co. (Kyounggi-do, Korea). [$^3$H]-Daunomycin (4.4 Ci/mmol) and [$^{14}$C]mannitol (50 mCi/mmol) were purchased from New England Nuclear Life Science Products (Boston, MA). Fetal bovine serum was purchased from Hyclone Laboratories (Logan, UT). Trypsin-EDTA was purchased from Gibco Laboratories (Gaithersburg, MD). NPEs which contain 4, 7, 9, 10 and 35 ethoxylates (i.e., NP-4, NP-7, NP-9, NP-10 and NP-35, respectively), Dulbecco's Modified Eagles's medium, nonessential amino acid solution, penicillin–streptomycin, Hank's balanced salt solution (HBSS), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 2-*N*-morpholinoethane sulfonic acids (MES), D-glucose and sodium bicarbonate were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade.

### 2.2. Cell culture

Caco-2 cells (passage 45–55, American Type Culture Collection, Rockville, MD) were grown in the

form of monolayers in Dulbecco's modified Eagles' medium, 10% fetal bovine serum, 1% nonessential amino acid solution, 100 units/ml penicillin, and 0.1 mg/ml streptomycin at 37 °C in an atmosphere of 5% $CO_2$ and 90% relative humidity. For the transport experiments, cells were seeded on permeable polycarbonate inserts (1 $cm^2$, 0.4 μm pore size, Corning Costar Co., Cambridge, MA) in 12-Transwell plates (Corning Costar Co.) at a density of 1–1.5 × $10^5$ cells/$cm^2$. The inserts were fed with complete media at 2-day intervals during the first week, and then daily until they were used for the transport experiments 18–25 days after the seeding (Li et al., 2001).

The integrity of the cell monolayers was evaluated by measuring transepithelial electrical resistance (TEER) values with an EVOM$^{TM}$ epithelial volt/ohmmeter (World Precision Instruments, Sarasota, FL). When the TEER value reached 300–600 Ω $cm^2$, the transport of [$^{14}$C]mannitol (10 μM) became <0.25% of the dose/h, corresponding to an apparent permeability ($P_{app}$) value of 5.6 × $10^{-7}$ cm/s. Cell inserts which showed such TEER values were used for the transport experiments. For the cellular uptake study, Caco-2 cells were grown on collagen-coated 12-well plates (4 $cm^2$) for 12–15 days and the medium was replaced at 2-day intervals (Li et al., 2001).

### 2.3. Transepithelial transport of mannitol and TEER

Effect of NPEs on the apical to basolateral (A–B) transport of mannitol (i.e., mannitol leakage) across Caco-2 cell monolayers, as well as the onset time and the reversibility of the effect, was investigated. Prior to the transport experiments, the cell monolayers were washed three times with incubation medium (pH 7.4, HBSS containing 25 mM HEPES and 25 mM D-glucose). After each wash, Caco-2 cell monolayers were incubated in incubation medium for 30 min at 37 °C, and the TEER value was then measured (Li et al., 2001; Maeng et al., 2002). Incubation medium on both sides of the cell monolayers was then removed by aspiration. For measurement of the apical to basolateral transport of mannitol, 0.5 ml of incubation medium containing [$^{14}$C]mannitol (10 μM) in the presence of various NPEs (i.e., NP-4, NP-7, NP-9, NP-10 or NP-35, 1 nM each) or various concentrations of NP-9 (i.e., 0.01–100 nM) was added to the apical side, and 1.5 ml of incubation medium without these compounds was added to the basolateral side. The inserts were moved to wells containing fresh incubation medium every 15 min for 1 h. After the experiments, TEER value of each well was checked. The radioactivity of a 1-ml aliquot of the basolateral side of each well was determined by liquid scintillation counter (Wallac 1409, Perkin-Elmer Life Science Inc., Boston, MA).

For measurement of onset time for NP-9 to affect the tight junction of biological membranes, mannitol leakage was measured after preincubation of the monolayer with 10 nM NP-9 for 2, 4, 6, 10, 20 or 60 min. The reversibility of the effect of NPEs on the tight junction was examined by measuring mannitol leakage for monolayer pretreated with 10 nM NP-9 for short term (i.e., 2 h) or long term (i.e., 72 h) period. A period of 72 h was selected based on our previous study in which the pretreatment of Caco-2 cells for 72 h, but not for 24 or 48 h, with a P-gp substrate, berberin, induced the expression of P-gp (Maeng et al., 2002). The pretreatment was performed by further incubating the cell monolayers, cultured on permeable inserts in Transwell plates for 18 days, at 37 °C for 2 h in transport medium or 72 h in culture medium containing 10 nM NP-9 in the apical side. After each pretreatment, the monolayers were replaced with fresh incubation medium without NP-9 at 30 min intervals for 2 h (i.e., four times), and the leakage of [$^{14}$C]mannitol (10 μM) in the A–B direction across the cell monolayer was measured for 1 h. Effect of the long term (72 h) pretreatment on cell toxicity was also examined by measuring protein concentration in the cells by Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard, and cell viability by trypan blue exclusion method (Han et al., 1999).

### 2.4. Transepithelial transport of daunomycin

Effect of pretreatment of Caco-2 cell monolayers with NP-9 on the transepithelial transport of daunomycin was measured. The pretreatment of the cell monolayers for 72 h was performed as mentioned in the uptake section. For measurement of the apical to basolateral transport of daunomycine, 0.5 ml of incubation medium containing [$^{3}$H]daunomycin (1 μM) was added to the apical side, and 1.5 ml of incubation medium without daunomycin was added to the basolat-

eral side. The subsequent procedures were identical to the measurement of A–B transport of mannitol. For the measurement of basolateral to apical (B–A) transport of daumomycin, 1.5 ml of incubation medium containing [$^3$H] daunomycin (1 μM) was added to the basolateral side, and 0.5 ml of incubation medium without daunomycin was added to the apical side. Incubation medium in the apical side was replaced with 0.3 ml of fresh incubation medium every 15 min for 1 h. A 300-μl aliquot of the apical side was taken after each replacement, and the radioactivity was measured by liquid scintillation counting.

### 2.5. Cellular uptake of daunomycin

Effect of NPEs on the cellular uptake of daunomycin, a P-gp substrate, was investigated in two ways, i.e., in the presence of NP-9 and after the pretreatment of the cell with NP-9. First, the cellular uptake of daunomycin, in the presence or absence of NP-9, was measured at 37 °C for Caco-2 cells grown for 15 days on 12-Transwell plates. After washing each well plate three times with 2 ml of incubation medium, the medium was removed and the uptake was initiated by the addition of 2 ml of incubation medium containing [$^3$H]daunomycin (1 μM) with (100–1000 nM) or without NP-9. At 5 min after the initiation, the medium was immediately removed by aspiration and replaced with fresh cold (4 °C) incubation medium containing 1 mM daunomycin three times in order to wash out any [$^3$H]daunomycin that might be adsorbed to the surface of the cell monolayers. One milliliter of a cell lysis solution (1% Triton-X 100 in 1 M NaOH) was then added to the well and lysed overnight at room temperature. The radioactivity in a 0.8-ml aliquot was determined by LSC.

Effect of pretreatment with NPEs on the cellular uptake of daunomycin was then investigated. The uptake for 5 min was measured as above described after the pretreatment of Caco-2 cell monolayers, which were cultured in 12-well plates for 15 days, were further cultured with culture medium containing NP-9 (1, 50, 100 and 100 nM) for 72 h at 37 °C.

### 2.6. Data analysis

In the transport studies with mannitol and daunomycin, the apparent permeability coefficients ($P_{app}$) were calculated in both apical to basolateral and basolateral to apical directions using the equation below:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A \times Co} \quad (1)$$

where the $dQ/dt$ (nmol/min) is the drug permeation rate, $A$ the surface area of the monolayer membrane (1 cm$^2$) and Co (μM) is the initial mannitol or daunomycin concentration in the donor compartment. Three monolayers were used in the determination of the means (±S.D.). The statistical significance of differences between treatments was evaluated using unpaired Student's $t$-test, and a value of $P < 0.05$ was considered to be statistically significant.

## 3. Results and discussion

### 3.1. The effect of ethoxylate number in NPE molecules on mannitol leakage

The A–B transport of mannitol (10 μM) was essentially linear for periods of up to 60 min regardless of the presence of various NPEs (i.e., NP-4, NP-7, NP-9, NP-10 and NP-35) in the apical side (Fig. 2A). The presence of NPEs (1 nM, each) in the apical side, generally led to an increase in the leakage of mannitol across Caco-2 cell monolayers, leading to a 1.3–3.3-fold increase in permeability compared to the control value (i.e., mannitol leakage in the absence of NPEs) (Fig. 2B). The effect was variable depending on the number of ethoxylate groups in the NPE molecules and a maximal effect was found for the 9 ethoxylates (i.e., NP-9). Thus, NP-9 was selected as a representative NPE in the subsequent experiments. A similar result was reported for a rat intestinal perfusion model, in which 1% (w/v) NPEs with 9–20 ethoxylates most increased the permeability of phenol red across the intestine (Swenson et al., 1994a).

### 3.2. The effect of NP-9 concentration on mannitol leakage and TEER values

The leakage of mannitol in the A–B direction across Caco-2 cell monolayers increased with increasing concentrations of NP-9 in the apical side from 0.01 to 100 nM (Fig. 3A). For example, more than

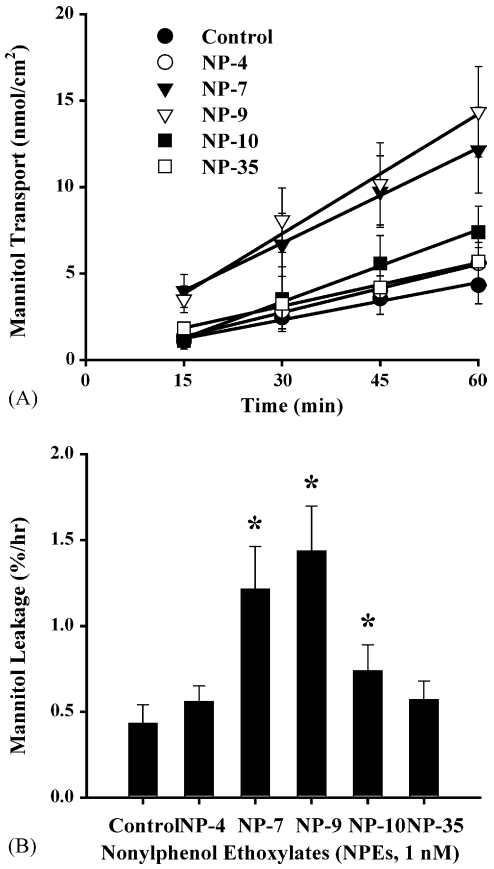


Fig. 2. Effect of 1 nM NPEs on apical to basolateral transport (A) and leakage (B) of [$^{14}$C]mannitol (10 μM) across Caco-2 cell monolayers. Each point and bar represents the mean ± S.D. of four experiments. *Significantly different from the control value ($P < 0.05$).

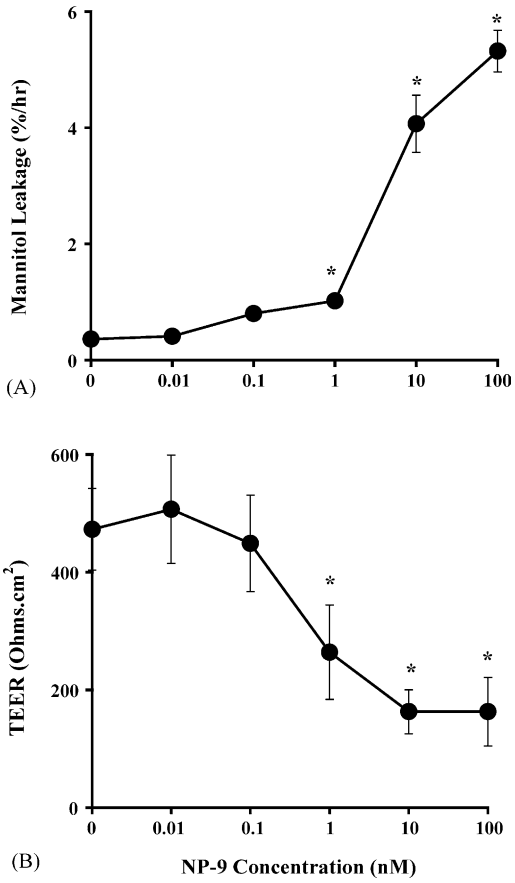


Fig. 3. Effect of NP-9 concentrations on [$^{14}$C]mannitol leakage (10 μM) (A) and TEER values (B) across Caco-2 cell monolayers. Each point and bar represents the mean ± S.D. of four experiments. *Significantly different from the control value ($P < 0.05$).

a 15-fold increase in mannitol leakage was observed in the presence of 100 nM NP-9. Consistent with these results, the TEER values were decreased with increasing concentrations of NP-9 in the apical side (Fig. 3B). The above results suggest that NPEs, as represented by NP-9, open the tight junctions and reduce TEER values across Caco-2 cell monolayers, thereby increasing the permeability of mannitol probably via the paracellular pathway. Thus, NPEs appear to lower the barrier function of biological membranes via opening the tight junction of the paracellular pathway of epithelial cells at least for hydrophilic small molecules.

### *3.3. The onset time for NP-9 to open the tight junctions*

In order to determine the onset time for NP-9 to open the tight junctions, the effect of NP-9 on the A–B transport of [$^{14}$C]mannitol (10 μM) across Caco-2 cell monolayers was examined for 1 h after preincubation of the cell monolayer as a function of incubation time (2, 4, 6, 10, 20 and 60 min) in the presence of 10 nM NP-9. Mannitol leakage increased with increasing NP-9 incubation times with a maximal rate for the first 10 min period (Fig. 4). Even when the incubation time of 6 min was used with 10 nM NP-9, a significant increase in the leakage of mannitol

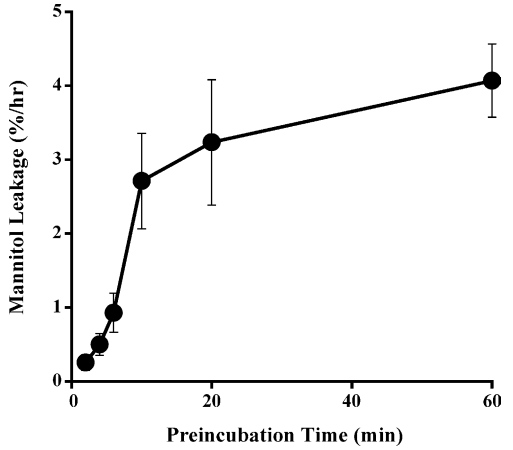


Fig. 4. Effect of NP-9 (10 nM) as a function of incubation time on [$^{14}$C]mannitol leakage (10 μM) across Caco-2 cell monolayers. Each bar represents the mean ± S.D. of four experiments. *Significantly different from the control value ($P < 0.05$).

was observed ($0.36 \pm 0.05\%\,h^{-1}$ versus $0.92 \pm 0.26\%\,h^{-1}$). This indicates that the effect of NP-9 on the tight junction of Caco-2 cell monolayers is manifested rapidly.

### *3.4. The reversibility of the effect of NP-9 on the tight junction*

The reversibility of the effect of NP-9 pretreatment on the tight junction of biological membranes was examined following pretreatment of Caco-2 cell monolayers with 10 nM NP-9 for short term (2 h) and long term (72 h) periods. After the short term exposure (i.e., 2 h incubation), mannitol leakages and TEER value across the monolayer were reversed to the control level (Fig. 5A and B), indicating the effect of NP-9 on the membrane is fairly reversible under the given pretreatment conditions (i.e., 2 h incubation). The above results appear to be consistent with the fact that surfactants generally enhance the permeability of xenobiotics across the intestinal wall in a reversible manner (Swenson et al., 1994b). However, the long term exposure (i.e., 72 h incubation) of NP-9 increased mannitol leakages (Fig. 5A), with a corresponding decrease in TEER values (Fig. 5B), suggesting that the effect of long term exposure NP-9 on mannitol leakage is irreversible. The long term exposure of NP-9, however, had no effect on the content of protein in the

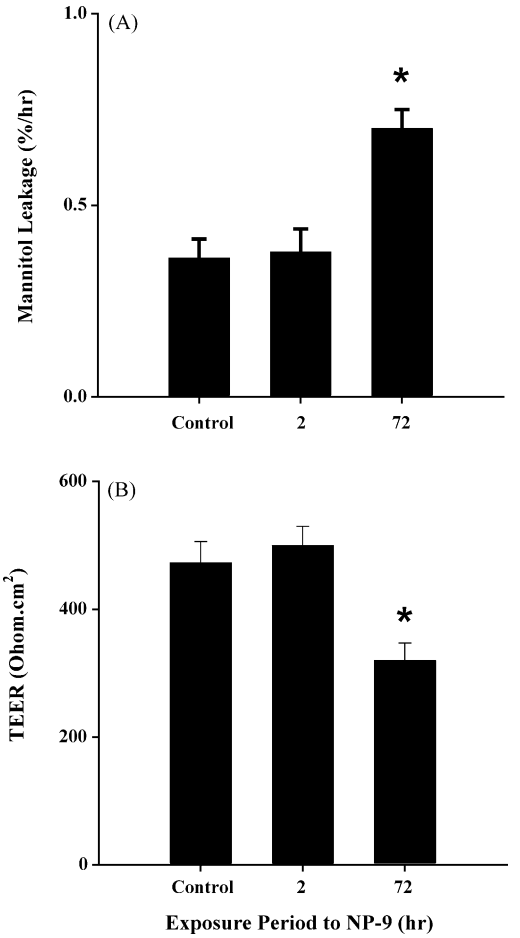


Fig. 5. Effect of exposure (2 and 72 h) to NP-9 (10 nM) on mannitol leakage (10 μM) (A) and TEER values (B) of the Caco-2 cell monolayers. Each point represents the mean ± S.D. of four experiments. *Significantly different from the control value ($P < 0.05$).

cells ($74 \pm 11$ μg/ml for control versus $80 \pm 5$ μg/ml for 72 h incubation), or the viability of the cells (>93% viability by the 72 h incubation). It is not clear from the above results, whether the NP-9 interacts directly with the junctional complexes or affects intracellular events. However, inhibition of $Ca^{2+}$ pumps by NPEs (Monteiro-Riviere et al., 2003) and subsequent disruption of tight junctions (Ma et al., 2000) in the absence of $Ca^{2+}$ ion is likely to represent the responsible mechanism. In short, the effect of NPEs on the tight junction appears to vary depending on the period of the pretreatment, i.e., reversible for short term exposure and irreversible for long term exposure to NP-9.

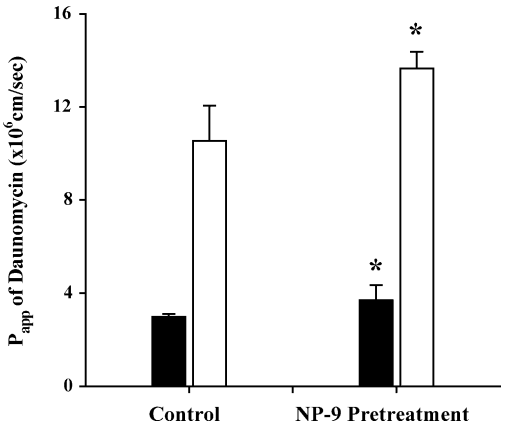


Fig. 6. Effect of pretreatment with 10 nM NP-9 for 72 h on the permeability of 1 μM [$^3$H]daunomycin across Caco-2 cell monolayers in the apical to basolateral (■) and basolateral to apical (□) directions. Each bar represents the mean ± S.D. of four experiments. *Significantly different from respective control values ($P < 0.05$).

### 3.5. The effect of pretreatment with NP-9 on the transepithelial transport of daunomycin

It is known that P-gp substrates frequently induce the expression of P-gp and accelerate the function of the transporter (Hauser et al., 1998). Thus, the issue of whether the pretreatment of Caco-2 cells with NP-9 induces the transport activity of P-gp was investigated. The vectorial transports of 1 μM daunomycin, a representative P-gp substrate (Hooiveld et al., 2001; Song et al., 2003), across Caco-2 cell monolayers were measured prior to and after pretreatment of the monolayer with 10 nM NP-9 for 72 h. In the absence of the pretreatment, the B–A permeability of daunomycin was approximately 3.5-fold larger than the A–B permeability (Fig. 6), consistent with the fact that daunomycin is a typical substrate of P-gp (Hooiveld et al., 2001; Song et al., 2003). Interestingly, both A–B and B–A permeabilities of daunomycin were slightly increased by the pretreatment (Fig. 6). However, this change appears to be attributable to the opening of tight junctions, as demonstrated by Figs. 3–5, rather than to P-gp associated changes, since induced expression or accelerated function of P-gp, for example, would have decreased the A–B permeability of daunomycin, contrary to the present case (i.e., Fig. 6). Therefore, no firm conclusion concerning the effect of P-gp expression can be made from the results of the transport studies, necessitating relevant uptake experiments.

### 3.6. The effect of presence of NP-9 on the uptake of daunomycin

The effect of NP-9 on the barrier function of P-gp was also investigated. The uptake of 1 μM [$^3$H]daunomycin increased in the presence of NP-9 in incubation medium with increasing NP-9 concentrations (100, 500 and 1000 nM), suggesting that NP-9 inhibits the P-gp-mediated efflux of daunomycin (Fig. 7A). This is consistent with the fact that NPEs are

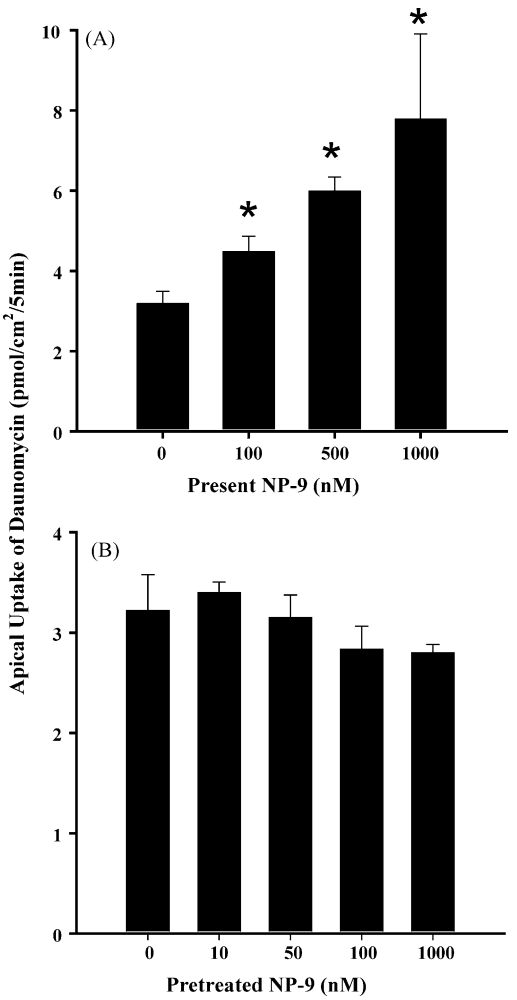


Fig. 7. Effect of the presence (A) and 72 h pretreatment (B) of/with various concentrations of NP-9 on the apical uptake of 1 μM [$^3$H]daunomycin for 5 min into Caco-2 cell monolyaers. Each bar represents the mean ± S.D. of four experiments. *Significantly different from the control value ($P < 0.05$).

P-gp substrates (Charuk et al., 1998; Loo and Clarke, 1998). Thus, the efflux function of P-gp would be expected to be perturbed by the presence of NPEs, thereby reducing the protection of the body from xenobiotics and hazardous environmental compounds (Charuk et al., 1998). NPEs have been reported to reverse the malarial quinoline resistance (Crandall et al., 2000; Ciach et al., 2003). The inhibition of quinoline efflux via P-gp from *P. falciparum* in the presence of NPEs might be associated with the reversal mechanism. The complete reversal of chloroquine resistance by verapamil (Martin et al., 1987), a representative P-gp substrate, supports this hypothesis.

### *3.7. The effect of pretreatment with NP-9 on the uptake of daunomycin*

In order to investigate the effect of NP-9 pretreatment in association with P-gp expression, the apical uptake of daunomycin into Caco-2 cells was measured. In the present study, the apical uptake of 1 μM daunomycin into Caco-2 cells for 5 min was not changed significantly by the 72 h pretreatment with NP-9 (1, 50, 100 and 1000 nM) (Fig. 7B), suggesting that pretreatments with NPEs did not significantly induce the expression of P-gp nor accelerated the function of the transporter in the cell. In accordance with this result, the pretreatment had no effect on the content of protein in the cells. Neither the viability of the cells was changed (>93% by the pretreatment), indicating negligible toxicity or damage to the cells for this pretreatment. Taken together with transport results (Fig. 6), the pretreatment of Caco-2 cells under the present conditions (at NP-9 concentration at or below 1000 nM, and for the pretreatment period of 72 h) does not appear to influence the expression of P-gp, but increases the paracellular permeability of daunomycin in both directions.

## 4. Conclusion

The presence of NPEs appears to rapidly widen the tight junction of epithelial cell membranes and to competitively inhibit the efflux function of the epithelial P-gp system, thereby reducing the ability of the body to protect itself against xenobiotics or environmental hazards that are transported via the paracellular pathway or the P-gp system. The effect of NPEs on the tight junction or paracellular pathway of Caco-2 cell monolayers varied depending on the pretreatment period, i.e., reversible for a short term (e.g., 2 h) treatment while irreversible for a long term (e.g., 72 h) pretreatment. NPEs do not appear to induce the expression of P-gp or accelerate the function of P-gp systems on the biological membranes. This is the first study that reports the effect of NPEs in a cell monolayer level. The relevance of the opening of the tight junction and competitive inhibition of the P-gp mediated transport in the cell monolayer system to their reported toxicity in the body (e.g., spermicidal effect) and reversal activity for malarial quinoline resistance awaits further study.

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