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In vitro tolerance of mononitrates and effects on aggregation of blood platelets

ENRICO LAMPA, AMELIA FILIPPELLI

Department of Experimental Medicine, Section of Pharmacology "Leonardo Donatelli", Second University of Naples, Naples - Italy

ABSTRACT: Long-term therapy with organic nitrates is frequently associated with a progressive reduction of hemodynamic effects (nitrate tolerance) (4-7). This induces a major limitation in terms of efficacy on nitrate therapy for myocardial infarction, stable angina and congestive heart failure. Recent studies have demonstrated that the development of in vitro tolerance is mainly, and quickly, induced by nitrates with high potency type and depends on the number of nitrate groups present on the molecule, pentaerythritol tetranitrate (PETN) or glyceryl trinitrate (GTN) are more potent than mononitrates (isosorbide-5-mononitrate (IS-5-MN) and isosorbide-2-mononitrate (IS-2-MN)). Mononitrates have been used for many years in cardiovascular therapy and they demonstrate a very interesting profile for better pharmacokinetic parameters in respect of the old nitrates. In delivering the exogenous nitric oxide (NO), they are an attractive therapeutic option, particularly with a view to slowing the progression of atherosclerosis and reducing the risk of thrombosis. The multiple actions of nitrates are the summarized inhibition of vascular smooth muscle cells (VSMCs) and migration, limiting the development of the complex plaque, inhibition of platelet activation, aggregation and adhesion to the area of endothelial damage, reducing the extent of thrombosis. In this study, the mononitrates induced tolerance in vitro as documented for other nitrates but to a lesser degree. The antiplatelet effects were demonstrated to be greater with IS-2-MN than IS-5-MN. Although the mononitrates induced tolerance, it has been demonstrated that the anti-aggregatory effects are not reduced after chronic treatment, and this is an important factor in protecting the ischemic patient from cardiac injury and the high risk of thrombosis in acute coronary syndrome. (Heart International 2007; 3: 122-8)

KEY WORDS: Nitrate tolerance, Platelet aggregation, Isosorbide-5-mononitrate, Isosorbide-2-mononitrate, Aortic rings, Nitric oxide

INTRODUCTION

The main mechanism underlying the anti-ischemic and cardioprotective effects of mononitrates is the relaxation of vascular smooth muscle cell (VSMC), determining in normal subjects and in patients with coronary disease dilation of the coronary arteries (coronary dilatory effect), systemic veins (venodilator effect), and systemic arterioles (arteriolar effect) (1-3).

The vasodilation action mechanism of exogenous nitrovasodilators is similar to that of nitric oxide (NO), the endogenous nitrovasodilator; they activate soluble guanylyl cyclase with the consequent production of cGMP (cyclic guanosine-3',5'-monophosphate). The peculiarity of nitrovasodilators to act directly on VSMCs, skipping the endothelium, is an important feature when compared to other vasodilators requiring the functional integrity of the endothelium.

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Long-term therapy with organic nitrates is frequently associated with a progressive reduction in hemodynamic and anti-aggregatory effects (nitrate tolerance) (4-8). This induces a major limitation in terms of efficacy on nitrate therapy for acute myocardial infarction, stable angina and congestive heart failure. Multifactorial mechanisms are involved in these phenomena:

– neurohumoral counterregulatory mechanism opposing NO-mediated vascular relaxation (pseudo-tolerance) (8);
– increases in phosphodiesterase 1A1 activity (9);
– desensitization of the sGC (10);
– increase in the production of reactive oxygen species (ROS) (11);
– impairment of glyceril trinitrate (GTN) biotransformation (true or classical tolerance) (12).

Recently, it has been demonstrated that the mitochondrial isoform of aldehyde dehydrogenase (ALDH-2) is primarily responsible for the biotransformation of GTN and that this process involves redox-sensitive thiol groups.

The clinical relevance of this concept was demonstrated by studies on individuals of East Asian origin with the mutation of ALDH-2 rendering the enzyme inactive, who show a significantly decreased responsiveness to GTN (13-15).

The list of enzymatic mechanisms of GTN metabolism and biotransformation includes glutathione S-transferases (16), the cytochrome p450 system (17), xanthine oxidoreductase (18) and, the above-mentioned mitochondrial ALDH-2 (12). A recent study demonstrated that the vasorelaxant responses of isolated porcine pulmonary arteries to different types of NO-based vasodilators increased from mononitrates to tetranitrates. The most potent nitrates were pentaerythritol tetranitrate (PETN), PEtriN, and GTN. These findings agree with previous organ-bath studies on isolated vessels from different species, such as rabbit aorta and bovine coronary arteries, resulting in pD2 values for the relaxant effects which are in the same range as those found in this study. The dinitrates and mononitrates were less effective than the tetranitrates and trinitrates, similar results were shown by other groups with rat and rabbit aortas. The dinitrate and mononitrate of isosorbide exhibit a lower relaxant response than the corresponding tetranitrates and trinitrates and again, the same results were obtained in other studies on different isolates of different species (19, 20). Moreover, the results confirmed that the mechanism underlying the vasorelaxant response is mediated by the activation of guanylyl cyclase; and therefore, by increasing the formation of cyclic GMP (cGMP). Investigations into the development of tolerance in vitro have demonstrated that dinitrates and mononitrates developed little or no tolerance compared to tetranitrates and trinitrates such as PETN, PEtriN and GTN (21, 22). Therefore, in vitro tolerance increases with the number of nitrate groups in the molecule and hence with the potency of the nitrates. Within the organic nitrates, only the high-potency compounds GTN, PETN and PEtriN undergo ALDH2-catalyzed bioactivation, and they are sensitive to the development of in vitro tolerance, unlike the low-potency dinitrates and mononitrates. However, the in vitro tolerance allows only a few insights into the mechanism of clinical tolerance caused by chronic treatment; additional factors may play an important role in vivo. There are various reports in the literature that therapy with PETN does not induce oxidative stress and this is supposed to be a key factor in preventing clinical tolerance. In vitro findings are in contrast to the therapeutic use of PETN, which requires high oral dosage and does not develop significant tolerance. The missing bioavailability for PETN due to poor absorption or a fast hydrolytic degradation to less vasoactive metabolites may explain the discrepancy between in vitro and in vivo. Therefore, according to the in vivo findings, future in vitro studies on the pharmacological effects of PETN should also include the effects of PEdiN and PEmonoN. These two metabolites may contribute to the moderate, long-acting, and tolerance-devoid activity of the PETN.

Mononitrates such as isosorbide-5-mononitrate (IS-5-MN) and isosorbide-2-mononitrate (IS-2-MN) are isosorbide dinitrate's (ISDN) pharmacologically active catabolites (23). Compared with ISDN, mononitrate pharmacokinetics are markedly different because of the greater systemic availability and volume of distribution of the mononitrates and slower clearance. Absorption of ISDN and mononitrates after oral dosing is nearly complete. The average bioavailability of ISDN is about 48%, but is highly variable (10-90%) due to first-pass metabolism and progressively increases during chronic therapy. Serum concentrations reach their maximum about 1 hr after ingestion. Most ISDN is eliminated renally as conjugated metabolites. The volume of distrib-

ution of ISDN is 2-4 L/kg. About 28% of circulating ISDN is protein bound. Under steady-state conditions, ISDN accumulates significantly in muscle (pectoral) and vein (saphenous) wall relative to simultaneous plasma concentrations (24, 25).

The IS-5-MN has an overall half-life of about 5 hr. The IS-2-MN has a half-life of about 2 hr. Peak plasma concentrations of the two active metabolites, IS-2-MN and IS-5-MN, are 98 and 364 ng/mL/65kg, respectively, at about 2 hr. Unlike other vasodilator organic nitrates in clinical use, IS-5-MN is notable for its relatively long t½, essentially complete oral absorption, lack of active metabolites, and low intersubject variability in kinetics (24). The potency of action of IS-2-MN is placed between ISDN and IS-5-MN and it shares the non-hemodynamic effects of other nitrates which have the capacity to inhibit the platelet aggregability as in vivo than in vitro (26-34).

Holmes et al (35) have shown that chronic therapy with either ISMN or transdermal nitroglycerin (TD-NTG) is associated with the development of vascular tolerance. Despite the induction of vascular tolerance, platelet responsiveness remains unaffected. Therefore, the development of vascular tolerance is unlikely to compromise the antiaggregatory effects of organic nitrates, or those of endogenous NO. This is the first human study to demonstrate differential susceptibility to tolerance induction at the vascular vs. platelet levels, but a previous investigation by Booth et al in rats, reached similar conclusions (36). The fact that platelets are less susceptible to tolerance induction than blood vessels is of potential clinical importance, as it would suggest ongoing protective effects against thrombosis in acute coronary syndromes irrespective of continuous nitrate administration.

Another interesting effect of mononitrate is that the anti-platelet activity in vivo appears and it is significant when high doses of mononitrate are used (31, 35). A further important conclusion from the above study is that there was no 'rebound' hyper-aggregability of platelets during 'nitrate-free' periods supports the lack of development of pseudo-tolerance at the platelet level during chronic nitrate exposure, such as might occur if plasma concentrations of pro-aggregating agents such as norepinephrine increased during chronic therapy (35).

In this study, we compared the in vitro effects of IS-2-MN and IS-5-MN on blood vessel tolerance and platelet aggregation.

In vitro tolerance of mononitrates

Preparation of aortic rings: Male Wistar rats were anesthetized with diethyl ether and sacrificed with a small animal guillotine. The thoracic aorta was immediately excised and placed in physiological salt solution (PSS) of the following composition (mM): NaCl 139.2, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.49, $NaHCO_3$ 11.9, $NaH2PO_4$ 0.4 and glucose 5.5, maintained at room temperature. The thoracic aorta was carefully cleaned of adhering fat, and connective tissue removed and cut into transverse rings (4 mm). Aortic rings were mounted, under 2 g resting tension, in an organ bath (Ugo Basile, Milan, Italy) containing 710 ml of solution and maintained at 37 °C, gassed with 95% O_2 and 5% CO_2. Isometric measurements were recorded with a Grass FTO3C transducer and displayed on PowerLab (AD Instruments, Castle Hill, Australia). The tissue was allowed to equilibrate for 60 min before experiments were carried out, during which time the resting tension was readjusted to 2 g, as required.

Experimental protocol: The aortic rings were submaximally contracted with 100 nM norepinephrine (NE). The absence of endothelium was verified by the inability of 1 mM acetylcholine (Ach) to induce relaxation. At the end of another equilibration period of 1 hr, the preparations were rendered tolerant to IS-2-MN and IS-5-MN. Briefly, to induce the tolerance, the rings were incubated for 45 min with IS-2-MN and IS-5-MN (3.5×10^{-2}M). Control rings that had not been exposed previously to nitrates, but were otherwise treated identically, were studied simultaneously. After an incubation period of 45 min (with or without nitrates), the aortic rings were allowed to equilibrate for 60 min and washed every 10 min with PSS. After that, the rings were contracted with NE (100 nM). When the contractions had reached a stable plateau, relaxation responses to IS-2-MN and to IS-5-MN were obtained.

Drugs: NE, Ach chloride, and all the other reagents and compounds used for Krebs' solution were obtained from Sigma Aldrich (Milan, Italy). IS-2-MN and IS-5-MN were generously provided by Luso Farmaco Institute of Italy (Milan, Italy).

Data analysis: Relaxation responses are expressed as a percentage of the initial tension induced by NE. For each vasodilator, the concentration necessary to produce 50% of its own maximal response (EC_{50}) was de-

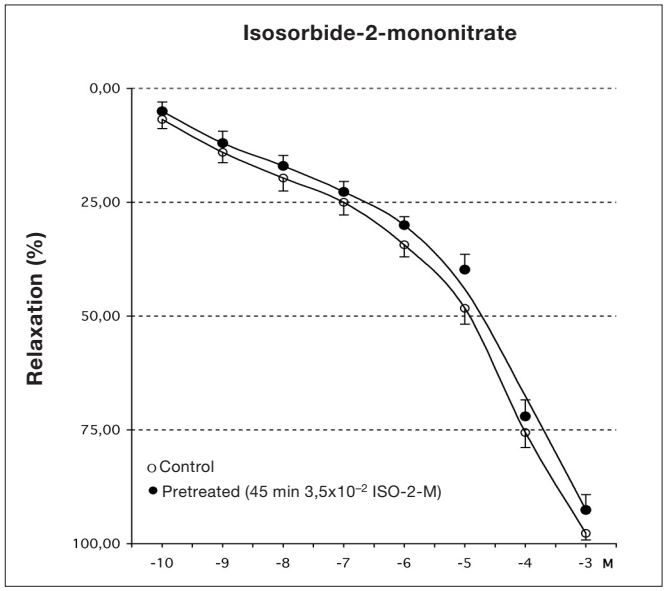

Fig. 1 - *Dose-effect curves for rat thoracic aortic rings preconstricted with NA 100 nM. Different concentrations of isosorbide-2-mononitrate (10^{-10} to 10^{-3} M) were added to preconstricted thoracic aortic rings that had been pretreated (•) or not (º) with 3.5 x 10^{-2} M isosorbide-2-mononitrate for 45 min. The concentrations (log M) of isosorbide-2-mononitrate are shown on the x axis, while the percentage relaxation is shown on the y axis.*

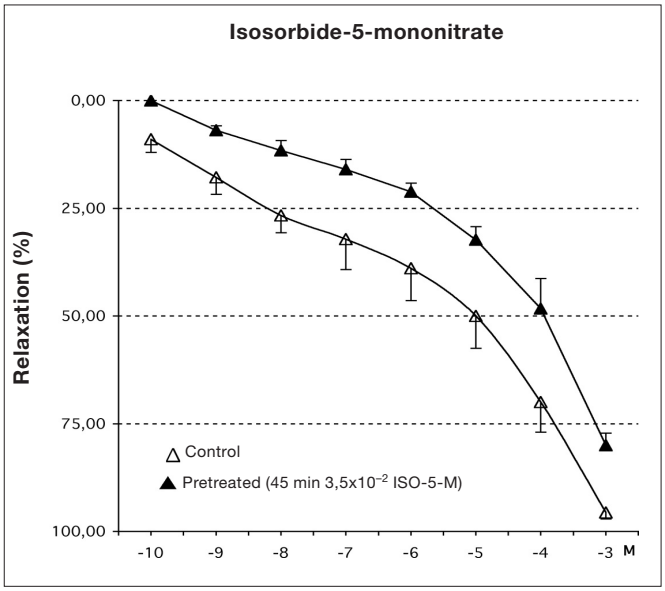

Fig. 2 - *Dose-effect curves for rat thoracic aortic rings preconstricted with NA 100 nM. Different concentrations of isosorbide-5-mononitrate (10^{-10} to 10^{-3} M) were added to preconstricted thoracic aortic rings that had be pretreated (▲) or not (Δ) with 3.5 x 10-2 M isosorbide-5-mononitrate for 45 min. The concentrations (log M) of isosorbide-5-mononitrate are shown on the x axis, while the percentage relaxation is shown on the y axis.*

termined. The EC_{50} values were converted to the negative logarithms and expressed as -log molar EC_{50}. Results are expressed as meansSEM and *n* refers to the number of animals from which blood vessels were taken. Agonist potencies and maximal effects were compared by the Student's t-test. Values were considered to be significantly different when $p<0.05$. The tolerance was defined as a ratio between EC_{50} obtained in the presence of nitrates and EC_{50} obtained in the absence of nitrates minus one.

Results: Figures 1 and 2 show the effects of incubation (45 min) with IS-2-MN and IS-5-MN (35 mM), respectively, on the concentration curve, of isolated rat aorta, contracted with 100 nM NE.

The incubation of aortic rings with IS-2-MN and IS-5-MN (35 mM) for 45 min and the subsequent washout with saline for 1 hr, and the addition of 100 nM NE induced a contraction. As shown in Table I and Figure 1, incubation with IS-2-MN for 45 min did not change the concentration response curve for the relaxant effect of IS-2-MN (100 pM 1 mM) on NE (100 nM) induced contraction, compared with the control and tolerance was equal to 0.53.

TABLE I - HALF MAXIMAL EFFECTIVE CONCENTRATION 50 (EC_{50}) AND TOLERANCE INDEX OF IS-2-MN (10^{-10}-10^{-3} M) AND IS-5-MN (10^{-10}-10^{-3} M) ON ISOLATED AORTIC RINGS FROM RATS PRE-CONTRACTED WITH 100 NM NE

Treatment	EC_{50}	Tolerance index (compared to the controls)
Controls	1.3x10-^{5}M	
IS-2-MN	2.0x10-^{5}M	- 0.53
Controls	1.5x10-^{5}M	
IS-5-MN	1.7x10-^{4}M	-10.3

Instead the cumulative addition of various concentrations of IS-5-MN (100 pM 1 mM) induced relaxation, and the relaxant effect was statistically changed (Tab. I, Fig. 2) compared with control and tolerance was equal to 10.3.

In conclusion, in our experimental model of in vitro tolerance induced by IS-2-MN or IS-5-MN, both drugs were able to induce tolerance on rat thoracic aorta, but IS-5-MN.

The results obtained in this in vitro experimental model show

that both active substances develop tolerance but IS-2-MN presents a lower tolerance induction than IS-5-MN.

Antiplatelet effect of mononitrates

In addition to their vasodilative activity, IS-2-MN and IS-5-MN are known to have an anti-platelet effect. It has been demonstrated that both drugs can exhibit an anti-platelet effect (1, 2). The aim of this study was to examine in vitro the effects of these two commonly used long-acting nitrate preparations on platelet aggregation compared with ASA.

Experimental protocol: Human platelet suspensions were prepared as described in the following. In this study, blood was collected from healthy human (females and males) volunteers who had taken no ASA and no other medicine that could interfere with platelet aggregation. After centrifugation at 180 g for 15 min at room temperature, the supernatant (platelet-rich plasma; PRP) was transferred to a new tube. The remaining plasma was re-centrifuged at 1200 g for 15 min to obtain platelet-poor plasma (PPP), used to get a final PRP adjusted to about 3×10^5 platelets ml^{-1}. Platelet aggregation was induced with collagen (10 µg/ml) and ADP (3 µM). The turbidimetric method was applied to measure platelet aggregation (37), using an aggregometer (II PA 3220). To assess and characterize the effect of nitrates, PRP was incubated with scalar concentrations (10^{-8}M to 10^{-3}M) of IS-2-MN or IS-5-MN. Samples were pre-warmed at 37° C for 15 min prior to induction of platelet aggregation with collagen or ADP and monitored after 5 min.

Spontaneous aggregation has been studied by placing the sample in an aggregometer with testing compounds at the different concentrations used and by recording the light transmission (%) for 5 min. The aggregation with or without the drugs was recorded as maximum peak. The percentage of platelet inhibition by drugs has been calculated as follows:

$$\% \text{ of inhibition} = \frac{\text{Aggregation control} - \text{Aggregation drugs-induced}}{\text{Aggregation control}} \times 100$$

Drugs: IS-2-MN and IS-5-MN were generously provided by the Luso Farmaco Institute of Italy.

Statistical analysis: Results are expressed as mean±SEM. Comparisons of responses were made

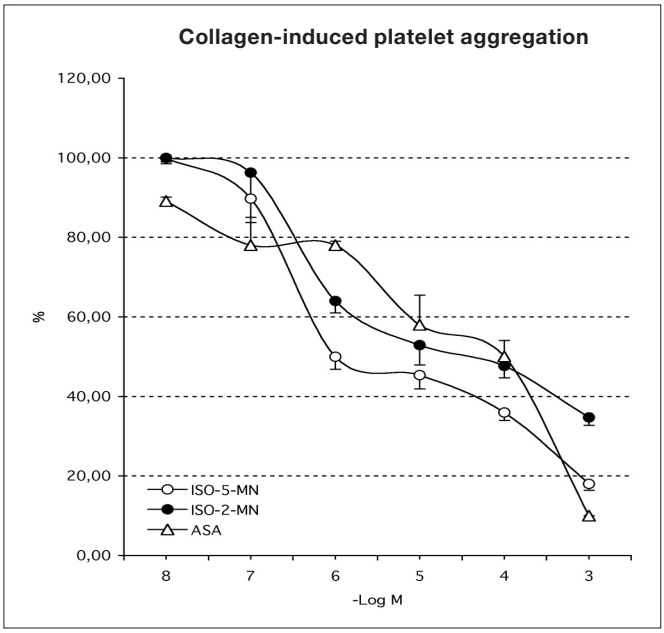

Fig. 3 - *Dose-effect curves of the inhibition of collagen-induced platelet aggregation obtained with acetylsalicylic acid (ASA), isosorbide-5-mononitrate (ISO-5-MN) and isosorbide-2-mononitrate (ISO-2-MN), considering the aggregation induced by collagen as 100%.*

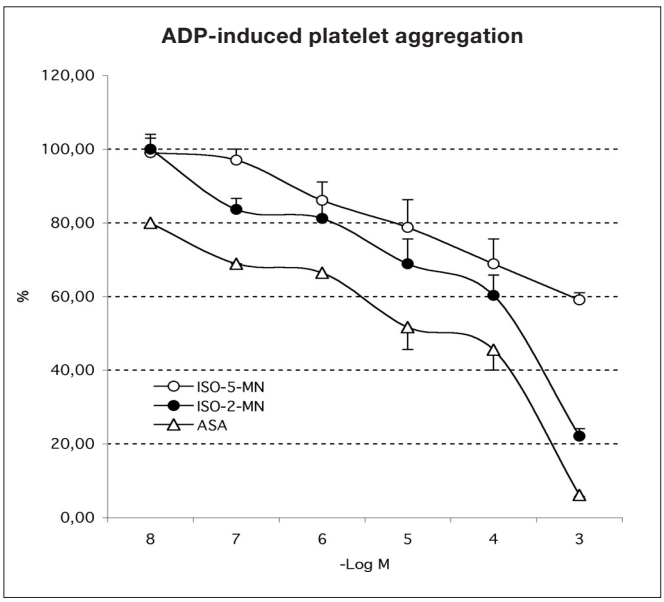

Fig. 4 - *Dose-effect curves of the inhibition of ADP-induced platelet aggregation obtained with acetylsalicylic acid (ASA), isosorbide-5-mononitrate (ISO-5-MN) and isosorbide-2-mononitrate (ISO-2-MN), considering the aggregation induced by ADP as 100%.*

using analysis of variance. Differences between two groups were assessed by the Student's t-test. Statistically significant differences were limited to $p<0.05$.
Results: IS-5-MN significantly inhibited the maximum intensity of platelet aggregation induced by collagen or ADP. IS-5-MN significantly inhibited platelet aggregation induced by collagen (Fig. 3) and ADP (Fig. 4) starting from 10^{-6}M concentration ($p<0.05$ at 10^{-6}M and $p< 0.01$ at other concentrations).
IS-2-MN significantly inhibited ($p<0.01$) platelet aggregation induced by collagen (Fig. 3) and ADP (Fig. 4).
The inhibition of platelet aggregation induced by collagen was already significant starting from 10^{-7}M concentration whereas inhibition induced by ADP was significant at 10^{-6} M concentration (Fig. 4).
In conclusion, IS-5-MN and IS-2-MN significantly inhibit the maximum intensity of platelet aggregation induced by collagen or ADP. IS-5-MN was shown strongly to inhibit maximal aggregation induced by ADP in comparison with aggregation induced by collagen. Instead, IS-2-MN inhibited aggregation induced by ADP was significantly reduced in both groups. In conclusion, organic nitrates possess important anti-platelet actions that are useful in the treatment of unstable angina and ischemic cardiopathy.

CONCLUSIONS

The pharmacokinetics of isosorbide mononitrates such as IS-5-MN and IS-2-MN are markedly different to those of the parent dinitrate (ISDN) and these differences follow from the greater systemic availability and volume of distribution of the mononitrates and slower clearance. Moreover, because tolerance remains the major limitation to the clinical utility of organic nitrates it can be better to recommend mononitrates because, despite a lower relaxant potency than the corresponding tetranitrates and trinitrates, they are less sensitive to tolerance development. A further important conclusion from this study is that chronic nitrate exposure dosage does not affect platelet responsiveness and this characteristic can have protective effects against thrombosis in acute coronary syndromes. The anti-platelet effect of mononitrate in vivo should be significant only using high doses of the drug, in this case, in Italy, the only mononitrate at high concentration of active substance is IS-5-MN 80 mg in a slow release formulation.

Address for correspondence:
Dr. Enrico Lampa
Department of Experimental Medicine
Section of Pharmacology "Leonardo Donatelli"
Second University of Naples
80100 Naples - Italy
enrico.lampa@unina2.it

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