

The Effect of Sibutramine, a Serotonin-Norepinephrine Reuptake Inhibitor, on Platelets and Fibrin Networks of Male Sprague-Dawley Rats: A Descriptive Study

Ciska van der Schoor, BSc Hons¹, Hester Magdalena Oberholzer, PhD¹,
Megan Jean Bester, PhD¹, and Mia-Jeanne van Rooy, MSc²

Department of Anatomy and ²Department of Physiology, Faculty of Health Sciences, University of Pretoria, Arcadia, South Africa

Correspondence: Hester Magdalena Oberholzer, Department of Anatomy, Faculty of Health Sciences, University of Pretoria, Private Bag x323, Arcadia 0007, South Africa. Tel: (+27)12 319 2533. Fax: (+27)12 319 2240. E-mail: nanette.oberholzer@up.ac.za

ABSTRACT

Sibutramine is used in the treatment of obesity due to its ability to influence feelings of hunger and satiety by inhibiting the re-uptake of serotonin and noradrenalin in the central nervous system (CNS). Sibutramine use has been associated with numerous adverse events in particular cardiovascular complications possibly due to the formation of thrombi. This ultrastructural descriptive study investigated the effect of sibutramine on blood coagulation, specifically the effect on morphology of platelets and fibrin networks using scanning electron microscopy. Male Sprague-Dawley rats treated with either a recommended therapeutic dose [low dosage 1.32 mg/kg] or a toxicological higher dose [high dosage 13.2 mg/kg] of sibutramine for 28 days were used and compared to control animals. Blood samples were collected and plasma smears were prepared for platelet evaluation. Following the addition of thrombin to the plasma samples, the morphology of the fibrin clots was evaluated. Platelet evaluation by scanning electron microscopy revealed morphology typical of a prothrombotic state with a characteristic excessive platelet activation in both low-dose (LD) and high-dose (HD) rats. The fibrin clots of sibutramine-treated rats, LD and HD revealed fused thick fibers with thin fibers forming a net-like structure over the thick fibers which differ considerably from the organized structure of the control animals. It can be concluded that sibutramine alters the ultrastructure of platelets and fibrin networks creating a prothrombotic state.

Keywords: Fibrin networks, plasma, platelets, prothrombotic state, sibutramine

Sibutramine hydrochloride monohydrate (N-[1-[1-(4-chlorophenyl)cyclobutyl]-3-methylbutyl]-N,N-dimethylamine hydrochloride monohydrate) is a neuropharmacological drug that is widely used as a weight-loss stimulant to treat obesity. It acts on the central nervous system (CNS) by inhibiting the neuronal re-uptake of the neurotransmitters noradrenaline (NA) and serotonin (5HT) [1,2]. These neurotransmitters are associated with the mechanism of satiety and hunger, and the inhibition of these

neurotransmitters leads to an increased satiety and a consequent reduction in food intake. Sibutramine received FDA approval in 1997 for the treatment of obesity since numerous clinical trials indicated that sibutramine, in conjunction with a low calorie diet, produced initial and sustained weight-loss [3]. These studies also reported that weight loss induced by sibutramine was of great clinical significance as it also showed improvement in glycemic control and enhancement of insulin sensitivity, lipid profiles

and significant reductions in cholesterol associated with cardiovascular risk in obese individuals [3,4].

Despite sibutramine's success as a weight-loss agent, it was removed from most markets based on the initial findings of the Sibutramine Cardiovascular OUTcome (SCOUT) trial performed by the FDA [5]. Serious adverse events had been reported including tachycardia, hypertension, and arrhythmias, and in some cases even death. Regardless of sibutramine's withdrawal as well as the potential dangers associated with its use, it is still available via the internet and many herbal products have been shown to contain far higher concentrations of sibutramine than that which is found in the prescription drug [6].

Sibutramine's therapeutic effects are mostly attributed to serotonin (5-HT) and norepinephrine (NE) transporter inhibition (SERT and NET, respectively) by its potent metabolites monodesmethylsibutramine (M1) and di-desmethylsibutramine (M2) at neuronal synapse sites centrally (CNS) and peripherally [1,7–10]. This inhibition ultimately leads to an increase in satiety mediated centrally by the β_1 -adrenergic and 5-HT_{2A/2C} receptors and peripherally by an increase in energy expenditure by enhancing the function of NE through β_3 -adrenoceptors [3,8,11,12].

Several studies have reported bleeding complications associated with the use of Selective Serotonin Reuptake Inhibitors (SSRIs) [13–16]. In contrast, Serotonin-Norepinephrine Reuptake Inhibitors (SNRIs) with a similar mechanism of action as sibutramine, generally are not associated with increased bleeding risks. However, ischemic events after treatment with these drugs have been reported, indicating an increased cardiovascular disease and stroke risk in this patient population [17,18]. Previous studies involving sibutramine, have shown that extracellular NE concentrations are increased and this effect is gradual and sustained. It has been reported that this gradual increase in extracellular NE induced by sibutramine, is mediated by noradrenergic activation of α_2 -adrenoceptors [19]. Platelets also express α_2 -adrenergic receptors [20]. Increased expression of these receptors is associated with an increased platelet activation and possible increased risk for thrombosis.

Little data is available on the effects of sibutramine on coagulation even though its most common associated risk includes cardiovascular complications. By implementing a suitable animal model with daily sibutramine administration, the coagulatory effects of this compound was investigated. This was achieved by evaluating the effects of a low physiological and high toxicological dosage on the ultrastructural morphology of platelets and fibrin networks.

MATERIALS AND METHODS

Implementation of the Sprague–Dawley rat model

Eighteen sexually mature male Sprague–Dawley rats (average weight 200–250 g) were used in this study and maintained at the University of Pretoria Biomedical Centre (UPBRC). These rats were provided with irradiated commercial Epol rat pellets and municipal water *ad libitum*. All experimental protocols complied with the requirements of the University of Pretoria's Animal Ethics Committee (ethical clearance number: h003-13). The animals were housed conventionally in cages complying with the sizes laid down in the SANS 10386:2008 recommendations. A room temperature of 22 °C (± 2); relative humidity of 50% (± 20) and a 12 h light/dark cycle was maintained. Enrichment was provided according to standard procedures employed at the UPBRC. Animals were allowed to acclimatize for seven days prior to project commencement, which was conducted over the following 28 days and were therefore housed for a total period of 35 days.

Sibutramine administration

Sibutramine hydrochloride monohydrate (BIO COM Biotech, Clubview, SA) solution was prepared by dissolving the white powder in sterile water. The concentrations to which the animals were exposed were extrapolated from the prescribed human dose (i.e. 15 mg/day) using the formula for dose translation based on Body Surface Area (BSA) as described by Reagan-Shaw et al. [21]. Animals were also treated with a 10-times higher dose than the extrapolated dose so that the full extent of possible sibutramine toxicity could be evaluated. Although sibutramine administration did not result in significant changes in weight, the average weight of animals were still used in calculating the concentrations required on a weekly basis. The experimental design is provided in Table 1.

Compound administration occurred daily via oral gavage. Animals were at all times restrained by proper handling method as required for oral gavage and this was done by qualified personnel at the UPBRC and thus no chemical or mechanical restraint was required.

Blood collection

On the day of termination, five to ten milliliters of blood was collected from each rat via cardiac puncture under Isoflurane anesthesia. All samples were collected in separate citrate tubes that inhibit coagulation. Following blood collection, the animals

TABLE 1. Sibutramine administration.

Experimental group	Treatment	Days
Control	0.5 mL/day sterile H ₂ O	28 days
Low dose (LD)	0.5 mL/day sibutramine (1.32 mg/kg)	28 days
High dose (HD)	0.5 mL/day sibutramine (13.2 mg/kg)	28 days

were terminated via Isoflurane overdose, according to standard methods employed by the UPBRC.

Platelet-rich plasma preparation

Blood samples were centrifuged at $300 \times g$ for two minutes to obtain platelet-rich plasma (PRP). 10 μ L of PRP was used to prepare smears on round glass cover slips (10 mm diameter). The cover slips were allowed to dry and were then washed in a phosphate-buffered saline (PBS) solution on a shaker for 20 min. The samples were then fixed in a 2.5% glutaraldehyde/formaldehyde (GA/FA) solution in .075 M phosphate buffer (pH 7.4) for 30 min and then washed three times in the same buffer allowing 3 min per wash. This was followed by secondary fixation in osmium tetroxide for 30 min and the samples were washed again as described above. The samples were then serially dehydrated in 30, 50, 70, and 90% ethanol, followed by three changes of absolute ethanol (three minutes each) and then dried using hexamethyldisilazane (HMDS). The cover slips were then mounted on aluminium stubs, coated with carbon and viewed with a Zeiss Ultra Plus FEG Scanning Electron Microscope (SEM) (Oberkochen, Germany).

Fibrin networks

Thrombin (provided by the South African National Blood Services) was used to prepare fibrin clots. The thrombin was prepared in a biological buffer containing 0.2% human serum albumin at a concentration of 20 U/mL. A volume of 10 μ L of thrombin was added to 10 μ L of rat PRP causing the conversion of fibrinogen to fibrin and the release of intracellular platelet components, such as transforming growth factor, platelet-derived growth factor, and fibroblastic growth factor into the coagulum. The PRP/thrombin mix was immediately prepared on a round glass cover slip to form the fibrin coagulum simulating the coagulation process in the body. The cover slips were then placed in a petri dish and kept at 37°C for 10 min. Finally, the cover slips with the coagula were placed in PBS and washed for 20 min on a shaker. This washing step was necessary to remove any blood proteins possibly trapped within the fibrin network.

Again, these samples were fixed in 2.5% GA/FA in 0.075 M phosphate buffer (pH 7.4) for 30 min after which sample processing was done as described for PRP samples. The cover slips were dried using HMDS, after which the coverslips were mounted on aluminium stubs and coated with carbon. The samples were viewed with a Zeiss Ultra Plus FEG SEM.

RESULTS

In this study, male Sprague–Dawley rats were exposed to different concentrations of sibutramine. Only male individuals were used in this study as female platelet reactivity and coagulation has been shown to vary during the oestrus cycle due to the influences of oestrogen and progesterone [22,23].

Since sibutramine is still commonly found as additive in slimming medications available on the internet or over-the-counter [24] despite the retraction of FDA approval, this study was aimed at determining the effects of sibutramine on blood coagulation and consequently the role in cardiovascular complications often observed in prolonged sibutramine use. In particular, the effects of sibutramine on platelets and fibrin networks were evaluated.

Figure 1 shows images acquired from smears prepared from the PRP of animals in the control group as well as LD and HD, which represent the groups exposed to low and high doses of sibutramine, respectively. Figure 1(A–C) depicts platelets of all three groups at low magnification to show general morphology whereas Figure 1(D–F) are micrographs of platelets at high magnification to study changes in morphology of the platelet membrane. Figure 1(A) shows a typical inert control platelet with a single pseudopod extending from the platelet membrane. At higher magnification (Figure 1D), the platelet membrane appears to be intact with a smooth surface and open canalicular pores are visible (indicated by white arrows). Figure 1(B and E) are representative of animals from LD. Multiple pseudopodia (thick white arrows) and membrane spreading (thin white arrow) can be seen. On higher magnification the membrane appears granular. Figure 1(C and F) show platelets from rats exposed to the higher dose of sibutramine, in HD. In Figure 1(C), a platelet with numerous pseudopodia is shown, with spontaneous formation of matted thick fibers associated with fibrin formation (white star). Also, platelet interaction is apparent. On high magnification, the membrane appears necrotic (white arrows) [25]. In some areas, membrane tears are also visible (white star).

Figure 2(A–C) is representative of the fibrin networks of the animals in the control and two experimental groups. Figure 2(A) shows a typical fibrin network of control animals where major thick

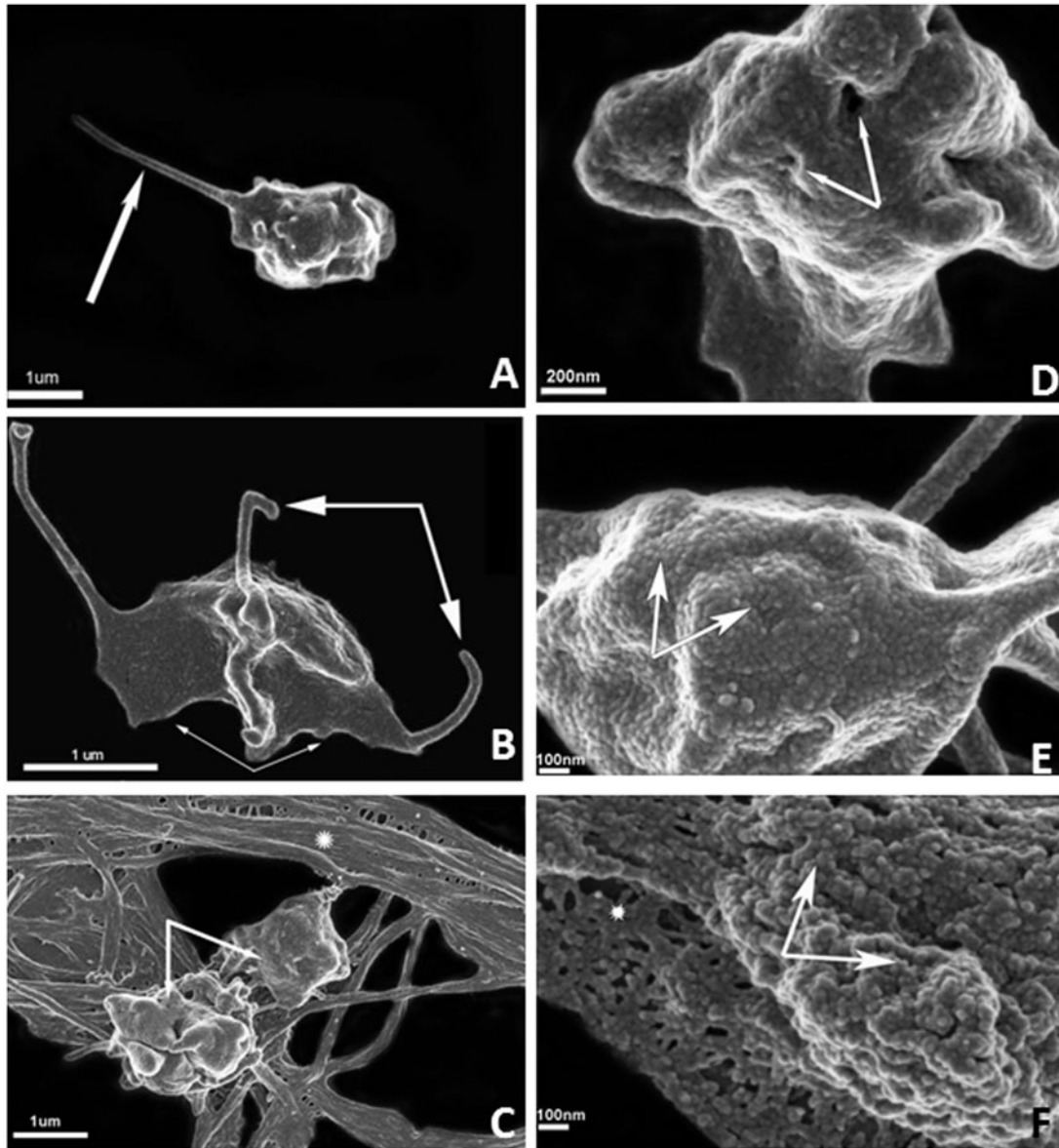


FIGURE 1. Platelets from animals in the three experimental groups. (A and D) low and high magnification, respectively, of a typical platelet structure from control rats (Arrow in A indicates a pseudopod and the arrow in D shows the open canalicular pores); (B and E) High and low magnification, respectively, of platelets of LD rats (Thick arrows show multiple pseudopodia and thin arrows show membrane spreading in B; Arrows in E indicate the granular appearance of the membrane); (C and F) Platelets from HD rats, low and high magnification, respectively (arrows indicate platelet interaction and the star indicates spontaneous fibrin formation in C; arrows in F indicate the necrotic appearance of the membrane and the star shows membrane tears).

fibers (thick white arrows) and minor thin fibers (thin white arrows) are present. The major, thick fibers are more abundant than the thin fibers, which is characteristic of control individuals [26]. Figure 2(B) represents fibrin networks of LD rats, which were exposed to the low dose of sibutramine. Major thick fibers are present but appear fused as indicated by the thick white arrows. Also, the minor thin fibers form a net-like structure (thin arrows) covering large parts of the thick fibers and therefore the clot. A similar morphological appearance was observed in samples obtained from HD rats as shown in Figure 2(C). Major thick fibers are visible (thick arrows) with thin fibers (thin arrows), covering the thick fibers as seen in

Figure 2(B). In some areas, the thin fibers are arranged in a denser net-like structure covering the thick fibers. This was not seen in the control group. Also, fused major thick fibers are present in both experimental groups (LD and HD) but this appearance was not observed in the control group.

DISCUSSION

Haemostasis involves the highly coordinated processes of platelet activation and blood clotting with the ultimate goal of vascular repair. The coagulation cascade is the vital component that maintains the

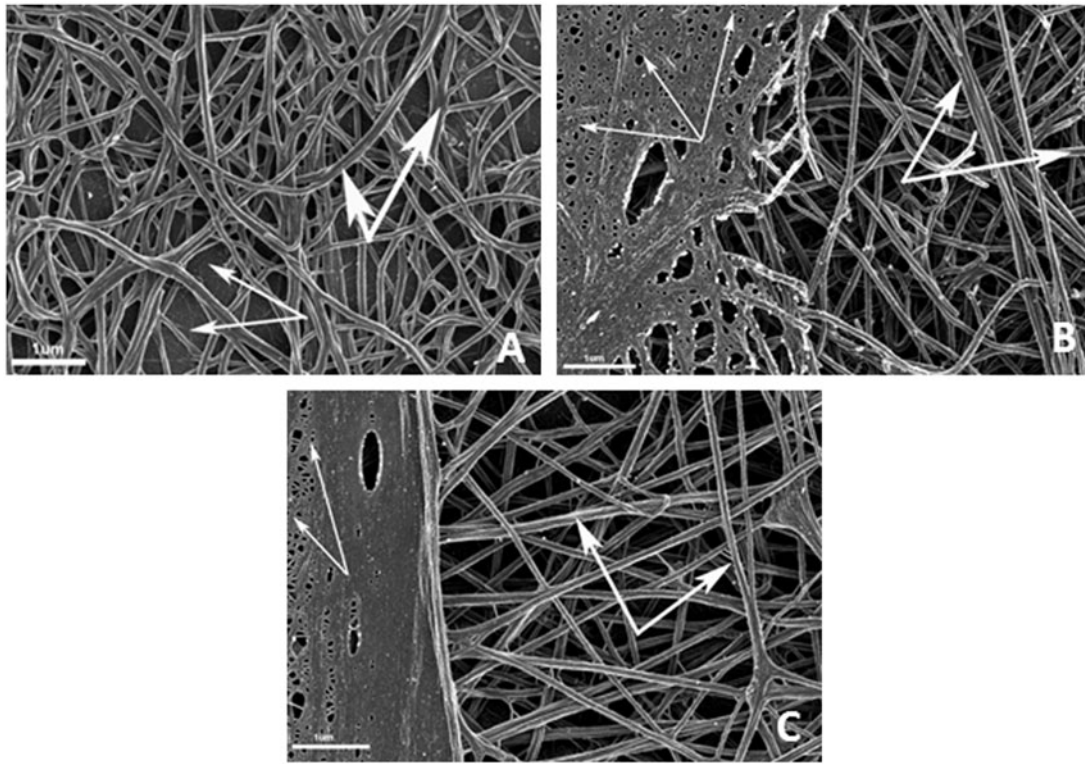


FIGURE 2. Fibrin networks of animals in the different experimental groups. (A) Control network with major thick (thick arrows) and minor thin (thin arrows) fibers; (B) LD rats showing fused thick fibers (thick arrows) and minor fibers forming a net-like structure (thin arrows); (C) HD rats showing minor fibers (thin arrows) covering the thick fibers (thick arrows).

balance required for hemostasis by activating platelets and forming a hemostatic platelet plug as well as stabilizing fibrin networks, contributing to blood clot formation [27,28].

Laboratory-based investigations of fibrin structures, platelet morphology, and the fibrin clot in general has become a popular and powerful research tool as these structural findings could differ in and provide essential information on various diseased states possibly contributing to the discovery of new therapeutic targets [29,30].

Results obtained in this study show different degrees of platelet activation between the control and experimental groups. Treatment of rats with sibutramine shows an increased degree of platelet activation characterized by the presence of more pseudopodia in conjunction with platelet spreading in the LD group, while spontaneous fibrin formation can be seen in the HD group. In this animal model, indications are that sibutramine causes the activation of platelets thereby increasing the thrombotic tendency. The fibrin networks differ between groups. When comparing the control fibrin network to that of the sibutramine groups, a dramatic difference in fibrin structure can be observed. Where the major fibers are more abundant in control individuals, minor fibers appear to become more prevalent in LD and increased in HD groups. Generally, increased thrombin concentrations are associated with the formation of stiffer

clots and increased thrombin activity on platelet surfaces has also been accredited to fibrin mesh architecture [31] as seen in the figures. In both experimental groups, the fibrin clots also presented with a matted, layered morphology and thicker, fused fibers. These characteristics are not observed in control individuals.

The fibrin morphology as described in this study strongly correlates with that seen in thromboembolic ischemic stroke, described by Pretorius et al. [25], which is associated with a hypercoagulable state [25,32,33] perhaps indicating a possible consequence of prolonged sibutramine use. Increased thrombin levels are known to produce thinner fibrin fibers that are more tightly packed. This can be seen in the matted, net-like arrangement of fibers in experimental animals. This suggests, as Pretorius et al. [25] also described, that the formation of this observed atypical fibrin morphology is due to the elevated concentrations of the responsible coagulation factors within collected samples. Further, supporting the possibility that sibutramine influences the coagulation potential of individuals and this is further supported by the observed spontaneous formation of fibrin networks in the PRP smears without the addition of thrombin to counteract the effects of the citrate in the tubes.

Between the LD and HD groups, elevated coagulation factors concentrations could also explain the observed differences in platelet morphology.

PRP smears prepared from samples collected from animals administered high doses of sibutramine, showed membrane blebbing and membrane tears, which is characteristic of cells undergoing necrosis. Again, a similar morphology has been described for platelets in procoagulant conditions such as stroke.

SNRIs, such as sibutramine, have been associated with an increased risk of bleeding to a much lesser extent than SSRIs. Additionally, various case reports have been published reporting ischemic events, including infarction [17,18]. Adrenergic receptors expressed on the surface of platelets increase platelet activation [20], and in a study conducted by Hallbäck et al. [34], results showed that SNRIs, such as venlafaxine, increased adhesion by possibly acting on these receptors, perhaps providing an explanation for the alterations in platelet morphology as observed in this study.

Norepinephrine has extensively been described as a trigger for the induction of human platelet aggregation [35–38]. Von Känel et al. [39] also showed that acute mental stress, during which NE levels are significantly increased, increases thrombin activity and fibrin turnover reliant on β 2-adrenergic receptor functioning and associated catecholamine activity [39].

The active metabolites of sibutramine, M1 and M2 possess potencies for the inhibition of reuptake of 5-HT and norepinephrine that are relatively similar to the existing SSRI's and SNRI's such as fluoxetine and desipramine, respectively [40]. To assess the potential of certain drugs to inhibit the reuptake of NE and 5-HT, Cheetham et al. [7,41] conducted two studies, which included sibutramine and its two metabolites. Results showed that the active metabolites M1 and M2 were as potent as many of the antidepressants currently in use. It is believed that through the inhibition of the cellular reuptake of NE and 5-HT, the extracellular synaptic concentrations are consequently increased, and the subsequent activation of α -adrenoceptors, β -adrenoceptors, and 5-HT_{2A/2C} receptors occurs [8,12,42–45]. Reports based on animal research have also showed that sibutramine administration leads to the down-regulation of pre- and post-synaptic adreno- and 5HT receptors [42,43,46–49], which further substantiate theories that sibutramine exerts its therapeutic effects by incidentally increasing the activity of norepinephrine and 5-HT at each particular receptor site.

CONCLUSION

Investigations of the morphological appearance of the platelets and fibrin networks of animals treated with sibutramine versus control, untreated, revealed differences that are consistent with changes described in

previous studies which have been associated with increased coagulability and disease [26].

DECLARATION OF INTEREST

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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