European Review for Medical and Pharmacological Sciences 2015; 19: 963-970

Effects of pioglitazone and glipizide on platelet function in patients with type 2 diabetes

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Abstract. – OBJECTIVE: Platelet hyper-reactivity is one of the most important causes of accelerated atherosclerosis and increased risk of thrombotic vascular events associated with type 2 diabetes mellitus (T2DM). This study aimed to investigate the effects of different add-on anti-diabetic therapies on platelet function in T2DM patients.

PATIENTS AND METHODS: A three-group parallel study was conducted in 120 patients with T2DM (HbA1c > 7%) undergoing treatment with metformin. Patients were randomly assigned to receive add-on therapy with glipizide or pioglitazone. Markers of PF (platelet PAC-1 binding, p-selectin expression and adenosine diphosphate-induced platelet aggregation) were measured at weeks 0, 4 and 24. Primary outcome was effects of pioglitazone and glipizide on platelet aggregation. Secondary outcome was the related influencing factors of platelet aggregation.

RESULTS: There were no significant differences in baseline characteristics between glipizide and pioglitazone groups. After 24 weeks, fasting blood glucose ($p < 0.01$) and HbA1c ($p < 0.01$) were higher in pioglitazone group than those in glipizide group. Fasting insulin ($p < 0.01$) and HOMA-IR ($p < 0.01$) were lower in pioglitazone group than that in glipizide group. Markers of platelet function were significantly decreased in both groups at 24 weeks (PAC-1: pioglitazone: -63.3%; glipizide: -45.9%; p-selectin: pioglitazone: -73.9%; glipizide: -54.9%; platelet aggregation: pioglitazone: -24.1%; glipizide: -13.4%; all $p < 0.01$ vs. baseline), but the decrease in platelet function was more significant in pioglitazone group ($p < 0.05$). Multiple linear regression analyses showed that platelet aggregation was independently associated with treatment groups ($p < 0.001$), Triglyceride ($p = 0.009$) and HDL-C ($p = 0.015$).

CONCLUSIONS: Add-on therapy with pioglitazone may be more effective than glipizide for inhibiting platelet activation in T2DM.

Key Words:
Type 2 diabetes, Glipizide, Pioglitazone, Platelet function, Add-on therapy.

Introduction

Platelet hyper-reactivity is one of the most important causes of accelerated atherosclerosis and increased risk of thrombotic vascular events in diabetic patients[1-3], manifesting by a 2- to 4-fold increase in coronary artery disease (CAD) risk. Increased plaque instability has been observed in diabetic patients[4], as well as delayed plaque healing and increased thrombosis[5].

Therefore, anti-platelet agents that reduce the cardiovascular risk have been used to investigate the importance of activated platelet function in atherosclerosis[6,7]. Previous studies in type 2 diabetes (T2DM) patients without CAD demonstrated that thiazolidinediones (TZD) (such as troglitazone) reduced platelet-dependent thrombus formation by improving glycemic control[8]. In CAD patients without T2DM, rosiglitazone has been shown to significantly reduce circulating platelet activity independently of its insulin-sensitizing effect[9]. Metformin is the treatment of choice for T2DM, although combination therapy is often necessary to achieve optimal glycemic control[10].

Results from the PROfix study demonstrated that metformin and pioglitazone treatment led to an improvement in platelet aggregation markers in patients with T2DM, but not in patients treated with metformin and glimeperide[11]. However, in animal studies, high concentrations of glipizide alone have been shown to inhibit platelet aggregation *in vitro* and in *vivo*[12]. However, the relative effects of different anti-diabetic combination therapies on platelet function have not yet been investigated adequately within a single study.

Thus, the objective of the present work was to compare different add-on anti-diabetic therapies (pioglitazone and glipizide) on platelet function in metformin-treated T2DM patients and to evaluate the potential of these different classes of drugs to protect patients from platelet aggregation and the associated risk of atherosclerosis.

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Patients and Methods

Study Design

This was a three-group parallel open-label study investigating the effects of adding pioglitazone, insulin or glipizide on metformin treatment in T2DM on platelets function in T2DM patients. No change was made to the protocol or assessed outcomes during the study. Patients using less than 1.5 g/d of metformin (Bristol-Myers Squibb, Shanghai, China) were increased to 1.5 g/d. All patients entered a 4-week run-in period, during which they continued their metformin monotherapy (at least 1.5 g/day). HbA1c levels were assessed after the run-in period and only patients with an HbA1c ≥ 7.0% were randomized. Patients were then randomized (equal allocation using computer-generated random tables generated by SPSS 10.0 and using sequentially numbered envelopes) to 24 weeks of add-on therapy with either glipizide (Pfizer Pharmaceutical Co., LTD., Dalian, China) (5-10 mg/day), pioglitazone (Sino-American Pharmaceutical Co., LTD., Hangzhou, China) (15-45 mg/day) or prandial insulin (individualized dosage). However, the effect of adding insulin on metformin treatment in T2DM patients on platelets function was not analyzed in the present paper, and will be presented in a subsequent article.

Patients

The study protocol was approved by the Anhui Provincial Hospital Clinical Research Ethics Committee, and all participants provided written informed consent before entering the study. Participants were enrolled consecutively from March 2008 to December 2011. All study procedures and data collections were performed in the Anhui Provincial Hospital. Inclusion criteria were: (1) diagnosed with T2DM (HbA1c > 7%) for less than 12 months; and (2) receiving metformin monotherapy for T2DM. Exclusion criteria were: (1) history of diabetic ketoacidosis; (2) family history of cancer; (3) heart failure; (4) impaired kidney function (Ccr < 80 ml/min); (5) impaired liver function (baseline alanine aminotransferase more than two times the upper limit of normal); (6) pregnant women or those of childbearing potential; (7) anemia; and (8) treatment using aspirin, clopidogrel, heparin, glucocorticoid, nonsteroidal anti-inflammatory drugs (NSAIDs), fibrates, ACE inhibitors or angiotension II receptor agonist during the last 2 weeks before entering the study.

Baseline Clinical Assessments

Age, gender, height, weight, blood pressure were measured. Body mass index (BMI) was calculated.

Fasting blood samples (10 to 12 h overnight fast) were obtained between 06:00 and 08:00 for the assessment of fasting blood glucose (FBG), fasting insulin levels, blood lipids and hemoglobin A1c (HbA1c). Markers of platelet activation included assessments of ADP-induced platelet aggregation, platelet PAC-1 binding and P-selectin expression. All assessments were performed at baseline (week 0), week 4 and week 24. Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated to estimate the degree of insulin resistance according to the following formula[13]: HOMA-IR= [Fasting insulin (mU/L) × Fasting glucose (mmol/L)]/22.5.

Platelet Aggregation

Platelet aggregation was assessed using 2 mL blood collected in 3.8% sodium citrate tubes. Samples were gently mixed with 3.8% sodium citrate (9:1), and centrifuged at 1,200 rpm for 10 minutes at room temperature, allowing collection of the upper platelet-rich plasma (PRP). Remaining blood samples were re-centrifuged at 3,000 rpm for 15 minutes to obtain platelet-poor plasma (PPP). The platelet count in the PRP fraction was adjusted to 2 to 3 $\times 10^8$ cells/mL. Adenosine diphosphate (ADP) (BD Biosciences, Franklin Lake, NJ, USA) (final concentration, 20 μM) was used to stimulate platelet aggregation[14-16]. Data were recorded for 5 minutes, and platelet aggregation was determined as the maximal percent change in light transmittance from baseline using PPP as a reference. Aggregation and light transmission of PPP were defined as 0% and 100%, respectively, and the PRP fraction was compared relatively to these levels[17-19].

Platelet Activation

Platelets were identified using peridinin chlorophyll protein (PerCP)-conjugated CD61 monoclonal antibody (BD Biosciences, Franklin Lake, NJ, USA). Platelets were discriminated from the other blood cells by their light scattering characteristics and gated using an electronic bit map. More than 98% of these cells were positive for CD61. The gated events were subjected to single color fluorescence. Platelet P-selectin expression was determined using a PE-conjugated anti-P-selectin antibody AC1.2 (PE-CD62P, BD Biosciences) and platelet activation was determined using fluorescein isothiocyanate (FITC)-conjugated PAC-1 antibody (BD Biosciences)[17,18,20].

Sample Size Estimation

Our preliminary study showed that pioglitazone administered for 1 month suppressed platelet aggregation. We hypothesized that platelet aggregation in patients treated with pioglitazone for 24 months was reduced to 0.65 ± 0.05. In patients treated with glipizide for 24 months, platelet aggregation was reduced to 0.70 ± 0.07. Analysis of the reduction in platelet aggregation in the metformin group using the Student's t-test with a two-sided significance level of 5% and a power of 90% revealed that a sample size of 33 patients per group is necessary to confer statistical significance on the results of this study. Assuming a 20% dropout rate, we proposed to recruit 40 patients per group.

Statistical Analysis

Primary outcome was the effects of pioglitazone and glipizide on platelet aggregation. Secondary outcome was the influencing factors of platelet aggregation. Statistical analysis was performed using SPSS 10 (SPSS Inc., Chicago, IL, USA). Data are presented as means ± standard deviations (SD). Statistical significance was evaluated by *t*-test or one-way analysis of variance (ANOVA) with least significant difference (LSD) test for post hoc analysis. Multiple stepwise linear regression analyses were performed using a model in which the dependent variable was ADP-induced platelet aggregation, with the following explanatory variables: groups (glipizide and pioglitazone groups), FBG, HbA1c, HOMA-IR, sex, age, BMI, P-selectin expression, PAC-1 binding, high-density lipoprotein cholesterol (HDL-C), triglycerides, low-density lipoprotein cholesterol (LDL-C), systolic blood pressure (SBP), diastolic blood pressure (DBP), fasting insulin levels and smoking status at baseline. *p*-values < 0.05 were considered statistically significant.

Results

The enrollment and outcomes of all patients included in this study are summarized in Figure 1. A total of 212 patients were screened, 120 were selected for randomization based on their HbA1c levels and 107 patients completed the 24-week study: 34 in the pioglitazone group, 37 in the insulin group, and 36 in the glipizide group. In the pioglitazone group, two patients developed marked lower extremity edema and withdrew from the study and four patients were lost to follow-up. In the glipizide group, four patients were lost to follow-up. All analyses were performed according to the originally defined groups.

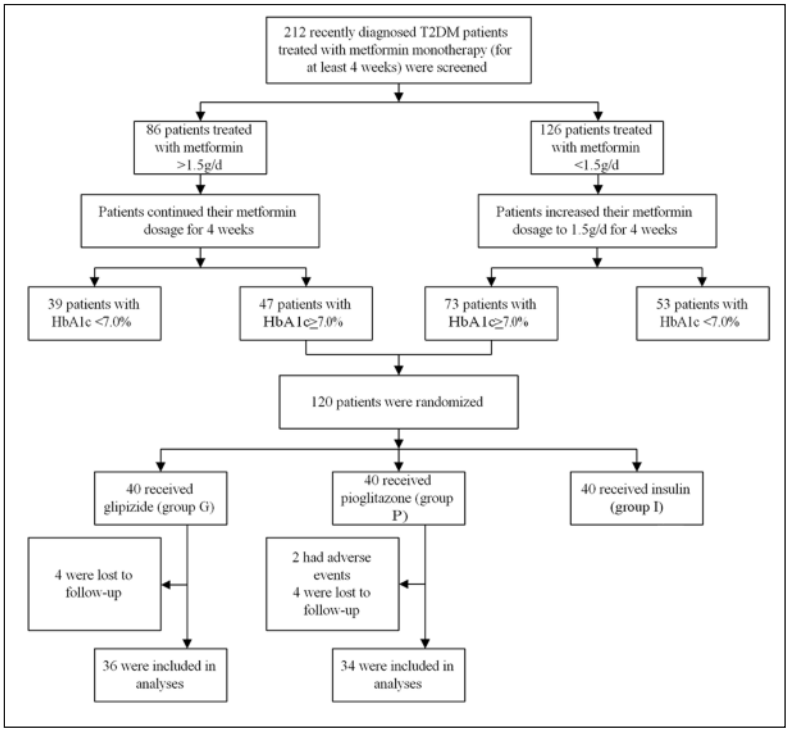

Figure 1. Enrollment and outcomes.

Baseline Characteristics Between the Pioglitazone and Glipizide Groups

Table I shows that both groups were well matched for baseline demographic characteristics, glycemia, fasting insulin levels, blood lipids, smoking status, and platelet function (indicated by ADP-induced platelet aggregation, P-selectin and PAC-1 expressions).

Changes in Metabolic Parameters at weeks 4 and 24

FBG was significantly decreased between baseline and week 4 in both groups (pioglitazone: -21.2%; glipizide: -29.4%; both $p < 0.01$ vs. baseline) (Table II), and this decrease was more significant in the glipizide group compared with that in the pioglitazone group ($p < 0.01$).

At week 24, compared with baseline, FBG and HbA1c levels were significantly decreased in both groups (FBG: pioglitazone: -23.2%; glipizide: -30.2%. HbA1c: pioglitazone: -16.5%; glipizide: -22.4%; all $p < 0.01$ *vs.* baseline). However, at week 24, FBG and HbA1c were both significantly higher in the pioglitazone group than those in the glipizide group ($p < 0.05$) (Table II).

Fasting insulin was significantly reduced between baseline and week 4 in both groups (pioglitazone: -15.4%; glipizide: -10.7%; both $p < 0.01$ *vs.* baseline). At week 24 fasting insulin was also significantly reduced compared to week 4 in the pioglitazone group (-18.1%; $p < 0.01$ *vs.* week 4), in the glipizide group the levels increased slightly from week 4 (+6.11%) but still remained significantly lower than at baseline ($p < 0.01$). At week 24 the difference between the groups was significant ($p < 0.01$) (Table II).

Compared with baseline, HOMA-IR was significantly decreased at week 4 in both groups (pioglitazone: -33.2%; glipizide: -36.0%; both $p < 0.01$ *vs.* baseline). The changes observed between weeks 4 and 24 were significantly greater in the pioglitazone group than that in the glipizide group (pioglitazone: -20.4%, $p < 0.01$; glipizide: +4.2%, $p > 0.05$ *vs.* week 4) (Table II).

Changes in Platelet Function at Weeks 4 and 24

Both treatments decreased ADP-induced platelet aggregation between weeks 0 and 4 (pioglitazone: -14.5%; glipizide: -12.2%; both $p < 0.01$ *vs.* baseline), and no significant difference was observed between the groups at weeks 4 (Table II). There were no significant changes in ADP-induced platelet aggregation between weeks

Table I. Comparison of baseline characteristics.

Characteristics	Pioglitazone (n=34)	Glipizide (n=36)	p
Male, n(%)	20 (58.8%)	21 (58.3%)	0.967
Mean age, years	54.15±4.91	53.56±3.61	0.566
BMI, kg/m^2	26.52±1.29	26.59±1.23	0.818
Triglyceride, mmol/L	2.03±0.44	2.10±0.41	0.470
HDL-C, mmol/L	1.07±0.20	1.16±0.19	0.048
LDL-C, mmol/L	2.90±0.51	3.04±0.58	0.304
SBP, mmHg	137.32±8.15	138.08±9.70	0.725
DBP, mmHg	87.35±6.10	85.19±8.42	0.226
FBG, mmol/L	9.33±0.76	9.14±1.38	0.486
HbA1c,%	8.73±0.80	8.65±0.75	0.670
HbA1c 7-9%, n (%)	21 (61.8%)	24 (66.7%)	0.804
HbA1c >9%, n (%)	13 (38.2%)	12 (33.3%)	0.804
Platelet aggregation, %	0.83±0.08	0.82±0.08	0.405
P-selectin expression, %	7.58±2.69	7.87±3.08	0.676
PAC-1 binding, %	7.30±2.51	7.74±1.99	0.422
HOMA-IR	5.05±1.02	4.84±1.04	0.385
Fasting insulin, mU/L	12.32±2.90	12.10±2.73	0.745
Statin use, n (%)	12 (35.3%)	15 (41.7%)	0.630
Non-smokers, n (%)	10 (29.4%)	14 (38.9%)	0.457

Note: The data was shown as means±standard deviation (SD). Patients with T2DM (HbA1c >7%) undergoing treatment with metformin were randomly assigned to receive add-on therapy with glipizide or pioglitazone. M/F: Male/Female; BMI: Body mass index; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; FBG: Fasting blood glucose; HbA1c: hemoglobin A1c; HOMA-IR: homeostatic model assessment of insulin resistance.

Table II. Changes in FBG, HbA1C and platelet function (indicated by ADP-induced platelet aggregation, P-selectin expression (CD62P) and PAC-1) at weeks 0, 4 and 24.

Group	n	Week	FBG (mmol/L)	Fasting insulin (mU/L)	HbA1C (%)	HOMA-IR	Platelet aggregation	P-selectin positive rate (%)	PAC-1 (%)
Piogli-tazone	34	0	9.33±0.76	12.32±2.90	8.73±0.80	5.06±1.02	0.83±0.08	7.58±2.69	7.30±2.51
		4	7.35±0.72$^{\Delta}$	10.42±2.22$^{\Delta}$		3.38±0.69$^{\Delta}$	0.71±0.08$^{\Delta}$	3.76±1.34$^{\Delta}$	4.20±1.65$^{\Delta}$
		24	7.17±0.68$^{\Delta}$	8.53±2.15$^{\Delta\blacktriangle}$	7.29±0.47$^{\Delta}$	2.69±0.60$^{\Delta\blacktriangle}$	0.63±0.05$^{\Delta\blacktriangle}$	1.98±1.30$^{\Delta\blacktriangle}$	2.68±1.42$^{\Delta\blacktriangle}$
Glipizide	36	0	9.14±1.38	12.10±2.73	8.65±0.75	4.84±1.04	0.82±0.08	7.87±3.08	7.74±1.99
		4	6.45±0.72$^{\Delta**}$	10.80±2.12$^{\Delta}$		3.10±0.76$^{\Delta}$	0.72±0.09$^{\Delta}$	3.69±1.67$^{\Delta}$	4.29±1.99$^{\Delta}$
		24	6.38±0.87$^{\Delta**}$	11.46±2.44$^{\Delta**}$	6.71±0.55$^{\Delta**}$	3.23±0.74$^{\Delta**}$	0.71±0.07$^{\Delta**}$	3.55±1.84$^{\Delta**}$	4.19±1.61 $^{\Delta*}$

Note: The data were expressed as means ± SD. $^{\Delta}p < 0.01$ *vs.* baseline (week 0); $^{\blacktriangle}p < 0.01$ *vs.* 4thweek; $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* Pioglitazone group.

4 and 24 in the glipizide group ($p > 0.05$). In the pioglitazone group, ADP-induced platelet aggregation continued to decrease between weeks 4 and 24 (-11.3%; $p < 0.01$), and was lower at 24 weeks than that in the glipizide group ($p < 0.01$).

The percentages of PAC-1 binding and P-selectin-positive platelets decreased significantly in both groups between weeks 0 and 4 (PAC-1: pioglitazone: -42.5%; glipizide: -44.6%. p-selectin: pioglitazone: -50.4%; glipizide: -53.1%; all $p < 0.01$ *vs.* baseline), with no significant differences being observed between the two groups (Table II). No significant changes in platelet PAC-1 binding or P-selectin were observed between weeks 4 and 24 in the glipizide group ($p > 0.05$). In the glipizide group, both markers were significantly lower at weeks 24 than those at weeks 4 (PAC-1: -36.2%; P-selectin: -47.3%; both $P < 0.01$ *vs.* week 4), resulting in significantly lower values compared with those in the glipizide group ($p < 0.01$).

Related Influencing Factors of Platelet Aggregation in T2DM

Multiple stepwise linear regression analyses showed that platelet aggregation was independently associated with groups (β coefficients = 0.092, 95% CI: 0.066-0.118 $p < 0.001$), Triglyceride (β coefficients = 0.041, 95% CI: 0.011-0.072, $p = 0.009$) and HDL-C (β coefficients = -0.084, 95% CI: -0.150--0.017, $p = 0.015$).

Discussion

The objective of the present study was to compare pioglitazone and glipizide add-on anti-diabetic therapies on platelet function in metformin-treated T2DM patients, and to evaluate the potential of these different classes of drugs to protect patients from platelet aggregation and the associated risk of atherosclerosis. Our results clearly demonstrated that add-on therapy with glipizide and pioglitazone attenuated platelet activation in T2DM patients, as measured by platelet PAC-1 binding, P-selectin expression, and ADP-induced platelet aggregation. Platelet function in both groups was attenuated slightly after 4 weeks of treatment. However, after 24 weeks, platelet function in patients receiving pioglitazone was suppressed to a greater degree than that in patients receiving glipizide, although HbA1c was higher in the pioglitazone group than that in the glipizide group. Furthermore, multiple regression analysis showed that add-on therapy with pioglitazone may be more effective than glipizide for inhibiting platelet activation in T2DM.

The role of platelets in thrombus formation is well known[6,7], and previous studies reported an association between platelet activation and the degree of insulin resistance reflected by HOMA-IR[21,22]. In T2DM, troglitazone was shown to decrease platelet function by improving insulin resistance[8], but the decreased platelet function by rosiglitazone was independent from insulin sensitivity in non-T2DM subjects[9]. A study showed that rosiglitazone had a greater effect on platelet function and HOMA-IR than gliclazide, and that attenuation of platelet activation in patients treated with rosiglitazone was partly due to improved insulin sensitivity[23]. Another study showed that metformin and pioglitazone had greater effects on platelet function than metformin and glimperide[11], which is similar to the present study.

Reduced oxidative stress and a possible direct effect mediated by the peroxisome proliferator-activated receptor gamma (PPARγ) were identi-

fied as two possible primary mechanisms of action[23]. Indeed, NO has a number of anti-thrombotic effects[24]. Furthermore, NO levels are lower in T2DM patients[25], and a previous study reported that rosiglitazone decreased asymmetric dimethylarginine levels, a natural inhibitor of NO synthetase[26]. In addition, PPARγ agonists have been shown to inhibit collagen-stimulation of platelet function by modulating early glycoprotein VI (GPVI) signaling[27]. Indeed, in a previous animal study, Bodary et al[28] showed that KK mice treated with glipizide were not protected from thrombotic events despite reduced glucose levels, while mice treated with pioglitazone were protected from thrombosis despite being fed a high-fat diet. In addition, soluble P-selectin levels, as well as platelet P-selectin, were decreased with pioglitazone treatment[28]. PPARγ is present in platelets and participate in CD40L decrease after platelet activation[29]. Other pathways, such as cNOS and thrombomodulin, could also be involved in the effects of pioglitazone[30], as well as leukocyte-associated factors modulating thrombin generation[31]. However, further studies are necessary to improve our understanding of these mechanisms.

Therefore, a decreased platelet activation with pioglitazone could not only improve glycemic control in T2DM patients, but could also decrease CAD events by a direct effect on platelet function and by indirect effects on the numerous aspects of macro-vascular complications of T2DM[32]. Furthermore, the use of a single drug could have a number of beneficial effects on diabetes and its complication[33], at least in early diabetes patients and in patients without much comorbidities. Most studies focus on only one member of the thiazolidinedione class, and we must be cautious before generalizing effects from a single member to the entire class.

Our findings were limited by the relatively short 24-week treatment period, thus precluding any comparison of the long term-effects of these agents that might be encountered in real-life clinical practice. The application of our findings is also restricted by the relatively small sample size based on experience at single center in China. We failed to collect data assessing coronary artery disease and alcohol and caffeine habits, factors that have been shown to influence platelet function, insulin sensitivity and resistance. We have to rely on the randomized selection of the groups and hope that these factors are not clinically significant between the groups. Further research to evaluate the effects of pre-incubation with pioglitazone and glipizide on platelet function in *vitro* may further elucidate the mechanisms involved in the antiplatelet effects of these agents.

Conclusions

We demonstrated that glipizide add-on therapy attenuated platelet function in T2DM patients, primarily by improving glycemic control. Compared with glipizide, the hypoglycemic effect of pioglitazone was poor, but platelet function at 24 weeks was more markedly improved. Further large-scale studies are required to confirm our results and to elucidate the mechanism of the observed effects. Results from the present study could provide clues for the treatment of other platelet-activated conditions.

Acknowledgements

The research was financially supported by the Natural Science Foundation of Anhui Province (09B117).

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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