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Synergism between metformin and analgesics/vitamin B_{12} in a model of painful diabetic neuropathy

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ABSTRACT

Metformin (a widely used antidiabetic drug) has demonstrated efficacy in models of painful diabetic neuropathy (PDN), as well as certain clinical efficacy in relieving/preventing PDN. This study aimed to determine the type of interaction between metformin and duloxetine/oxycodone/eslicarbazepine acetate [ESL]/vitamin B_{12} in relieving diabetic pain hypersensitivity. Antihyperalgesic efficacy was determined using a Von Frey apparatus in mice with streptozotocin-induced PDN. We examined metformin's efficacy following oral (acute and prolonged 7-day treatment) and local (spinal and peripheral) application. The examined analgesics were administered in a single oral dose, whereas vitamin B_{12} was intraperitoneally administered for 7 days. In combination experiments, metformin (prolonged treatment) and analgesics/vitamin B_{12} were co-administered in fixed-dose fractions of their ED_{50} values and the type of interaction was determined using isobolographic analysis. Metformin produced dose-dependent antihyperalgesic effects in diabetic mice after oral (acute and prolonged 7-day treatment) and local spinal/peripheral application. Two-drug metformin combinations with analgesics/vitamin B_{12} also dose-dependently reduced mechanical hyperalgesia. The isobolographic analysis revealed that metformin synergises with analgesics/vitamin B_{12}, with a 6–7 fold dose reduction of both drugs in the examined combinations. In conclusion, metformin reduces hyperalgesia in diabetic animals, most likely by acting at the spinal and peripheral level. Additionally, it synergizes with duloxetine/oxycodone/ESL/vitamin B_{12} in reducing hyperalgesia. Metformin co-treatment may increase analgesic efficacy and enable the use of lower (and potentially safer) analgesic doses for treating PDN. Combined metformin-vitamin B_{12} use may provide more effective pain relief and mitigate metformin-induced vitamin B_{12} deficiency.

1. Introduction

Diabetic neuropathy is a debilitating complication of diabetes mellitus that affects about 50 % of diabetic patients, and is seen with similar prevalence in patients suffering from type 1 and 2 diabetes [1]. Among the manifestations of diabetic neuropathy, neuropathic pain (i.e. painful diabetic neuropathy [PDN]), characterized by spontaneous pain (usually in the form of burning pain of the feet) and painful hypersensitivity (hyperalgesia/allodynia), represents one of the most troublesome symptoms which is associated with reduced quality of life, comorbidities (depression, anxiety and cardiovascular diseases) and increased mortality [1,2]. PDN develops because of the negative impact of hyperglycemia on peripheral nerve structure/function (e.g. increase in expression/activity of sodium channels), and changes in central nociceptive pathways (e.g. increased spinal nociceptive processing and reduced descending inhibitory modulation) [1,2]. Contemporary guidelines recommend the use of antidepressant or antiepileptic drugs (duloxetine or gabapentinoids) as first-line agents to relieve pain associated with PDN, whereas in refractory cases opioids may be considered [3,4]. However, the efficacy and/or safety of currently available treatment options are often limited, and development of novel treatment strategies is needed. Combination therapy with mechanistically

Abbreviations: %AH, percent of antihyperalgesic activity; AMPK, AMP-activated protein kinase; AUC, area under the curve; ED_{50}, dose that produces an antihyperalgesic effect of 50 %; $ED_{50\ add}$, theoretical additive ED_{50} for drug mixture; $ED_{50\ mix}$, experimental ED_{50} for drug mixture; ESL, eslicarbazepine acetate; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; OGTT, oral glucose tolerance test; PDN, painful diabetic neuropathy; PWT, paw withdrawal threshold; TRPA1, transient receptor potential cation channel, subfamily A, member 1.

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different analgesic drugs could overcome these limitations, given that multiple mechanisms contribute to the generation of PDN, and simultaneously targeting several mechanisms may provide more effective pain relief. Additionally, combination therapy could enable the use of lower doses of individual analgesics, thereby reducing the incidence of dose-dependent side effects [5].

Metformin is considered to be the drug-of-choice for the initial treatment of type 2 diabetes, because of its high antihyperglycemic efficacy, favorable safety profile and certain cardioprotective properties [6]. Apart from its role in treating type 2 diabetes, there is an abundance of preclinical data that metformin can attenuate inflammatory/neuropathic pain, [7], as well as some clinical data that it can relieve/prevent neuropathic pain [8–10]. Of particular value are the findings of Pop-Busui et al. [9] who demonstrated that metformin treatment (in combination with pioglitazone) is associated with the lowest prevalence of diabetic neuropathy among several different treatment options. Interestingly, there is also evidence that metformin can alleviate painful hypersensitivity in models of type 1 diabetes and its effects are seen after acute/short-term application [11,12]. These findings suggest that the analgesic properties of metformin are independent of its antihyperglycemic effects, and that metformin could prove useful in the treatment of PDN in patients with type 1 diabetes.

Here, we aimed to expand the current data on the antihyperalgesic effects of metformin in a model of type 1 diabetes (streptozotocin-induced PDN) by examining its effects after systemic and local application. We also examined the type of interaction between metformin and three mechanistically different analgesic drugs, duloxetine and oxycodone (two drugs with demonstrated clinical efficacy in alleviating PDN) [3,4], and eslicarbazepine acetate (ESL; a novel sodium channel-blocking antiepileptic drug, that has demonstrated efficacy in a model of PDN) [13]. Additionally we investigated the type of interaction between metformin and vitamin B_{12}, seeing as this vitamin has shown neuroprotective properties in PDN models [14,15], clinical efficacy in relieving PDN [16,17] and that metformin use can lead to vitamin B_{12} deficiency (and potentially to worsening of neuropathy) [6].

2. Material and methods

2.1. Animals

The experiments were performed on male 2–3 month old C57BL/6 mice, weighing 20–30 g at the beginning of the study. The animals (source: Military Academy Breeding Farm, Belgrade, Serbia) were housed under standard laboratory conditions: temperature of 22 ± 1 °C, 60 % relative humidity and maintained on a 12/12 h light/dark cycle with freely available food and water. Experimental groups consisted of 5–9 mice. All experiments were approved by the Institutional Animal Care and Use Committee of the Faculty of Pharmacy, University of

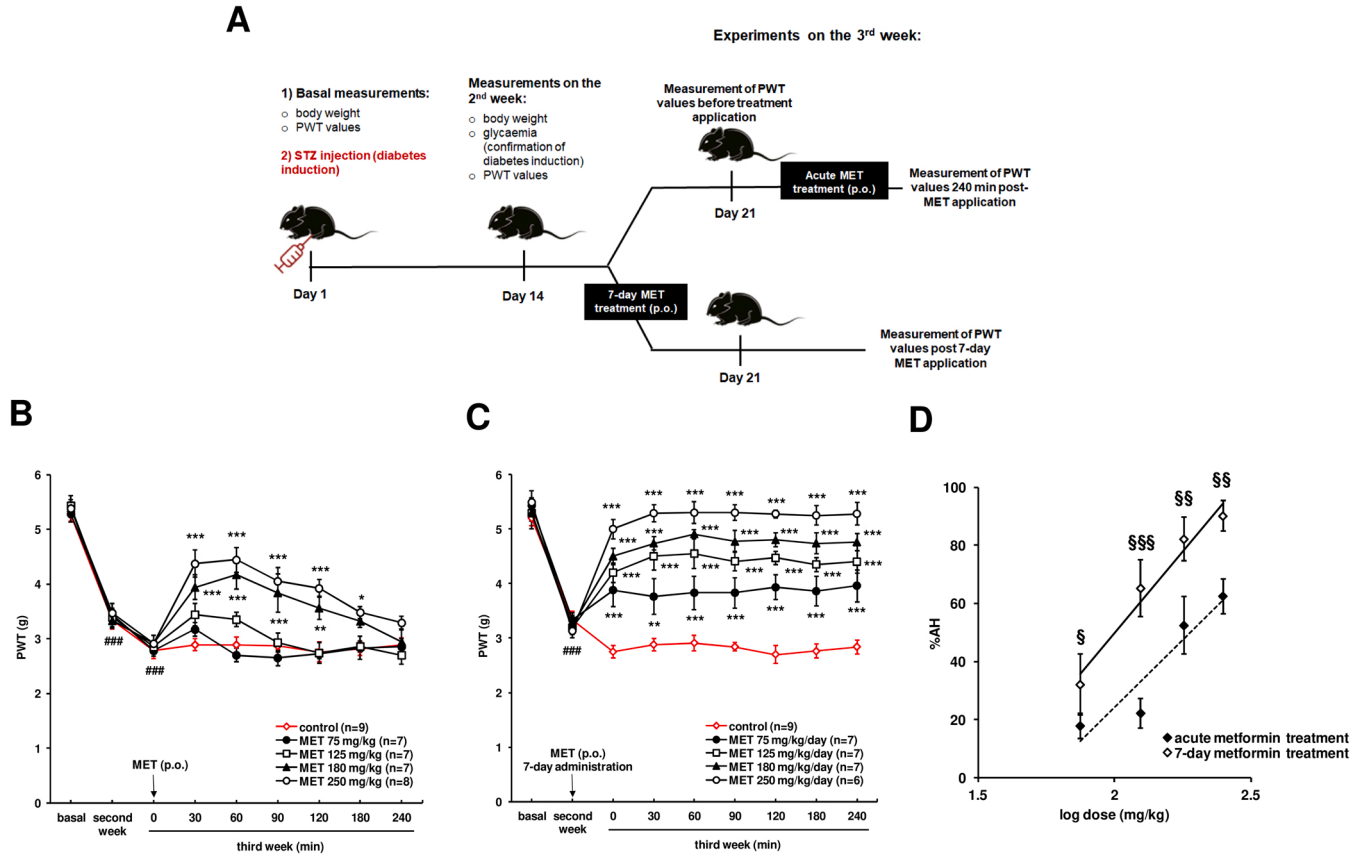

Fig. 1. (**A**) Experimental protocol used for examining the antihyperalgesic effects of metformin (**MET**) after systemic acute and prolonged 7-day administration in the model of streptozotocin (STZ)-induced painful diabetic neuropathy in male, 2–3 month old C57BL/6 mice. (**B-C**) The effects of MET after acute administration (**B**) and prolonged 7-day administration (**C**) on mechanical paw withdrawal thresholds (PWT) of diabetic mice. PWTs were measured before (basal), 2 weeks and 3 weeks after the STZ injection. In all experimental groups there was a significant reduction of PWT values 2 and/or 3 weeks after diabetes induction compared to their respective basal values ($^{###}P < 0.001$; two-way repeated measures ANOVA, followed by Tukey HSD *post hoc* test). MET was applied orally (p.o.) in a single dose, 3 weeks after the induction of diabetes (**B**), or for 7 days starting 2 weeks after the induction of diabetes (**C**) (denoted by arrows). Each point represents the mean ± SEM of the PWT (g). Statistical significance ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; two-way repeated measures ANOVA, followed by Tukey HSD *post hoc* test) was determined by comparison with the control group. (**D**) Log-dose response curves for the maximal antihyperalgesic effects (%AH) produced by MET after systemic acute and prolonged 7-day administration in the model of painful diabetic neuropathy ($^{§}P < 0.05$, $^{§§}P < 0.01$, $^{§§§}P < 0.001$; two-way ANOVA followed by Tukey HSD *post hoc* test compared with the same dose after acute administration).

Belgrade, Belgrade, Serbia (permit number: 323–07–03984/2017–05), and were carried out in accordance with EU Directive 2010/63/EU for animal experiments.

2.2. Drugs and their administration

We examined the efficacy of metformin following systemic and local (spinal and peripheral) administration. For studying systemic effects, metformin (tablet mixture: Glucophage®, Merck Healthcare KGaA, Germany) was suspended in distilled water and applied orally (p.o.) using an oral gavage for mice in a volume of 10 mL/kg, either as a single dose (3 weeks after diabetes induction) or as a prolonged 7-day treatment (starting 2 weeks after diabetes induction) (Fig. 1A). In experiments where the local antihyperalgesic efficacy of metformin was examined, metformin (pure substance: a gift from Galenika AD Beograd, Serbia) was dissolved in saline and applied intrathecally (i.t.) or intraplantarly (i.pl.) as a single dose in a volume of 10 μL/mouse using a microliter syringe and a 30 G needle (3 weeks after diabetes induction) (Fig. 2A). For spinal administration, mice were briefly anesthetized using sevoflurane (Sevorane®, Aesica Queenborough Limited, UK), following which their backs were shaved and disinfected with 70 % ethanol. The needle was then inserted into the L5-L6 region and the solution was gradually injected (correct placement of the needle was verified when the mouse flicked its tail following lumbar puncture) [18]. Local i.pl. administration was performed without anaesthesia on lightly restrained mice. The metformin solution was injected into the right hind paw.

Duloxetine (tablet mixture: Cymbalta®, Lilly S.A., Spain), oxycodone (tablet mixture: OxyContin®, Mundipharma GmbH, Germany), and ESL (tablet mixture: Zebinix®, Bial Portela & CA, Portugal) were suspended in distilled water and applied p.o. as a single dose in a volume of 10 mL/kg (3 weeks after diabetes induction). Vitamin B_{12}, in the form of hydroxocobalamin (solution for injection: OHB12, Galenika AD Beograd, Belgrade, Serbia), was diluted to the appropriate concentration in saline and applied intraperitoneally (i.p.) in a volume of 10 mL/kg for 7 days (starting 2 weeks after diabetes induction) (Supplemental figures

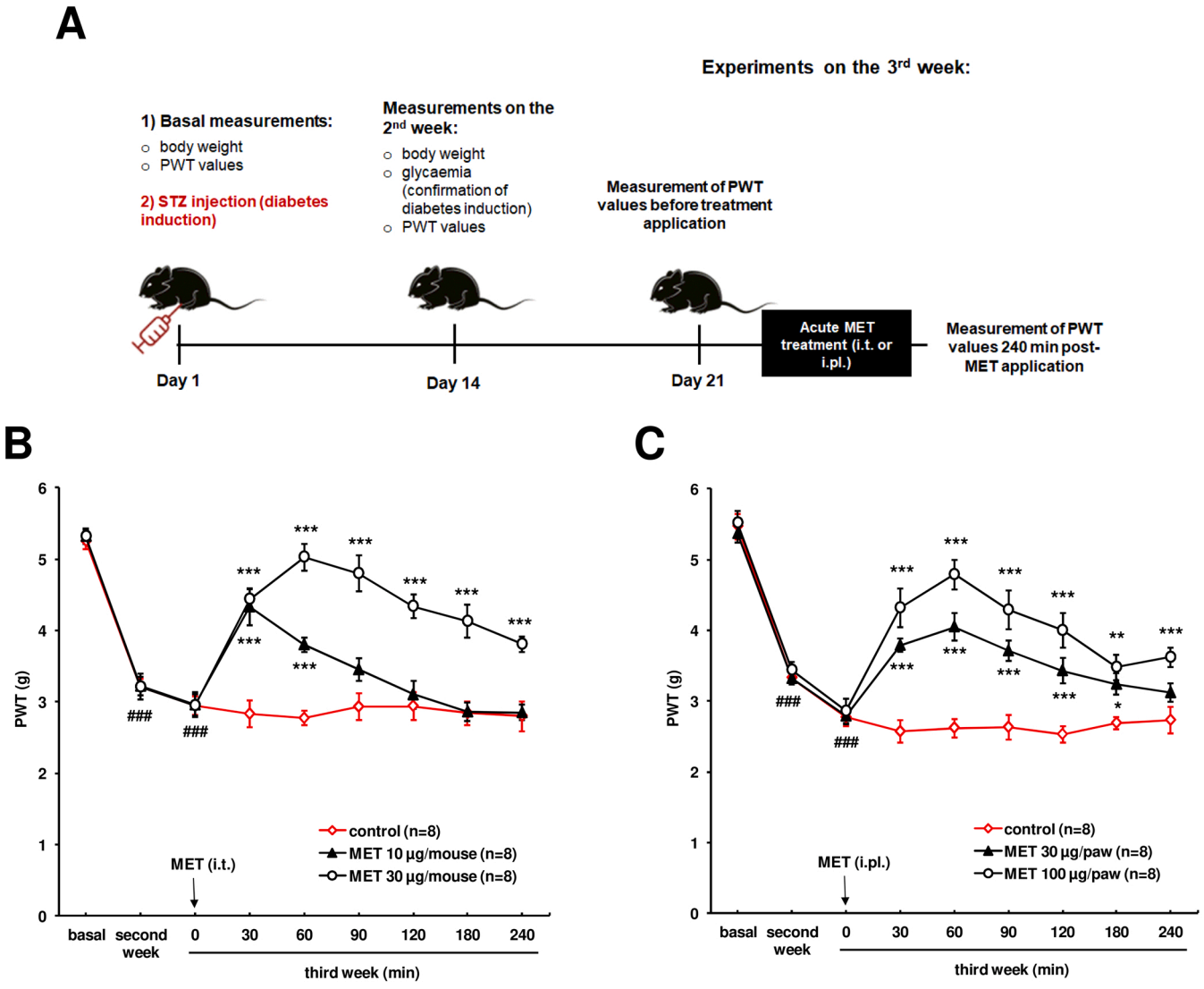

Fig. 2. (**A**) Experimental protocol used for examining the antihyperalgesic effects of metformin (**MET**) after local spinal and local peripheral application in the model of streptozotocin (STZ)-induced painful diabetic neuropathy in male, 2–3 month old C57BL/6 mice. (**B-C**) The effects of metformin (**MET**) after local spinal (**B**) and local peripheral application (**C**) on mechanical paw withdrawal thresholds (PWT) of diabetic mice. PWTs were measured before (basal), 2 weeks and 3 weeks after the STZ injection. In all experimental groups there was a significant reduction of PWT values 2 and 3 weeks after diabetes induction compared to their respective basal values ($^{###}P < 0.001$; two-way repeated measures ANOVA, followed by Tukey HSD *post hoc* test). MET was applied intrathecally (i.t.) or intraplantarly (i.pl.) in a single dose, 3 weeks after the induction of diabetes (denoted by arrows). Each point represents the mean ± SEM of the PWT (g). Statistical significance ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; two-way repeated measures ANOVA, followed by Tukey HSD *post hoc* test) was determined by comparison with the control group.

1A and 2A). In combination experiments, metformin was applied p.o. as a 7-day treatment (starting 2 weeks after diabetes induction) whereas duloxetine, oxycodone and ESL were applied in a single dose (3 weeks after diabetes induction). In the case of the metformin-vitamin B_{12} combination, both drugs were applied for 7 days, starting 2 weeks after diabetes induction (metformin was applied p.o. and vitamin B_{12} i.p.) (Figs. 4A and 5A).

Streptozotocin (Sigma-Aldrich Chemie GmbH, Munich, Germany) was dissolved in citrate buffer (0.03 M; pH 4.5), immediately before i.p. injection (10 mL/kg body weight).

antihyperalgesic effects were assessed in six time points (30, 60, 90, 120, 180 and 240 min following metformin administration), 3 weeks after the induction of diabetes, by measuring PWT values (*PWT post-treatment application*). Control group animals received the appropriate amount of vehicle (distilled water p.o., saline i.t. or saline i.pl.) instead of metformin (Figs. 1A and 2A). The antihyperalgesic effects (%AH) produced by metformin after acute administration were calculated using the following formula:

$$\%\mathbf{AH} = \frac{\mathbf{PWT}_{post-treatment\ \ application} - \mathbf{PWT}_{3\ \ weeks\ \ after\ \ diabetes\ \ induction\ \ (before\ \ treatment\ \ application)}}{\mathbf{PWT}_{basal} - \mathbf{PWT}_{3\ \ weeks\ \ after\ \ diabetes\ \ induction\ \ (before\ \ treatment\ \ application)}} \ \ x \ \ \mathbf{100}$$

2.3. Induction and assessment of diabetes

Diabetes in C57BL/6 mice was induced with a single injection of streptozotocin (150 mg/kg; i.p.) [19]. Two weeks after streptozotocin application, blood was sampled from the tail vein and glucose levels were measured using an automatic analyser (GlucoSure Autocode, Prizma, Serbia). Mice with a non-fasting blood glucose level higher than 13.9 mmol/L (250 mg/dL) were considered diabetic and were used for further experiments [19].

2.3.1. Assessment of mechanical pain hypersensitivity (mechanical hyperalgesia)

The development of mechanical hypersensitivity in diabetic mice was assessed by measuring paw withdrawal thresholds (PWT) using an electronic Von Frey anesthesiometer (IITC Life Science, Woodland Hills, CA) [19]. Mice were placed in individual plastic boxes on top of a metallic mesh floor. The mechanical stimulus was delivered using a

In experiments where we examined the antihyperalgesic effects of metformin following local i.pl. administration, measurements of antihyperalgesic effects were made on the right hind paw where we injected the metformin solution. Additionally, we measured PWT values on the contralateral left hind paw. These measurements were done in order to determine whether the effects of metformin following i.pl. administration are of local nature or are due to resorption from the site of injection and systemic effects.

In experiments where metformin was applied as a prolonged 7-day treatment, the p.o. treatment was started 2 weeks after diabetes induction and the measurement of antihyperalgesic effects were made on the third week (approximately 24 h after the last metformin dose). For the sake of uniformity, we monitored the effects of metformin during 240 min (the same as in the case with acute administration). Control group animals received an appropriate amount of vehicle for 7 days (Fig. 1A). The %AH was calculated using the following formula:

$$\%\mathbf{AH} = \frac{\mathbf{PWT}_{post-treatment\ \ application} - \mathbf{PWT}_{2\ \ weeks\ \ after\ \ diabetes\ \ induction\ \ (before\ \ treatment\ \ application)}}{\mathbf{PWT}_{basal} - \mathbf{PWT}_{2\ \ weeks\ \ after\ \ diabetes\ \ induction\ \ (before\ \ treatment\ \ application)}} \ \ x \ \ \mathbf{100}$$

plastic, semiflexible filament coupled with a force transducer. The force was applied to the plantar surface of the hind paw and the pressure was gradually increased until the animal briskly withdrew its paw, which was automatically recorded on a digital screen of the apparatus. PWTs (in grams) were measured before diabetes induction (basal values), 2 weeks and 3 weeks after the injection of streptozotocin. In every time point at least four PWT measurements were made and the mean value was used for further calculations. Following the streptozotocin injection, there is a progressive decline in PWT values.

The level of mechanical hyperalgesia (%HA) was calculated for each diabetic animal using following formula:

$$\%\mathbf{HA} = \frac{\mathbf{PWT}_{basal} - \mathbf{PWT}_{2\ \ or\ \ 3\ \ weeks\ \ after\ \ diabetes\ \ induction}}{\mathbf{PWT}_{basal}} \ \ x \ \ \mathbf{100}$$

2.3.2. Examination of the antihyperalgesic effects of metformin following systemic and local application

In this study we examined the antihyperalgesic effects of metformin following systemic p.o. (acute and prolonged 7-day treatment) and local application (acute i.t. and i.pl. treatment). In cases where metformin was applied in an acute manner (single p.o., i.t., or i.pl. administration), the

Values of %AH larger than 100 % or negative %AH values were rounded to 100 % and 0 %, respectively.

The doses of metformin expected to produce an antihyperalgesic effect of 50 % (ED_{50} values) with 95 % confidence limits were estimated from corresponding log dose-response curves determined at the time of peak effect for both systemic acute and prolonged 7-day metformin administration [20].

2.3.3. Examination of the antihyperalgesic effects of analgesics/vitamin B_{12} and two-drug metformin-analgesic/vitamin B_{12} combinations

Duloxetine, oxycodone and ESL were p.o. applied in a single dose, 3 weeks after the induction of diabetes and their antihyperalgesic effects were measured 30, 60, 90, 120, 180 and 240 min following their administration (Supplemental figure 1A). The antihyperalgesic effects were calculated using the same formula used to calculate the antihyperalgesic effects following acute metformin administration (see above). Vitamin B_{12} was applied i.p. for 7 days, starting 2 weeks after diabetes induction. Its antihyperalgesic effects were measured in the third week following diabetes induction, approximately 24 h after the

last i.p. injection (Supplemental figure 2A). The antihyperalgesic effects produced by vitamin B_{12} were calculated with the same formula used to calculate the antihyperalgesic effects produced by metformin following prolonged 7-day administration (see above).

In combination experiments where we examined the effects of two-drug combination of metformin and analgesics/vitamin B_{12}, metformin was applied for 7 days, duloxetine/oxycodone/ESL in a single dose and vitamin B_{12} as a 7-day treatment (as in the case with individual administration) (Figs. 4A and 5A). The antihyperalgesic effects produced by combinations were calculated using the same formula as the one used for calculating the antihyperalgesic effects produced by metformin following prolonged 7-day administration (see above).

As in the case of metformin, ED_{50} values for analgesics/vitamin B_{12} and two-drug metformin-analgesic/vitamin B_{12} combinations were determined with linear regression analysis of log dose-response curves [20].

2.4. Analysis of the type of interaction between metformin and analgesics/vitamin B_{12}

The type of interaction between metformin and the selected drugs was determined using isobolographic analysis at the ED_{50} level of the effect, as described previously [19,21]. In brief, we determined the ED_{50} value for each individual drug from the corresponding log dose-response curves. In the next step, we examined the antihyperalgesic efficacy of two-drug combinations of metformin and duloxetine/oxycodone/ESL/vitamin B_{12}, which were co-applied in fixed-dose fractions of their individual ED_{50} (1/16, 1/8, 1/4 and 1/2 of the ED_{50} values). The experimental ED_{50} ($ED_{50\ mix}$) values for drug combinations were determined by linear regression analysis of log dose-response curves and compared to their theoretical additive ED_{50} values ($ED_{50\ add}$). In cases where the $ED_{50\ mix}$ of the drug combination was significantly lower than the $ED_{50\ add}$ value a supra-additive (synergistic) interaction between the drugs exists [21].

Additionally, we calculated the interaction index (γ) for each combination to describe the magnitude of interaction [19,22].

2.5. Analysis of the duration of effect of analgesics and metformin-analgesic combinations

In order to compare the duration of antihyperalgesic effects produced by an analgesic after individual acute administration (duloxetine, oxycodone, ESL) to the duration of effects produced by the same analgesic when it was combined with metformin, the slopes of %AH-ΔAUC regression lines were calculated as previously described [19,23]. The areas under the time-PWT curves (AUC), for PWT values measured on the third week after diabetes induction (0–240 min) were determined for the control (AUC_C) group and every tested dose of a drug/drug combination (AUC_D), and the differences were calculated as $\Delta AUC = AUC_D - AUC_C$ [19,23]. The ΔAUC for each tested drug/drug combination dose is presented as a function of its maximal %AH ($\Delta AUC = slope \times \%AH + intercept$). In the %AH-ΔAUC regression line, the slope is the relative measure of the duration of the drug/drug combination effects; the treatment with a significantly greater slope exerts an effect of longer duration than that with a smaller slope [19,23,24]. The slopes produced by an analgesic after individual administration was statistically compared with the slope for the combination of that analgesic and metformin (using the test for parallelism; see below) in order to determine if there is a change in the duration of effects. In addition, a high correlation coefficient of the %AH-ΔAUC regression line indicates that the duration of the effect of the drug/drug combination treatment is dose dependent [19,23,24].

2.6. Oral glucose tolerance test (OGTT)

We performed the OGTT, as previously described [25], in order to assess if the effects of metformin on painful hypersensitivity are possibly dependent on its antihyperglycemic efficacy. Using the OGTT we examined the effects of prolonged 7-day metformin treatment on fasting glycaemia and glucose levels following oral glucose administration. Briefly, mice were fasted for 6 h, following which we measured fasting glucose levels in blood sampled from tail veins. Then, glucose (2 g/kg; dissolved in distilled water) was administered p.o. in a volume of 10 mL/kg and blood glucose values were measured in three time points (30, 60 and 120 min after the administration of glucose solution). In these experiments we had 3 experimental groups: healthy control mice (treated with distilled water for 7 days), diabetic control mice (treated with distilled water for 7 days, starting 2 weeks after diabetes induction) and a group of diabetic mice which received the highest dose of metformin tested for antihyperalgesic efficacy (250 mg/kg; p.o. for 7 days, starting 2 weeks after diabetes induction). The OGTT was performed approximately 24 h after the last dose of vehicle/metformin was administered (Supplemental figure 3A).

2.7. Rotarod test

We used the rotarod test to assess the effects of the highest tested doses of metformin (administered as a single dose), analgesics (duloxetine, oxycodone, ESL) and their two-drug combinations on motor coordination or sedation. In the case of two-drug combinations metformin was administered for 7 days (starting 2 weeks after diabetes induction), whereas analgesics were administered acutely (as a single dose, 3 weeks after the induction of diabetes). The test was performed as described previously [19]. We used a rotarod apparatus (Treadmill for mice 7600; Ugo Basile, Milano, Italy) consisting of a rod rotating at a constant speed of 15 rpm. Diabetic animals were trained to remain balanced on the rotating rod for 2 days. The day after the training session, the effects of different treatments on motor performance were assessed. Only diabetic animals that could remain balanced on the rod for 60 s on two consecutive trials (separated by 30 min pause between trials) were used in the experiments. Following treatment application, latencies to remain on the rotating rod were recorded at six time points during 240 min (which correspond to time points when antihyperalgesic effects were measured). The cut-off time was 60 s.

2.8. Statistical analysis

The statistical analysis was performed using SigmaPlot 11 (Systat Software Inc., Richmond, CA, USA). The results are presented as mean group values ± SEM. Data from the model of streptozotocin-induced PDN (PWT values) and the OGTT were analysed using two-way repeated measures ANOVA (type treatment was the between-subject factor, and time was the within-subject factor). Differences between antihyperalgesic effects produced by metformin after systemic acute and prolonged 7-day treatment were analysed using a two-way ANOVA (the factors were duration of treatment and metformin dose). Data from the rotarod test was analysed using Mann-Whitney *U*-test. Pharmacological computations were done according to Tallarida [20]. The difference between theoretical ED_{50} ($ED_{50\ add}$) and experimental ED_{50} ($ED_{50\ mix}$) for drug combination was examined by a modified *t*-test [21]. The slopes of two %AH-ΔAUC regression lines (for an analgesic after individual administration and the same analgesic combined with metformin) were compared using the test for parallelism, which uses the Student *t*-test to compare the slopes of two regression lines. A *P* value of less than 0.05 was considered statistically significant in all tests that were used.

Table 1
Parameters of the isobolographic analysis for metformin combinations with analgesics/vitamin B_{12} in the model of streptozotocin-induced painful diabetic neuropathy.

Drugs	ED_{50}[a] in mg/kg ± SEM (95 % confidence limits)			Maximal antihyperalgesic effects
Metformin (7-day administration)	100.4 ± 7.6 (55.1 – 130.3)			32.1–90.1 %
Duloxetine	2.5 ± 0.4 (0.7 – 4.8)			35.3–86.0 %
Oxycodone	0.6 ± 0.1 (0.2 – 1.1)			37.0–78.4 %
Eslicarbazepine acetate	71.9 ± 10.1 (14.8 – 189.9)			31.0–80.1 %
Vitamin B_{12}	0.3 ± 0.01 (0.2 – 0.3)			22.2–90.2 %
Drug combinations	**$ED_{50\ add}$[b] in mg/kg**	**$ED_{50\ mix}$[c] in mg/kg**	γ[d]	**Maximal antihyperalgesic effects**
Metformin + duloxetine	51.5 ± 3.7 (39.8 – 58.3)	17.0 ± 2.1 (7.7 – 33.2)*	0.33	24.8–81.5 %
Metformin + oxycodone	50.5 ± 3.2 (40.2 – 56.0)	16.0 ± 0.1 (15.7 – 16.3)*	0.32	29.4–75.2 %
Metformin + eslicarbazepine acetate	86.1 ± 6.3 (70.0 – 99.1)	24.8 ± 1.3 (19.1 – 30.9)*	0.29	22.6–93.9 %
Metformin + vitamin B_{12}	50.4 ± 1.6 (46.3 – 54.2)	16.9 ± 1.2 (12.0 – 23.2)*	0.33	22.8–83.2 %

[a] ED_{50} = Effective dose required to produce 50 % antihyperalgesic activity.
[b] $ED_{50\ add}$ = Theoretical additive ED_{50} for drug mixture.
[c] $ED_{50\ mix}$ = Experimental ED_{50} for drug mixture.
[d] $\gamma = ED_{50\ METFORMIN\ COMBINED\ WITH\ ANALGESIC/VITAMIN\ B12} / ED_{50\ METFORMIN\ GIVEN\ ALONE} + ED_{50\ ANALGESIC/VITAMIN\ B12\ COMBINED\ WITH\ METFORMIN} / ED_{50\ ANALGESIC/VITAMIN\ B12\ GIVEN\ ALONE}$. Values near 1 indicate an additive interaction, values larger than 1 imply an antagonistic interaction and values smaller than 1 indicate a synergistic interaction [22].
* $P < 0.05$ between $ED_{50\ add}$ and $ED_{50\ mix}$ (modified t-test), indicates a synergistic interaction [21].

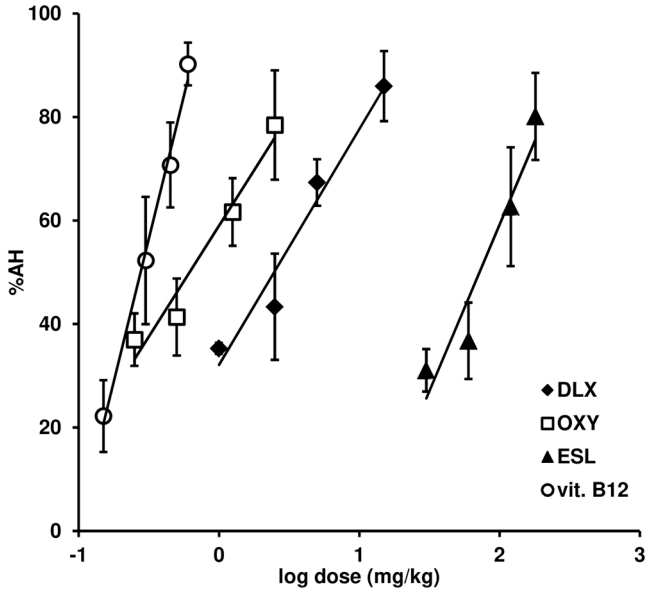

Fig. 3. Log dose-response curves for the maximal antihyperalgesic effects produced by duloxetine (**DLX**), oxycodone (**OXY**), eslicarbazepine acetate (**ESL**) and vitamin B_{12} (**vit. B12**) in the model of streptozotocin-induced painful diabetic neuropathy (determined 3 weeks after the induction of diabetes, at the time of peak effects). Each point represents the mean (±SEM) of the percentage of antihyperalgesic effects (%AH) obtained in 5–9 animals.

3. Results

3.1. Antihyperalgesic effects of metformin in the model of streptozotocin-induced PDN

In all experimental groups used for the assessment of the antihyperalgesic effects of metformin there was a significant decrease of PWT values 2 and/or 3 weeks after the induction of diabetes compared to their respective basal values ($P < 0.001$; Figs. 1 and 2). The level of mechanical hyperalgesia that developed in all experimental groups was similar, 34.4–42.6 % (2 weeks after diabetes induction) and 43.7–49.0 % (3 weeks after diabetes induction).

Systemic administration of metformin reduced mechanical hyperalgesia in diabetic mice in a significant and dose-dependent manner after acute and prolonged 7-day administration (both $P < 0.001$; Fig. 1, B and C). The maximal antihyperalgesic effects of a single systemic dose of metformin (75–250 mg/kg; p.o.) were 17.9–62.5 %, whereas the antihyperalgesic effects achieved after prolonged 7-day administration (75–250 mg/kg/day p.o.) were 32.1–90.1 %. When we compared the effects of the same metformin doses after acute and prolonged administration, we found that the antihyperalgesic effects of metformin achieved after 7-day administration were significantly higher than after acute administration ($P < 0.001$; Fig. 1D). The ED_{50} for metformin was 189.5 ± 25.6 mg/kg after acute administration of a single dose and 100.4 ± 7.6 mg/kg/day after prolonged 7-day administration (Table 1).

Similar to systemic application, local i.t. (10 and 30 μg/mouse) and local i.pl. (30 and 100 μg/paw) application of metformin also produced a significant and dose-related antihyperalgesic effect in mice with streptozotocin-induced PDN ($P < 0.001$ for both routes; Fig. 2, B and C). The maximal antihyperalgesic effects achieved after i.t. metformin application were 60.5 % for the 10 μg dose and 85.0 % for the 30 μg dose. Following i.pl. administration into the right hind paw, the maximal antihyperalgesic effects produced by metformin were 48.6 % for the 30 μg dose and 75.8 % for the 100 μg dose. At the same time, local i.pl. injection of metformin into the right hind paw did not have a significant effect on PWT values measured on the contralateral left hind paw ($P = 0.674$; not shown), indicating that the measured antihyperalgesic effects after i.pl. application were not due to systemic effects of metformin.

3.2. Antihyperalgesic effects of analgesics and vitamin B_{12} in the model of streptozotocin-induced PDN

In all experimental groups used for the assessment of the antihyperalgesic effects of analgesics and vitamin B_{12}, there was a significant decrease in PWT values 2 and/or 3 weeks after diabetes induction (all $P < 0.001$; Supplemental figures 1 and 2), with hyperalgesia values of 36.4–45.2 % (2 weeks after diabetes induction) and 43.3–49.9 % (3 weeks after diabetes induction).

Application of a single dose of duloxetine (1–15 mg/kg; p.o.), oxycodone (0.25–2.5 mg/kg; p.o.) and ESL (30–180 mg/kg; p.o.), 3 weeks after diabetes induction, produced significant and dose-dependent antihyperalgesic effects in diabetic mice (all $P < 0.001$; Fig. 3 and

Supplemental figure 1, B-D). The maximal antihyperalgesic effects produced by analgesics are presented in Table 1. The ED_{50} values for duloxetine, oxycodone and ESL in the model of streptozotocin-induced PDN were calculated by linear regression analysis of log dose-response curves (Fig. 3) and are presented in Table 1.

Prolonged 7-day administration of vitamin B_{12} (0.15–0.60 mg/kg/

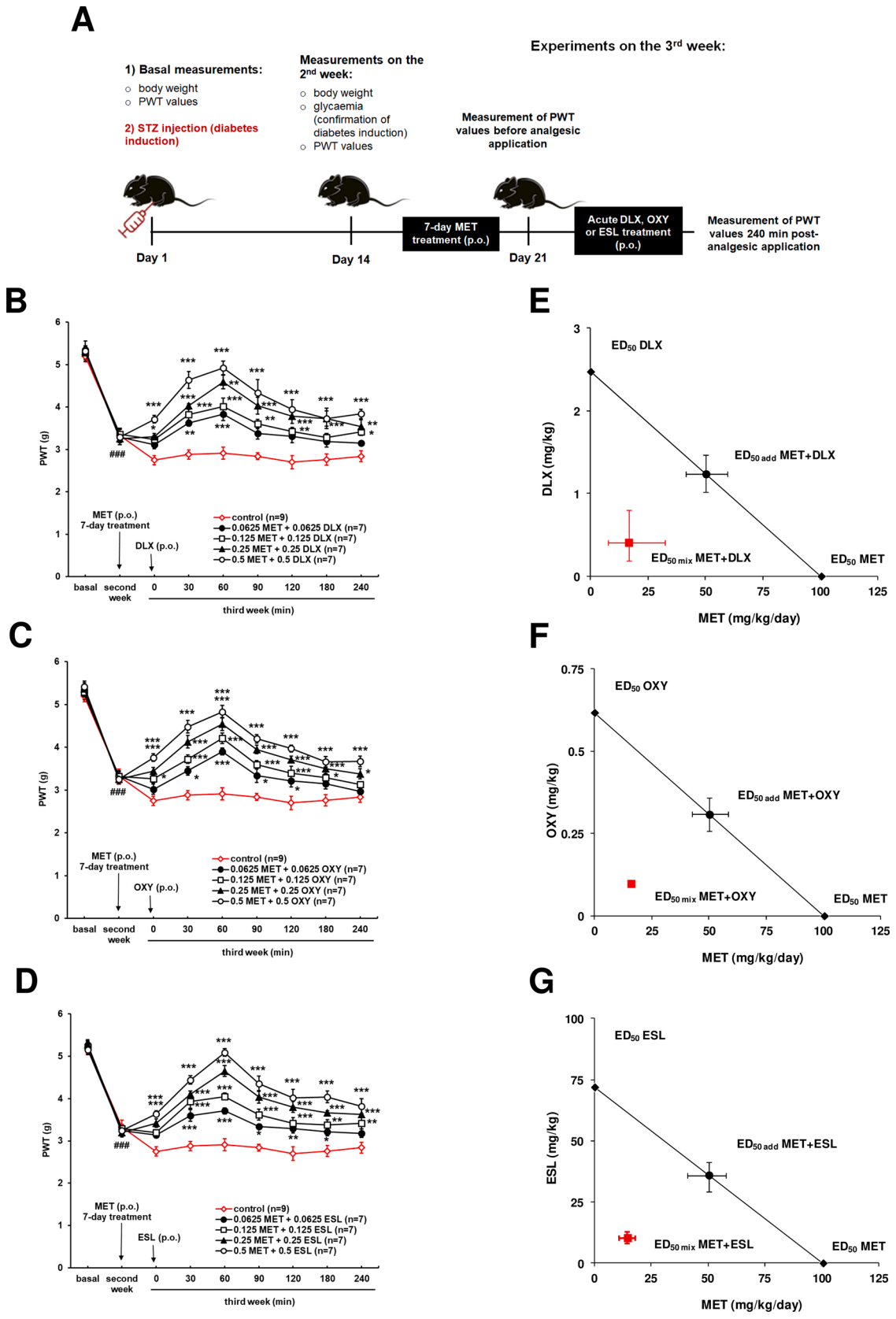

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Fig. 4. (**A**) Experimental protocol used for examining the antihyperalgesic effects of metformin (**MET**) combinations with duloxetine (**DLX**), oxycodone (**OXY**) and eslicarbazepine acetate (**ESL**) in the model of streptozotocin (STZ)-induced painful diabetic neuropathy in male, 2–3 month old C57BL/6 mice. (**B-D**) The effects of the **MET+DLX** (**B**), **MET+OXY** (**C**) and **MET+ESL** (**D**) combinations on mechanical paw withdrawal thresholds (PWT) of diabetic mice. PWTs were measured before (basal), 2 weeks and 3 weeks after the STZ injection. In all experimental groups there was a significant reduction of PWT values 2 weeks after diabetes induction compared to their respective basal values ($^{\#\#\#}P < 0.001$; two-way repeated measures ANOVA, followed by Tukey HSD *post hoc* test). MET, DLX, OXY and ESL were applied orally (p.o.). MET was administered for 7 days, starting 2 weeks after the induction of diabetes, whereas DLX, OXY and ESL were applied in a single dose, 3 weeks after the induction of diabetes (denoted by arrows). All the drugs were administered in fixed-dose fractions of their individual ED_{50} values (1/16 = 0.0625, 1/8 = 0.125, 1/4 = 0.25, 1/2 = 0.5; e.g. 0.5 MET + 0.5 DLX indicates that animals received one half of the ED_{50} of MET and one half of the ED_{50} of DLX). Each point represents the mean ± SEM of the PWT (g). Statistical significance ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; two-way repeated measures ANOVA, followed by Tukey HSD *post hoc* test) was determined by comparison with the control group. (**E-G**) Isobolograms for the MET+DLX (**E**), MET+OXY (**F**) and MET+ESL (**G**) combinations in the model of painful diabetic neuropathy. Individual ED_{50} values for each drug are plotted on the axes. The straight line connecting the ED_{50} values is the theoretical additive line, and the point on this line is the $ED_{50\ add}$ (theoretical additive ED_{50}). There is a significant difference ($P < 0.05$; modified *t*-test) between the $ED_{50\ add}$ and $ED_{50\ mix}$ (experimental ED_{50} for drug mixture) in every combination, indicating a synergistic drug interaction for the combinations tested.

day; i.p.) also reduced mechanical hyperalgesia in a significant and dose-dependent manner ($P < 0.001$; Fig. 3 and Supplemental figure 2B). The antihyperalgesic effects produced by vitamin B_{12} and its ED_{50} value are presented in Table 1.

3.3. Antihyperalgesic effects of metformin-analgesic/vitamin B_{12} combinations in the model of streptozotocin-induced PDN and isobolographic analysis

As in previous cases, there was a significant reduction of PWT values in all experimental groups used for the assessment of the antihyperalgesic effects of two-drug combinations of metformin and analgesics/vitamin B_{12} 2 weeks after the application of streptozotocin (all $P < 0.001$; Figs. 4, B-D and 5B). The level of hyperalgesia that had developed was similar in all groups, and was in the range of 36.4–40.4 %.

Two-drug combinations of metformin and duloxetine/oxycodone/ESL/vitamin B_{12}, administered in doses that correspond to fixed fractions of their individual ED_{50} values (1/16, 1/8, 1/4 and 1/2 of ED_{50} values) produced significant and dose-dependent antihyperalgesic effects in the model of streptozotocin-induced PDN (all $P < 0.001$; Fig. 4, B-D and 5B). The maximal antihyperalgesic effects produced by metformin combinations with analgesics/vitamin B_{12} are presented in Table 1.

Experimentally determined $ED_{50\ mix}$ and theoretical $ED_{50\ add}$ values for metformin-analgesic/vitamin B_{12} combinations in the model of PDN are presented in Table 1. In all examined combinations the $ED_{50\ mix}$ values were significantly lower than the $ED_{50\ add}$ values (all $P < 0.05$) and the interaction index was lower than 1 (Figs. 4, E-G and 5C, Table 1) indicating that metformin interacts in a synergistic manner with duloxetine, oxycodone, ESL and vitamin B_{12} in this pain model. Based on the values of the interaction index, there was an approximately 6-fold reduction of doses of both drugs in metformin combinations with duloxetine/oxycodone/vitamin B_{12}, and a 7-fold reduction of doses of both drugs in the metformin-ESL combination (compared to the doses needed to produce the same level of effect after individual administration). In addition, the 95 % confidence intervals of the $ED_{50\ mix}$ and $ED_{50\ add}$ values (presented on isobolograms) of the metformin-analgesic/vitamin B_{12} combinations do not overlap, which confirms the synergistic nature of interaction between metformin and analgesics/vitamin B_{12} (Figs. 4, E-G and 5C).

3.4. Analysis of the duration of antihyperalgesic effects of analgesics and two-drug metformin-analgesic combinations in the model of streptozotocin-induced PDN

The duration of antihyperalgesic effects of acutely administered analgesics (duloxetine, oxycodone, ESL), as well as their combinations with metformin are expressed as slopes of regression lines which describe the dependence of the ΔAUC parameter on the maximal antihyperalgesic effect produced by the analgesic/metformin-analgesic combination (see Methods; Table 2).

Based on the values of the slopes (Table 2) after individual analgesic administration and combined administration of analgesics with metformin, we found that the duration of effects of duloxetine were significantly shorter when it was co-administered with metformin compared to the duration of its antihyperalgesic effects when it was administered individually ($P = 0.027$). The duration of antihyperalgesic effects of ESL was also shorter when it was combined with metformin, however the difference between slopes was borderline significant ($P = 0.058$). In the case of oxycodone, its duration of antihyperalgesic effects was unchanged when it was combined with metformin ($P = 0.725$).

The duration of effects of metformin and vitamin B_{12} after prolonged 7-day administration was not determined, because these drugs were administered in a prolonged manner and the antihyperalgesic effect they produced was relatively constant when measured 3 weeks after diabetes induction.

3.5. Effects of metformin treatment in the OGTT

In diabetic mice, we detected a significant increase in both fasting glycaemia values and glycaemia values following oral glucose application compared to the same values obtained in healthy control mice ($P < 0.001$; Supplemental figure 3B). Prolonged 7-day administration of metformin in the highest dose that was tested for antihyperalgesic effects (250 mg/kg/day; p.o.) failed to produce a significant effect on fasting glycaemia, as well as glycaemia values 30, 60 and 120 min following glucose application ($P = 0.973$; Supplemental figure 3B).

3.6. Effects of metformin, analgesics and metformin-analgesic combinations in the rotarod test

Acute administration of the highest tested doses of metformin (250 mg/kg; p.o.), duloxetine (15 mg/kg; p.o.), oxycodone (2.5 mg/kg; p.o.) and ESL (180 mg/kg; p.o.) had no significant effect on motor performance of diabetic mice in the rotarod test (all $P > 0.198$ for every tested drug and time point; Fig. 6A). Additionally, application of the highest tested doses of metformin-analgesic combinations to diabetic mice, which consisted of 1/2 of the ED_{50} of metformin (administered in a prolonged manner) and duloxetine/oxycodone/ESL (administered in an acute manner) did not produce a significant effect in the rotarod test (all $P > 0.272$ for every tested combination and time point; Fig. 6B).

4. Discussion

We demonstrated that acute and prolonged systemic application of metformin produces dose-dependent antihyperalgesic effects in the model of streptozotocin-induced PDN, with comparable efficacy to duloxetine, oxycodone, ESL and vitamin B_{12}. The highest tested dose had no significant effect on motor performance in the rotarod test (acute metformin application), nor on fasting/post-glucose glycaemia values determined in the OGTT (prolonged metformin application), indicating that metformin`s antihyperalgesic effects were not due to motor

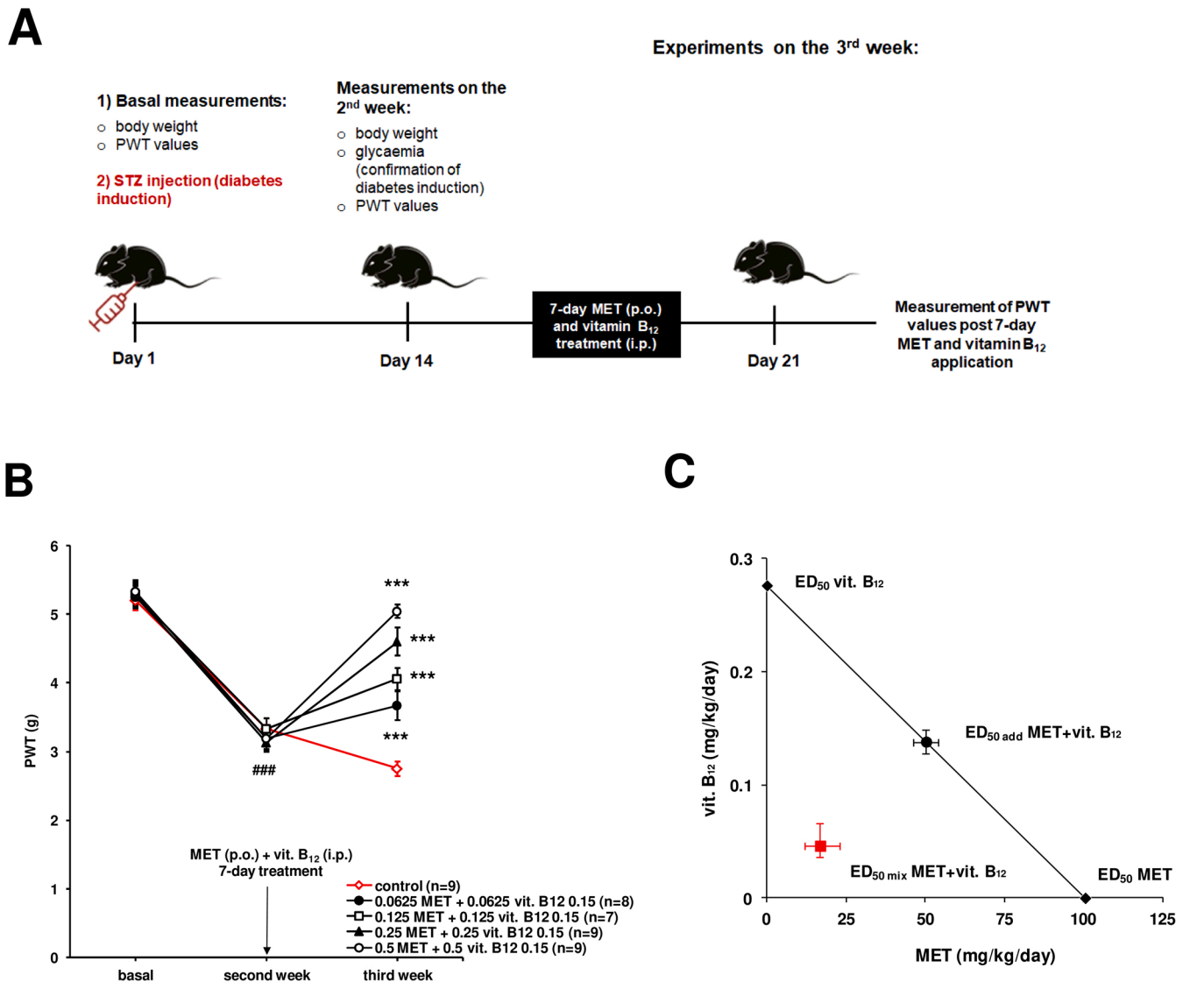

Fig. 5. (**A**) Experimental protocol used for examining the antihyperalgesic effects of the metformin (**MET**) combination with vitamin B_{12} (**vit. B_{12}**) in the model of streptozotocin (STZ)-induced painful diabetic neuropathy in male, 2–3 month old C57BL/6 mice. (**B**) The effects of the **MET+vit. B_{12}** on mechanical paw withdrawal thresholds (PWT) of diabetic mice. PWTs were measured before the induction of diabetes, 2 weeks and 3 weeks after the STZ injection. In all experimental groups there was a significant reduction of PWT values 2 weeks after diabetes induction compared to their respective basal values ($^{###}P < 0.001$; two-way repeated measures ANOVA, followed by Tukey HSD *post hoc* test). MET was applied orally (p.o.) and vit. B_{12} intraperitoneally (i.p) for 7 days, starting 2 weeks after the induction of diabetes (denoted by arrow). Both drugs were administered in fixed-dose fractions of their individual ED_{50} values (1/16 = 0.0625, 1/8 = 0.125, 1/4 = 0.25, 1/2 = 0.5; e.g. 0.5 MET + 0.5 vit. B_{12} indicates that animals received one half of the ED_{50} of MET and one half of the ED_{50} of vit. B_{12}). Each point represents the mean ± SEM of the PWT (g). Statistical significance ($^{***}P < 0.001$; two-way repeated measures ANOVA, followed by Tukey HSD *post hoc* test) was determined by comparison with the control group. **(C)** Isobologram for the MET+vit. B_{12} combination in the model of painful diabetic neuropathy. The ED_{50} values for each drug are plotted on the axes. The straight line connecting each ED_{50} value is the theoretical additive line, and the point on this line is the $ED_{50\ add}$ (theoretical additive ED_{50}). There is a significant difference ($P < 0.05$; modified *t*-test) between the $ED_{50\ add}$ and $ED_{50\ mix}$ (experimental ED_{50} for drug mixture), indicating a synergistic drug interaction between MET and vit. B_{12}.

impairment or changes in glucose levels. Our results are in line with previous reports on the efficacy of metformin in reducing mechanical hypersensitivity in models of PDN after systemic acute and prolonged administration [11,12,26]. Additionally, we found that local spinal and peripheral application of metformin is capable of reducing mechanical hyperalgesia in diabetic animals. These findings suggest that after systemic metformin application both central and peripheral effects could contribute to the overall antihyperalgesic efficacy. Our results align well with the findings of Wang et al. [26] who demonstrated the antihyperalgesic efficacy of metformin in *db/db* mice (model of type 2 diabetes) following local peripheral administration, as well as Ling et al. [27] who found that spinal administration of metformin can reduce mechanical/thermal hypersensitivity of mice with oxaliplatin-induced neuropathic pain.

Metformin's efficacy in alleviating painful hypersensitivity in neuropathic pain models (including models of PDN) has been attributed to its ability to activate the AMP-activated protein kinase (AMPK), an enzyme that has a pivotal role in controlling multiple cellular functions, including neural excitability [7,11]. The activity of this enzyme is lowered in the model of streptozotocin-induced PDN (in dorsal root ganglia and peripheral nerves), and metformin application is capable of restoring its activity [12,28]. Activated AMPK negatively modulates several downstream targets to reduce neuropathic painful hypersensitivity. Among these targets, the role of mitogen-activated protein

Table 2
Parameters of the duration of antihyperalgesic action of analgesics and metformin-analgesic combinations in the model of streptozotocin-induced painful diabetic neuropathy.

Drug/drug combination	Slope[a] ± SEM (95 % confidence limits)	r[b]
Duloxetine	5.1 ± 0.6 (2.6 – 7.6)	0.99
Oxycodone	4.2 ± 0.4 (2.4 – 6.0)	0.99
Eslicarbazepine acetate	5.1 ± 1.0 (0.9 – 9.2)	0.97
Metformin + duloxetine	3.0 ± 0.2 (2.1 – 4.0)[c]	0.99
Metformin + oxycodone	4.0 ± 0.2 (3.2 – 4.8)	1
Metformin + eslicarbazepine acetate	2.8 ± 0.1 (2.4 – 3.3)[d]	1

[a] Slope of the %AH-ΔAUC regression line is a relative measure for the duration of the effect produced by a drug/drug combination; treatments with a significantly greater slope has a longer duration of antinociceptive effect [19,24].
[b] r = Correlation coefficients of the %AH-ΔAUC regression lines; values near 1 indicate that the duration of the effect of a drug/drug combination treatment is dose-dependent [19,24].
[c] $P < 0.05$; compared with the slope for duloxetine alone, test for parallelism.
[d] $P = 0.058$; compared with the slope for eslicarbazepine acetate alone, test for parallelism.

kinases (MAPKs) and mammalian target of rapamycin (mTOR) kinase appears to be most widely studied [7]. These kinases are thought to have a critical role in the development of painful hypersensitivity in neuropathic pain states [29,30]. In models of neuropathic pain (induced by nerve/spinal cord injury) metformin reduced pain hypersensitivity by negatively modulating the activity of MAPKs and/or the mTOR kinase [7]. The same mechanism could also account for metformin's efficacy in the model of streptozotocin-induced PDN, seeing as increased activity of these kinases has been demonstrated in dorsal root ganglia and in the spinal cord of animals with streptozotocin-induced PDN and their inhibition can reduce hypersensitivity in this model [31,32]. Our results align well with this hypothesis, as we demonstrated that both spinal and peripheral metformin application is capable of reducing hyperalgesia in animals with streptozotocin-induced PDN. Additionally, the efficacy of metformin in alleviating PDN has also been attributed to its ability to reduce the activity of TRPA1 channels (*via* AMPK activation), which are important for transducing nociceptive stimuli [26].

One of the main findings of this study is that metformin interacts synergistically with three mechanistically different analgesics to reduce mechanical hypersensitivity in diabetic animals. We detected a 6-fold (metformin combinations with duloxetine/oxycodone) and 7-fold (metformin-ESL combination) decrease in doses of both drugs needed to reduce hyperalgesia compared to doses that produce the same level of effect after individual administration. These results are in line with our previous study [23], where we demonstrated that metformin synergizes with different analgesics (ibuprofen, aspirin, tramadol and pregabalin) in reducing inflammatory hyperalgesia. To the best of our knowledge no previous study examined the interaction between metformin and analgesics in experimental models of neuropathic pain. However, there are studies which demonstrated the beneficial effects of metformin combinations with antidiabetic drugs/hypolipemics on other behavioral manifestations of diabetic neuropathy (such as reduced grip strength and reduced sensitivity to thermal stimuli) [33,34].

A synergistic interaction between two drugs may be a result of a pharmacodynamic (i.e. the drugs possess different mechanisms of action) and/or a pharmacokinetic interaction (i.e. due to changes in pharmacokinetic properties of individual drugs). Although we did not perform a pharmacokinetic analysis, we assume that pharmacokinetic interactions are not of significance for the examined combinations for several reasons. First, the duration of antihyperalgesic effects of acute treatments was either unchanged (oxycodone) or shorter (duloxetine and ESL) when these drugs were applied in combination with metformin (in cases of pharmacokinetic interactions a prolongation of duration would be expected). Second, metformin was applied in a prolonged manner, with the last dose being administered approximately 24 h before the analgesics. Lastly, metformin is generally not prone to pharmacokinetic interactions, as it is neither metabolized, nor bound to plasma proteins in a significant manner [35]. Therefore, the finding of synergistic interactions between metformin and duloxetine/oxycodone/ESL can in most part be explained by an existence of a beneficial pharmacodynamic interaction between these drugs, i.e. metformin reduces hyperalgesia *via* a mechanism (AMPK activation) different from the other tested drugs, which will be further discussed.

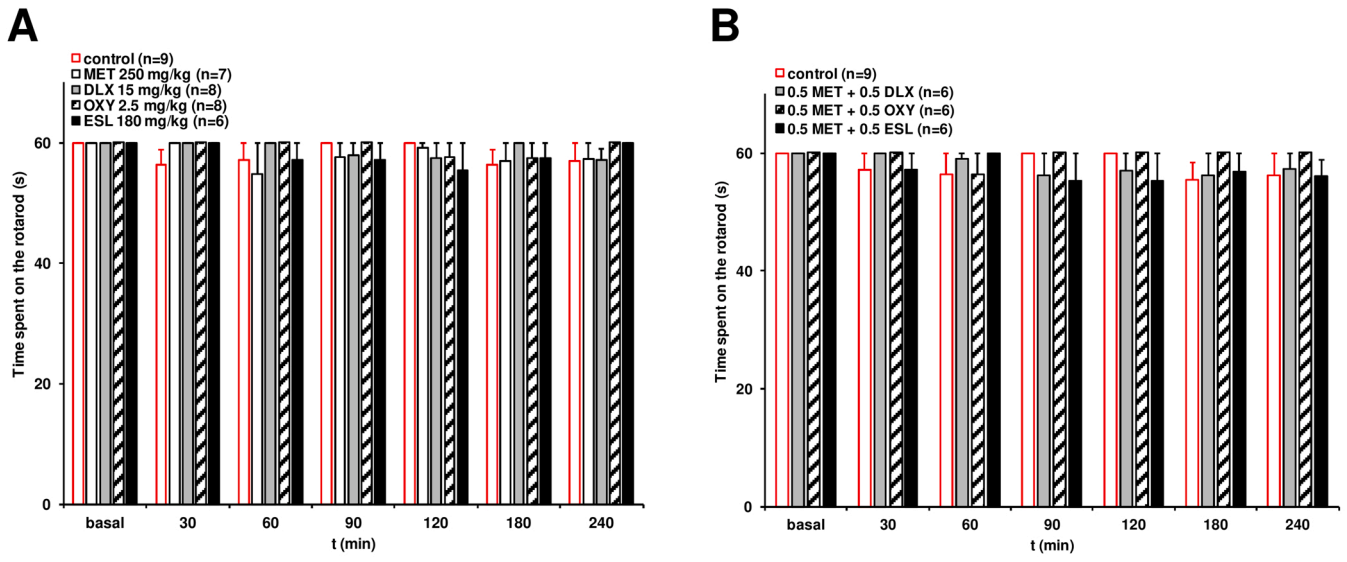

Fig. 6. Time course of the effects of the highest tested doses of metformin (**MET**), individual analgesics (**A**) and two-drug combinations of MET with analgesics (**B**) on motor performance of diabetic mice, expressed as time spent on the rotarod (s). Basal (predrug) time spent on the rotarod was obtained before acute treatment administration. MET, duloxetine (**DLX**), oxycodone (**OXY**) and eslicarbazepine acetate (**ESL**) were administered orally in single dose (**A**). In the case of two-drug combinations, drugs were administered in doses that are equal to 0.5 of their ED_{50} determined in the model of streptozotocin-induced painful diabetic neuropathy (e.g. 0.5 MET + 0.5 DLX indicates that animals received one half of the ED_{50} of MET and one half of the ED_{50} of DLX). MET was applied orally for 7 days, whereas DLX, OXY and ESL were administered orally in a single dose (**B**). Each column represents the mean ± SEM of time spent on the rotarod. Statistical significance (Mann-Whitney *U*-test) was determined by comparison with the control group values.

Duloxetine is a serotonin noradrenaline reuptake inhibitor and represents a first-line drug for treating PDN [3]. In line with its main mechanism of action, the efficacy of duloxetine in reducing painful hypersensitivity in animals with PDN has been linked to its ability to increase serotonergic and noradrenergic neurotransmission at the spinal level [36,37]. Oxycodone is an opioid analgesic with demonstrated clinical efficacy in treating PDN [4]. In the model of streptozotocin-induced PDN, oxycodone was shown to reduce painful hypersensitivity by activating μ and κ-opioid receptors at different levels of pain processing pathways [38,39]. Oxycodone's ability to activate κ-opioid receptors is thought to be one of the reasons for its higher efficacy in alleviating neuropathic pain compared to other opioids (such as morphine) [40]. The efficacy of ESL in alleviating PDN can be attributed to its main mechanism of action (blockade of voltage-gated sodium channels) [41], seeing as there is an increase in expression of different types of voltage-gated sodium channels in dorsal root ganglia of diabetic animals, and reduction of their activity can alleviate painful hypersensitivity in animals with streptozotocin-induced PDN [42,43]. In addition, ESL was shown to produce its antinociceptive effects, in part, *via* serotonin, adrenergic, cholinergic, cannabinoid and opioid receptors [13,44]. These mechanisms could also contribute to its efficacy in reducing PDN seeing as increase in monoamine [36,37,45], cannabinoid [46], opioid neurotransmission [38,39] and/or activation of receptors for these neurotransmitters can reduce painful hypersensitivity in PDN models.

We also demonstrated that metformin-analgesic combinations do not produce significant motor-impairing effects in diabetic mice, indicating that metformin does not potentiate the sedative properties of these drugs. Additionally, the finding of synergistic interactions could suggest that, during combined use with metformin, lower doses of the examined analgesics may be used for alleviating PDN in diabetic patients, which may lead to a reduction of dose-dependent side effects. This is of particular importance for oxycodone, seeing as higher opioid doses are associated with serious adverse effects, such as addiction (considered to be the most significant adverse effect when opioids are used for treating chronic non-malignant pain), physical dependence and overdose [47].

Another significant finding of this study is that metformin synergizes with vitamin B_{12} in reducing mechanical hyperalgesia in diabetic animals. There was an approximately 6-fold of reduction of doses of both drugs in the metformin-vitamin B_{12} combination compared to the doses needed to produce the same level of effect after individual administration. As previously discussed, we suppose that the basis of this beneficial interaction are different, but complementary mechanisms of antihyperalgesic efficacy. Previous studies have found that vitamin B_{12} is capable of alleviating different structural, electrophysiological and molecular changes related to neuropathic pain in the model of streptozotocin-induced diabetes [14,15,48]. These experimental findings are consistent with results from clinical studies which demonstrated that vitamin B_{12} can alleviate manifestations of diabetic neuropathy, including pain [16,17]. The efficacy of vitamin B_{12} in reducing PDN has been linked to increased expression of insulin-like growth factor 1 (a neurotrophic factor important for the maintenance of structure/function of axons) [48].

The finding of a beneficial interaction between metformin and vitamin B_{12} is of special value because chronic metformin use can lead to a deficiency in this vitamin (probably by interfering with its resorption) [6,49]. Low and borderline low vitamin B_{12} levels are seen in about 20 % of patients following prolonged metformin use [49]. The risk of this adverse effect is dose and time-dependent, and tends to be more prevalent in vegetarians [50]. Moreover, metformin-induced vitamin B_{12} deficiency has been linked to worsening of diabetic neuropathy by some studies, and the risk is even highlighted in the recent guidelines of the American Diabetes Association [6]. Therefore, combined use of metformin and vitamin B_{12} can reduce the risk of vitamin B_{12} deficiency and worsening of neuropathy as a potential adverse effect of metformin therapy.

5. Conclusions

Metformin produces antihyperalgesic effects in a model of PDN, after acute and prolonged administration. The antihyperalgesic effects of metformin are probably mediated by spinal and peripheral actions. Additionally, metformin synergises with mechanistically different analgesics (duloxetine, oxycodone and ESL) and with vitamin B_{12} in producing antihyperalgesic effects. These findings indicate that metformin therapy may increase analgesic efficacy and enable the use of lower analgesic doses for treating PDN (which could increase their safety). Combined metformin and vitamin B_{12} may enable higher antihyperalgesic efficacy and mitigate the risk of vitamin B_{12} deficiency and worsening of diabetic neuropathy.

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CRediT authorship contribution statement

Uroš Pecikoza: Investigation, Formal analysis, Visualization, Writing – original draft. **Maja Tomić:** Conceptualization, Formal analysis, Writing – review & editing. **Katarina Nastić:** Investigation, Formal analysis. **Ana Micov:** Investigation, Formal analysis. **Radica Stepanović-Petrović:** Conceptualization, Formal analysis, Writing – review & editing, Supervision, Funding acquisition.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Data Availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.113441.

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