Tyrame [N-(3-hydroxy-1:3:5(10)-estratrien-17 β -yl)-4-hydroxy-phenethylamine], antithrombotic aminoestrogen that decreases microvesicle formation

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Abstract

Background: Estrogens that are used as contraceptives or in replacement therapy are associated with an increase in the risk for developing thrombosis, mainly during the first year of treatment and in women with associated risk factors. **Objective:** To synthesize, characterize and identify the anticoagulant, antiplatelet aggregation and microvesicle-reducing effect of the new aminoestrogen Tyrame. **Material and methods:** CD1 strain mice were used, which had Tyrame (0, 1 and 2 mg/100 g) subcutaneously administered. At 24 h, a blood sample was obtained to determine whole-blood clotting time, microvesicles concentration and inhibitory effect on platelet aggregation. **Results:** Blood clotting time increased up to 1.5 times in comparison with the control. Platelet aggregation inhibition had different magnitude depending on the agonist agent employed, and was complete with collagen. Both effects had a dose-dependent relationship. The microvesicles decreased up to six times with respect to the control. **Conclusions:** Tyrame reduces platelet aggregation and microvesicle formation, which emphasizes its potential therapeutic utility as an estrogen free of thrombotic effects.

KEY WORDS: Tyrame. Aminoestrogens. Thrombosis. Microvesicles.

Tyrame [N-(3-hidroxi-1:3:5(10)-estratrien-17 β -il)-4-hidroxi-fenetilamina], aminoestrógeno antitrombótico que disminuye la formación de microvesículas

Resumen

Antecedentes: Los estrógenos empleados como anticonceptivos o en la terapia de sustitución se asocian a un incremento en el riesgo de desarrollar trombosis, principalmente durante el primer año de tratamiento y en mujeres con factores de riesgo asociados. **Objetivo:** Sintetizar, caracterizar e identificar el efecto anticoagulante, antiagregante plaquetario y reductor de las microvesículas del nuevo aminoestrógeno tyrame. **Material y métodos:** Se emplearon ratones cepa CD1 a los que se les administró por vía subcutánea tyrame (0, 1 y 2 mg/100 g). A las 24 h se tomó una muestra de sangre para determinar el tiempo de coagulación en sangre total, la concentración de microvesículas y el efecto inhibidor de la agregación plaquetaria. **Resultados:** El tiempo de coagulación en sangre se incrementó hasta 1.5 veces

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con respecto al control. La inhibición de la agregación plaquetaria tuvo diferente magnitud dependiendo del agente agonista, siendo completa con colágena. Ambos efectos siguieron una relación dependiente de la dosis. Las microvesículas disminuyeron hasta seis veces con respecto al control. **Conclusiones:** el tyrame disminuye la agregación plaquetaria y la formación de microvesículas, lo que acentúa su posible utilidad terapéutica como un estrógeno sin efectos trombóticos.

PALABRAS CLAVE: Tyrame. Aminoestrógenos. Trombosis. Microvesículas.

Introduction

Millions of women use estrogens, alone or in combination with progestogens, as oral contraceptives or in hormone replacement therapies¹. However, different studies show no benefit for secondary prevention of coronary heart disease in women^{2,3}.

Exogenous estrogens do not confer cardiovascular protection and are associated with an increased risk for developing venous and/or arterial thrombosis, especially during the first year of treatment in women with associated risk factors, such as a mutation in the prothrombin gene G20210A⁴ and factor V Leiden⁵.

Estrogens alter several hemostatic mechanisms, thus generating a prothrombotic state⁶.

Activated platelets have tissue factor (TF) and circulating coagulation factors concentrated on the negative phospholipids of their membrane, and thus facilitate interactions between them, with thrombin being generated.

Until a few decades ago, platelet function was assumed to conclude after being activated. Currently, there is evidence that, after activation and/or apoptosis, platelets and other cells form microvesicles (MV), which are fragments of membranes that lack symmetry, and an important characteristic is that they expose phosphatidylserine and TF on their surface. The membrane fragment adopts a circular shape; these fragments have a size that can vary between 100 and 1,000 nm, preserve characteristics of the cells they are formed from, and through circulation, they can reach territories that, in some cases, could be inaccessible to the cells that generate them. MVs participation in many pathological states, including both arterial and venous thromboses, has been recently pointed out.

Among MVs particularities, their possibility of transporting miRNA, mRNA, DNA and proteins between the cell that gives them origin and other cells and initiating responses in the tissues they access to, such

as Akt phosphorylation and endothelial nitric oxide synthase (eNOS) expression, stand out.

MVs are at an angular point in hemostasis physiological equilibrium. Their activity can be prothrombotic if they carry TF⁷ and/or profibrinolytic⁸ if they carry plasminogen activators within their structure. Both mechanisms explain the concept of TF and plasmin that are formed in blood and that have turned around coagulation and fibrinolysis traditional concepts.

Monocyte MVs can transport TF and be an alternative source thereof⁹.

On the other hand, MVs can offer plasminogen activators and be a source of plasmin.

This scenario shows the importance of MVs; their presence is associated with the arterial and venous thrombotic diseases that induce deterioration of the vascular endothelium and that culminate in the formation of a thrombus, as well as in chronic metabolic and autoimmune states that favor them such as, among others, cardiovascular and cerebrovascular diseases.

Our group has developed estrogens with substitutions in estrogen ring 17 beta position, which to a variable degree decrease coagulation, platelet activity and NO synthesis, which favors sustained vasodilation and thus prevents thrombosis¹⁰. In addition, dimeric compounds buame and bisebudia (buame dimeric compound) were synthesized, which showed an inhibitory effect on platelet aggregation and adenosine triphosphate (ATP) decreased release by platelets¹¹.

All these immediate effects on platelet activity guide to recognizing a non-genomic effect of these estrogens on both cell organelles and cell membrane biological membranes. This effect is clearly observed when verifying that the ATP released from platelet aggregation decreases with the effect of aminoestrogens, which supports the mechanism that decreases the formation of MVs and exosomes, since ATP is found within platelet dense granules.

It is also possible for an interaction mainly at aminoestrogens beta estrogen receptor to be recognized¹².

With the purpose to provide new aminoestrogenic compounds with anticoagulant and anti-platelet aggregation activity, and to study their effect on MV production, we synthesized a new compound, tyrame, whose alcohol-amine structure includes tyramine within its molecule.

Material and Methods

Reagents

Estrone, tyramine, anhydrous toluene, absolute ethanol, absolute methanol, deuteriochloroform (CDCl_3), dimethylsulfoxide- d_6 (DMSO-d_6), and sodium borohydride were purchased from Sigma-Aldrich Chemical Co. ADP, epinephrine and collagen were from the Chrono-Par® brand.

All other reagents used were of the highest possible purity.

Tyrame synthesis

It was obtained¹³ at the Institute of Chemistry of the National Autonomous University of Mexico (UNAM – *Universidad Nacional Autónoma de México*). As previously described, anhydrous toluene was used as solvent, followed by a reduction reaction with sodium borohydride in methanol. Its characterization and chemical purity was determined by the usual analytical and spectroscopic methods. Figure 1 shows the synthesis of tyrame.

Tyrame anticoagulant effect

The experiments that are detailed below were submitted for approval to UNAM's Faculty of Medicine Research and Ethics Commission, with protocol number O44-2011. The guidelines for the use of live laboratory animals published in "Guide for the Care and Use of Laboratory Animals" were followed.

Tyrame anticoagulant effect was determined in whole blood using CD1 strain male mice, which were kept under controlled periods of light and darkness, controlled temperature and with food and water ad libitum.

The animals were assigned to the different groups making a balanced distribution based on their weight, and the group that had DMSO administered was considered the control group. Tyrame was dissolved in

Figure 1. Tyrame synthesis. A mixture of estrone (540 mg, 2 mM) and tyramine (300 mg) in 350 mL of anhydrous toluene was heated under reflux, with stirring, for 48 hours, using a Dean-Stark trap. The reaction mixture was concentrated until dryness with vacuum, and the resulting solid was cooled to room temperature. The glassy solid product was dissolved with hot anhydrous methanol (350 mL) and the solution was cooled to room temperature. Subsequently, sodium borohydride (400 mg) was added. The mixture was then heated under reflux for 20 minutes. The solution was poured into ice water, and the white crystals that were obtained were filtered and dried. Recrystallization from methanol-water yielded of 209 mg of the white pure product.

DMSO and subcutaneously administered at 0, 1 and 2 mg/100 g of weight in order to generate dose-response curves 24 h after administration.

Anticoagulant effect in whole blood was determined as described by González et al., 2010¹⁰.

Anti-platelet aggregation effect

Platelets from donors of the Ignacio Chávez National Institute of Cardiology Blood Bank were used; the donors met the Blood Bank requirements. For each determination, 9 mL of blood from 4 donors were used; the blood was received in Vacutainer® tubes containing 0.129 M sodium citrate as anticoagulant. Platelets were isolated by centrifugation at 100 x g for 1 min, separated from the blood pack, with platelet rich plasma (PRP) being obtained. The blood remnant was centrifuged at 250,000 x g for 15 min. Platelets were adjusted to 250,000/ μL .

Anti-platelet aggregation activity was determined as described by González et al., 2010¹⁰.

Microvesicle formation decrease

To determine circulating MV concentration, the blood of the mice treated with 4 g/100 g of the anti-coagulant effect experiment in whole blood was used. The sample was obtained under anesthesia with intramuscular ketamine (50 mg/kg) as inducer, and phenobarbital (10 mg/kg) as long-acting anesthetic. The blood sample (2 mL) was obtained from the abdominal aorta and placed in a tube with sodium citrate (0.105 M). The mice were sacrificed with a phenobarbital overdose.

Figure 2. Tyrame [N-(3-hydroxy-1:3:5(10)-estratrien-17 β -yl)-4-hydroxy-phenethylamine], yield: 35%, white crystals; melting point 171 °C. Elemental analysis in percentage (C, 80.51; H, 9.50; N, 4.60). Calculated for C₂₆H₃₁NO₂ (C: 80.49; H: 9.46; N: 4.69). Mass spectrum (m/z): M⁺ 391 (base peak 284); IR (KBr wafer, in cm⁻¹) ν -O-H 3485. ¹H Nuclear magnetic resonance (CDCl₃-DMSO-d₆ in ppm), 0.70 (s, 3H, 18CH₃), 1.16-2.25 (m), 6.46 (d, 3H, J = 5 Hz, H₄, H₂₂, H₂₆), 6.58 (dd, 4H, J = 5 Hz and J = 17 Hz, H₂₁, H₄, H₂₅, H₂₃), 7.04 (d, 3H, J = 17 Hz, H₁, H₂₂, H₂₆), 8.78 (s, 2H, O₁ and O₂). The mass spectrum shows the expected molecular ion [M]⁺ at 391 m/z. The base peak is at 284 m/z.

The infrared spectrum showed a ν -O-H vibration band that begins at around 3,500 cm⁻¹; however, due to strong hydrogen bonding, the band is very broad. Bands corresponding to ν -C_{aliphatic}-H and ν -C_{aromatic}-H are also present. There is a band of around 1,607 cm⁻¹, which is assigned to vibrations of C-C-C aromatics.

The X-ray structure exhibits anomalous scattering effects. In the crystalline state, it shows several H bonds originating from water molecules between O(1)-H(1A) of the H₂O group with the nitrogen atom N(1)#1 of the neighboring molecule. There are also contacts between H and O(1) (2.626 Å); H(19)-C(2) (2.889 Å); C(2)-H(16) (2.889 Å) and H(20)-H(4) (2.374 Å) of a neighboring molecule.

Due to the aromatic character of ring A, C(1), C(2), C(3), C(4), C(10), C(9), C(5), C(8) and O(1) should be coplanar. However, the angle between the planes C(1)-C(2)-C(3)-O(1)-C(4)-C(10)-C(9) and C(8)-C(9)-C(10)-C(5) is 3.19 (0.17)°. Ring B has a half chair conformation. Ring C shows a chair conformation with substituents at C(17) and C(13) in an axial position. Ring D shows a 13- β envelope conformation (torsion angle C(17)-C(13)-C(14)-C(15) = 45.76 (0.21)°). The ring D/side chain juncture geometry is trans to C(13) and gauche to C(16).

Bond lengths and angles are as reported for other compounds with similar characteristics. Ellipsoids are calculated with 50% probability.

Statistical analysis

The results are expressed as means $\pm$ standard deviation and are statistically analyzed with an ANOVA test using Dunnett as post hoc test. A p-value < 0.05 is considered to be statistically significant.

Results

Tyrame characterization

Figure 2 shows tyrame hydrate X-ray structure and data on the mass, nuclear magnetic resonance and infrared spectra.

Table 1. The compounds were dissolved in dimethyl sulfoxide (DMSO), calculated for 100 g of mouse weight, and were subcutaneously administered. At 24 h, clotting time in whole blood was determined. First, the dorsal vein of the mouse was dilated (placing the tail in a water bath, at 37°C for 3 min). Subsequently, a cross section was made to obtain $\pm$ 25 μ l of whole blood, which was collected in a capillary previously marked at two ends. The blood is allowed to circulate between the two marks in an equipment that limits the movement to 60°. When the capillary filling starts, a stopwatch is activated, when the blood circulation stops, the stopwatch is stopped and the time is recorded (in seconds)

Compound	Dose	Time in s
Tyrame	DMSO	124.3 $\pm$ 19
	1 mg/100 g	162.7 $\pm$ 11.6
	2 mg/100 g	191.0 $\pm$ 8.4*

*p $\leq$ 0.05 is considered statistically significant, ANOVA with post hoc Dunnett, SPSS v21.0.

Table 2. For each determination, 500 μ L of platelet-rich plasma were placed in a platelet aggregation tube and incubated for 5 min at 37 °C; then, 10 μ L of tyrame at different concentrations (5-500 mM) were added, and immediately after, the agonist agent, 10 μ M ADP, 10 μ M epinephrine, or 2 g/mL collagen. Aggregometry was performed using a Lumi-Aggregometer (Model 560 CA, with accompanying software Model 810 AGGRO/LINK Chrono-log, Havertown, PA, USA). The agonist agent activates the platelets by inducing a change that immediately briefly prevents the passage of light; as the response becomes irreversible, the trace is modified until reaching a plateau. The percentage of platelet aggregation achieved by dimethyl sulfoxide (DMSO) is considered to be 100%, and the results are expressed as a percentage with regard to the control group. The results are expressed as the mean $\pm$ standard deviation. The platelet aggregation results obtained using the DMSO solvent (control group) and the inducing agent are considered as 100% aggregation and 0% inhibition. The results obtained for each agonist agent with tyrame different concentrations were calculated as the percentage of aggregation with regard to the control, with the percentage of inhibition thus being obtained. For example, a percentage of inhibition of 78.4% means that the percentage of aggregation was 21.6%. Inducing agents are prepared according to the manufacturer's instructions (Sigma)

Compound	Concentration (μ M)	Platelet aggregation inhibition (%)		
		ADP 10 μ M	Epinephrine 10 μ M	Collagen 1 mg/mL
Tyrame	5	8.9 $\pm$ 10.5	10 $\pm$ 16.0	3 $\pm$ 7.5
	50	30.6 $\pm$ 11.9*	86 $\pm$ 6.5*	8 $\pm$ 15.3
	500	78.4 $\pm$ 14.6*	97.5 $\pm$ 6.2*	96.9 $\pm$ 5.4*

*p $\leq$ 0.05 is considered statistically significant, ANOVA with post hoc Dunnett, SPSS v21.0.

Figure 3. MVs were isolated by differential centrifugation. The citrated blood sample (0.105 M) was centrifuged twice (1,500 x g for 15 min and 13,000 x g for 2 min) to obtain platelet-poor plasma. 500 μ L of sample were centrifuged (20,000 x g for 90 min at 4 °C), the MVs were washed and re-suspended with HEPES buffer (10 mM NaCl, 140 mM HEPES; pH 7.4). MVs were broken by heat shock and protein concentration was determined at 280 nm on a spectrophotometer. The results are expressed as protein concentration. MV concentration decreased six times at 24 h of treatment (4 g/100 mg). The result is expressed in mg of protein/mL, read in a spectrophotometer at 280 nm. When MVs are formed, the receptors, proteins, DNA, RNA, miRNA and other molecules present in the original cell are thought to be organized within their structure.

Tyrame anticoagulant effect in whole blood

Table 1 shows the clotting times results in whole blood.

Tyrame anti-platelet aggregation effect

Table 2 shows the platelet aggregation inhibitory effect.

MV formation inhibitory effect

Figure 3 shows tyrame inhibitory effect on MV formation.

Conclusions

The results show that tyrame has a discrete anticoagulant effect 24 h after its administration, with a behavior similar to that shown by other aminoestrogens being observed. However, its anti-platelet aggregation effect is superior, especially when collagen is used as inducing agent.

This anti-platelet aggregation effect is reflected on a marked decrease in circulating MVs.

This effect highlights and suggests the possible advantages of the use of tyrame as an estrogen free of thrombotic risk because it reduces the formation of MVs, which would be therapeutically useful in different types of cancer¹⁴, cardiovascular disorders¹⁵ and autoimmune diseases¹⁶ where MVs play an important role in their pathophysiology.

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Conflict of interests

The authors declare that they have no conflicts of interest.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures that were followed adhered to the ethical standards of the responsible committee for experimentation on human beings and were in agreement with the World Medical Association and the Declaration of Helsinki.

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

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