EXPERIMENTAL STUDY

Carvedilol – a ß-blocker with considerable antiaggregatory effect on human blood platelets

Petrikova M, Jancinova V, Nosal R, Majekova M, Fabryova V

Institute of Experimental Pharmacology, Slovak Academy of Sciences, Bratislava, Slovakia. **exfapegi@savba.sk**

Abstract

Background: **Activated blood platelets play a key role in the genesis of many pathological states. Several studies have documented that ß-blockers can influence platelet aggregation. Carvedilol, a third generation non-selective agent with vasodilatory properties, is successfully used in pathological states accompanied with platelet hyperreactivity, however information on its antiplatelet activity is lacking.**
Objectives: **The aim of this study was to analyse the in vitro effect of carvedilol on aggregation of human blood platelets, to compare this effect with the effect of propranolol and atenolol, and to determine whether its suggested antiaggregatory effect was accompanied with reduced thromboxane B_2 formation. Moreover, some physico-chemical parameters of the drugs tested were calculated and compared.**
Methods: **Platelets were isolated by differential centrifugation and platelet aggregation was measured by the turbidimetric method. The amount of thromboxane B_2 was measured by the radioimmunoassay method. Physico-chemical parameters of the drugs tested were calculated using the computer programme Hyperchem.**
Results: **Carvedilol and propranolol inhibited platelet aggregation in the rank order of stimuli: PMA > thrombin > A23187 > epinephrine. The reduction was accompanied by inhibition of thromboxane B_2 formation. In comparison to propranolol, carvedilol was more effective, with the exception for aggregation stimulated with ADP. Atenolol did not affect any platelet function tested. From the drugs studied, the molecule of carvedilol was found to possess the highest partition coefficient, the highest index of molar refractivity, and the lowest dipole moment.**
Conclusion: **Our study found carvedilol to be more potent than propranolol and atenolol in inhibiting platelet aggregation and thromboxane B_2 production. This may be due to the different structure and more convenient physico-chemical parameters of the carvedilol molecule.** ***(Tab. 2, Fig. 8, Ref. 28.)***
Key words: carvedilol, beta-blockers, blood platelets, aggregation, physico-chemical parameters.

Platelet aggregation is a critical processs for maintaining normal haemostasis. However, exaggerated platelet aggregation contributes to the pathogenesis of cardio- and cerebrovascular diseases and their complications (1, 2). Several drugs from different pharmacological groups can correct increased platelet aggregability (3–5), e.g. ß-blockers whose ability to inhibit aggregation was confirmed under in vitro conditions by many authors (6–11).

Extensive series of in vitro studies (12, 13) demonstrated that the inhibitory effect of ß-adrenergic antagonists on platelet aggregation is independent of their ability to antagonize ß-adrenergic receptors and results from the structure and from the interference of this drugs with the microenvironment of platelet membranes (14).

Carvedilol is a third generation non-selective ß-blocker with vasodilatory properties through α_1-blockade. Cardioprotection, observed both experimentally (15–17) and in patients (18, 19), was ascribed to the anti-oxidant activity of carvedilol and of its metabolites. Despite the fact that carvedilol is successfully used

Institute of Experimental Pharmacology, Slovak Academy of Sciences, Bratislava, and Department of Haematology and Transfusiology, University Hospital, Kramare, Bratislava, Slovakia

Address for correspondence: M. Petrikova, Institute of Experimental Pharmacology SAS, Dubravska 9, SK-841 04 Bratislava, Slovakia. Phone: +421.2.59410671

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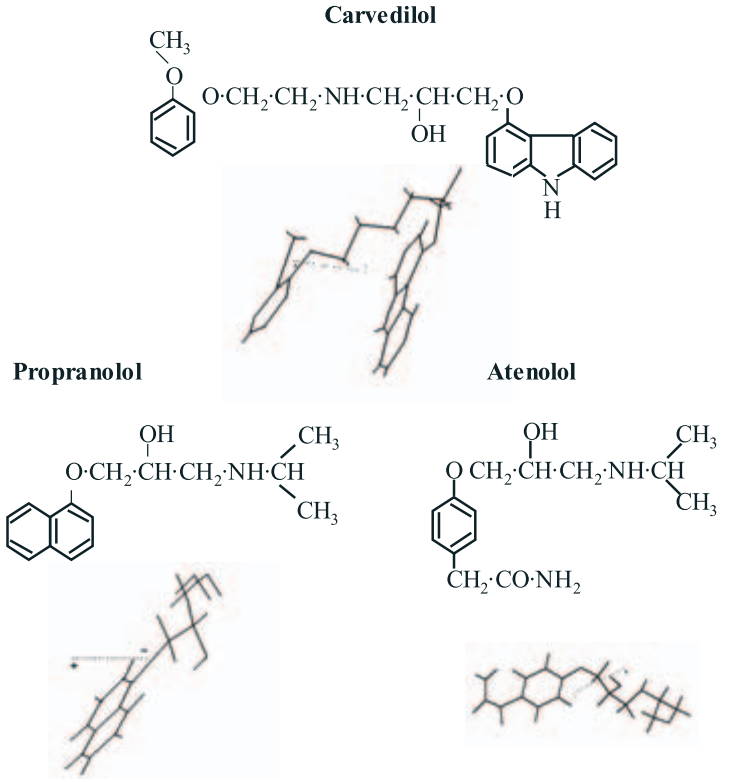

Fig. 1. Chemical formulas and optimal structures of carvedilol, propranolol and atenolol. Marks -/+ denote the orientation of dipole moment.

in the therapy of cardio- and cerebrovascular diseases, i.e. in pathological states accompanied with platelet hyperreactivity, information concerning its antiplatelet activity is rather rare (20).

This study investigated the in vitro effects of carvedilol, propranolol and atenolol on aggregation of human blood platelets stimulated with different stimuli and on thromboxane B_2 formation. The findings were analysed and compared with some physico-chemical parameters characterising the affinity of the drugs to platelet membranes – partition coefficient, dipole moment and molar refractivity.

Materials and methods

Materials

Adenosine-5'-diphosphate (ADP) was from Serva (Germany), Ca^{2+}-ionophore A23187 (A23187), epinephrine bitartrate salt (EPI) and 4ß-phorbol-12ß-myristate-α-13-acetate (PMA) were from Sigma-Aldrich Chemie (Germany), human thrombin (THROM) from Imuna (Slovakia). Carvedilol (1-[carbazolyl-(4)-oxy]-3-[(2-methoxy-phenoxyethyl) amino]-propanol, CAR) was a kind gift from Dr. Sven Hauptmann, Roche Mannheim (Germany), propranolol (1-isopropylamino-3-(1-naphthyloxy)-2-propanol, PRO) was obtained from ICI (England), atenolol (4-{2-hydroxy-3-[(methylethyl)amino]propoxy}benzenacetamide, ATE) was obtained from AstraZeneca (England) and thromboxane B_2 kits from the Institute of Radioisotopes (Hungary). All other chemicals of analytical grade were from available commercial sources. Tyrode's solution (pH=7.4) contained 136.9 mmol/l NaCl, 2.7 mmol/l KCl, 11.9 mmol/l $NaHCO_3$, 0.4 mmol/l $NaH_2PO_4.2H_2O$, 1 mmol/l $MgCl_2.6H_2O$ and 5.6 mmol/l glucose.

Platelet preparation

Platelets were isolated as described earlier (10). In brief, fresh blood of healthy volunteers was anticoagulated with 3.8 % trisodium citrate (ratio blood:citrate = 9:1) and centrifuged at 200xg and 22 °C for 15 min. Platelet rich plasma (PRP) was removed and an aliquot was re-centrifuged for 30 min at 980xg to obtain platelet poor plasma (PPP). After adjustment of platelet count to $2x10^5$/µl by autologous PPP (Thrombocounter C, Coulter Electronics, England), PRP was used for the study of ADP- or epinephrine-stimulated aggregation.

For all other studies, isolated platelets were prepared as follows: PRP was mixed with a solution containing 4.5 % citric acid and 6.6 % glucose (50 µl/ml PRP) and centrifuged at 980xg for 10 min. Platelets were resusupended in an equal volume of Tyrode's solution containing 5.4 mmol/l ethylenediamine tetraacetic acid (EDTA), pH=6.5. After 10 min of stabilisation at room temperature, the suspension was centrifuged for 6 min at 980xg and platelets were resuspended in the same buffer free of EDTA (pH=7.4) to obtain $2x10^5$ (aggregation) or $1x10^4$ (thromboxane) platelets per 1 µl.

Platelet aggregation

Platelet aggregation was measured turbidimetrically according to Nosal et al (10) in a dual channel aggregometer (Chrono-

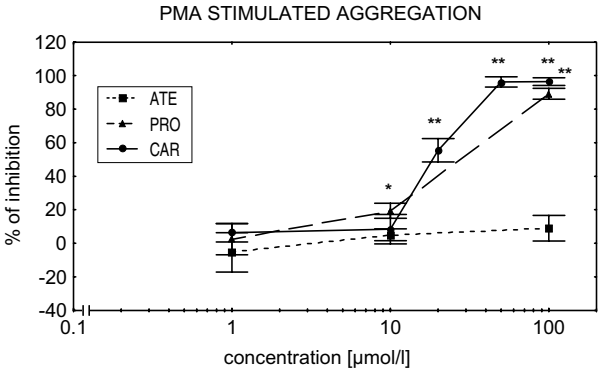

Fig. 2. Inhibition of phorbolmyristate acetate- (PMA)-stimulated platelet aggregation by carvedilol , propranolol and atenolol. Mean±SEM, n=6, * p<0.05, ** p<0.01.

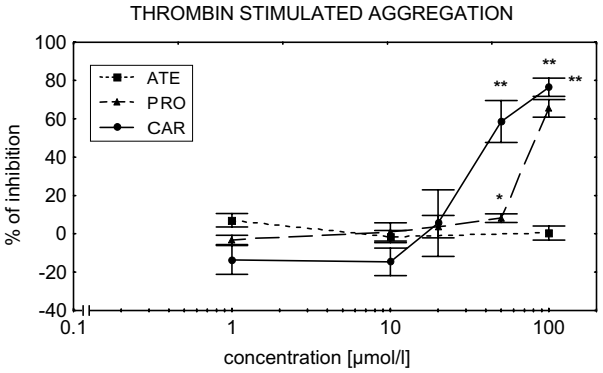

Fig. 3. Inhibition of thrombin-induced platelet aggregation in the presence of carvedilol, propranolol and atenolol. Mean±SEM, n=6, * p<0.05, p<0.01.**

log aggrometer, USA). After 2 min of stabilisation at 37 °C, platelets (450 µl) were incubated with the ß-blocker tested (20 µl) for 30 s. Aggregation was initiated by addition of 20 µl of epinephrine (final concentration 4 µmol/l), ADP (2 µmol/l), PMA (50 nmol/l), thrombin (0.05 NIH U/ml) or Ca^{2+}-ionophore A23187 (1.8 µmol/l) and measured as the amplitude of the aggregation curve in the 60th (in the case of PMA at the 120th) second of stimulation. To eliminate changes in aggregability not associated with the presence of the drugs tested, each sample was run in parallel with a control and the antiaggregatory effect was expressed as a percentage of inhibition.

Thromboxane B_2 (TXB_2) generation

After the treatment of isolated platelets (450 µl, $1x10^4$/µl) with 20 µl of the drugs tested (30 s at 37 °C), production of TXB_2 was initiated by 20 µl of thrombin (0.05 NIH U/ml) or Ca^{2+}-ionophore A23187 (1.8 µmol/l). The incubation was terminated after 5 min by an addition of indomethacine (100 µmol/l), by cooling and centrifuging (14000xg for 2 min at 4 °C) the samples. The amount of TXB_2 in supernatant was measured by the radioimmunoassay (RIA) method using the RIA multidetector counter JNG 402 Tesla (Slovakia).

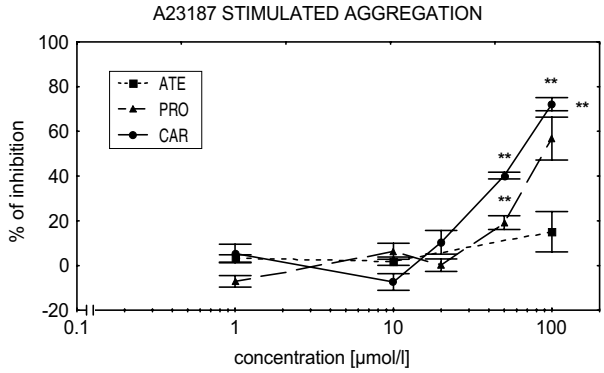

Fig. 4. Inhibition of Ca^{2+}-ionophore A23187-induced platelet aggregation in the presence of carvedilol, propranolol and atenolol. Mean±SEM, n=6, ** p<0.01.

Calculation of physico-chemical parameters

Physico-chemical parameters were calculated for optimum structures of carvedilol, propranolol and atenolol molecules along with dipole moments (Fig. 1). Partition coefficients, molar refractivity and dipole moments of the drugs tested were calculated using the computer programme Hyperchem. The fragmentation method (21, 22) was applied for the determination of molar refractivity indices and of octanol: water partition coefficients.

Data analysis

Commercially available computer statistic programmes were used for all calculations (means, standard errors of the mean-SEM, correlation coefficients). Mean inhibitory concentrations were estimated by a non-linear model fitting procedure; statistical significance of differences between means was established by Student's t-test and $p<0.05$ was taken as statistically significant.

Results

Effects on platelet aggregation and physico-chemical parameters of drugs tested

Carvedilol (20 and 50 µmol/l) decreased the aggregation of isolated human platelets activated with PMA by 55 % and 96 %, respectively; the inhibitory effect of propranolol started at 10 µmol/l concentration by 19 % reduction, atenolol did not show any significant inhibition of aggregation (Fig. 2).

Thrombin-induced aggregation was significantly lowered in the presence of 50 and 100 µmol/l carvedilol (by 59 % and 76 %), whereas the same concentrations of propranolol caused 8 % and 65 % inhibition.

Both drugs tested decreased the aggregation stimulated with A23187 at 50 and 100 µmol/l concentration – carvedilol by 40 % and 72 %, propranolol by 19 % and 57 %, respectively. Pretreatment of platelets with atenolol had no effect on the aggregation of isolated platelets stimulated with thrombin or A23187 (Figs 3, 4).

Significant inhibition of epinephrine PRP aggregation was achieved with 50 µmol/l carvedilol (31 %) and propranolol (24 %)

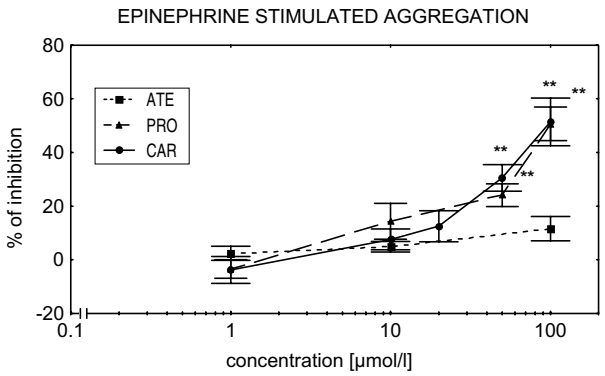

Fig. 5. Inhibition of epinephrine-stimulated platelet aggregation by carvedilol, propranolol and atenolol. Mean±SEM, n=6, ** p<0.01.

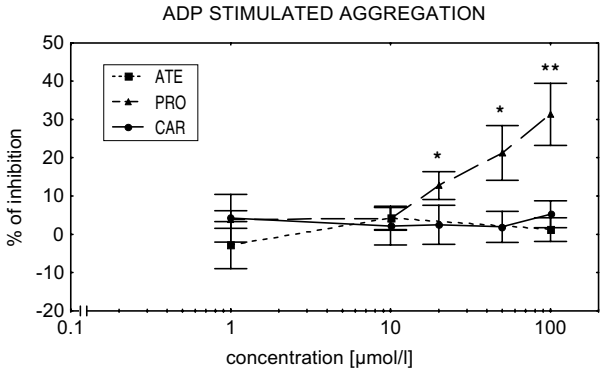

Fig. 6. Inhibition of ADP-induced platelet aggregation in the presence of carvedilol, propranolol and atenolol. Mean±SEM, n=6, * p<0.05, ** p<0.01.

and it rose to 51 % reduction at 100 µmol/l concentration of each drugs. Aggregation stimulated with ADP was reduced in the presence of 20, 50 and 100 µmol/l propranolol by 13 %, 21 % and 31 %, respectively, whereas carvedilol and atenolol were found to be ineffective at these concentrations (Figs 5, 6).

Calculation of mean inhibitory concentrations (i.e. concentrations yielding 50 % inhibition of aggregation) revealed that both liphophilic drugs tested, carvedilol and propranolol, reduced the platelet aggregation, depending on the stimulus used, in the following rank order of potency: PMA > thrombin > Ca^{2+}-ionophore A23187 > epinephrine > ADP (Tab. 1). In comparison to propranolol, the antiaggregatory effect of carvedilol was more pronounced (with an exception for ADP-induced aggregation). On the other hand, the hydrophilic drug atenolol, applied at the same concentrations, was without effect on the aggregation induced with different stimuli, the fact of which corresponded with the liposolubility (indicated by the logarithm of partition coefficient), index of molar refractivity and dipole moment of these drugs (Tab. 2).

Effects on thromboxane B_2 formation

The more pronounced antiplatelet activity of carvedilol (compared to propranolol and atenolol) was further demonstrated by the more effectively inhibited formation of thromboxane B_2 – a stable metabolite of thromboxane A_2 (Fig. 7). In the concentration of 10 µmol/l, carvedilol decreased thrombin-stimulated thromboxane formation by 64 %, whereas propranolol and atenolol were ineffective. In the presence of 1 µmol/l propranolol, even the potentiation of thromboxane synthesis by 33 % was observed.

Generation of thromboxane B_2 in platelets stimulated with A23187, not affected by propranolol or by atenolol, was completely blocked by carvedilol in the concentration of 100 µmol/l (Fig. 8).

Discussion

Our results show that both lipophilic drugs, carvedilol and propranolol, inhibited the response of human platelets to all aggregating agents tested (with the exception for ADP). Their dose-dependent reduction of aggregation decreased depending on the stimulus used in the following order (in parentheses the respective mean inhibitory concentrations of carvedilol and propranolol are given in µmol/l): PMA (19 and 34) > thrombin (55 and 77) > Ca^{2+}-ionophore A23187 (58 and 81) > epinephrine (86 and 118). In accordance with the findings of other authors (20), carvedilol was least effective in platelets stimulated with ADP; in concentrations up to 100 µmol/l no significant inhibition of aggregation was found. The presented results suggest that the mechanism of platelet activation by ADP differs from that of other stimuli used (23).

Carvedilol, even at high concentrations, did not impair blood platelets and both lipophilic ß-blockers studied were slightly diverse in the mode of their antiaggregatory effects.

Tab. 1. Inhibitory effect of carvedilol, propranolol and atenolol on PMA-, thrombin-, A23187-, epinephrine- and ADP-stimulated aggregation. Mean inhibitory concentrations (IC_{50}).

Stimulus of aggregation	IC_{50} [µmol/l] Carvedilol	Propranolol	Atenolol
PMA	18.76±1.13	33.50±12.16	not effective*
Thrombin	54.46±6.10	77.44±17.66	not effective
A23187	57.85±6.31	81.23±17.18	not effective
Epinephrine	86.04±15.08	117.99±37.06	not effective
ADP	not effective	150.31±9.66	not effective

* in concentrations up to 100 µmol/l

Tab. 2. Calculated physico-chemical parameters of the drugs tested.

	Partition coefficients [log P]	Molar refractivity	Dipole moments [Debyes]
Carvedilol	3.29	115.79	0.798
Propranolol	2.80	76.82	1.712
Atenolol	0.56	73.5	2.89

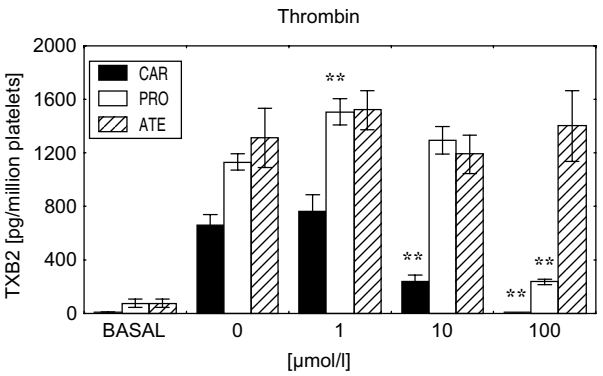

Fig. 7. Thromboxane B_2 (TXB_2) formation in platelets stimulated with thrombin. Effect of carvedilol (full columns), propranolol (open columns) and atenolol (striped columns). Mean±SEM, n=6, ** p<0.01. BASAL = unstimulated platelets.

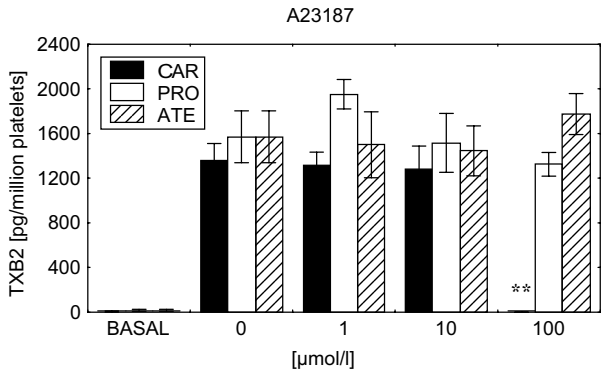

Fig. 8. Thromboxane B_2 (TXB_2) formation in platelets stimulated with Ca^{2+}-ionophore A23187. Effect of carvedilol (full columns) propranolol (open columns) and atenolol (striped columns). Mean±SEM, n=6, ** p<0.01. BASAL = unstimulated platelets.

The fact that effective concentrations occurred in the micromolar range, while nanomolar concentrations were sufficient for adrenoceptor blocking effect (24), indicated that, similar to other ß-blockers, the reduced platelet aggregation seems to result from the interference of carvedilol with platelet membrane organisation rather than from the blockade of α- and ß-adrenergic receptors (13).

Carvedilol and propranolol, drugs with comparatively high lipophility, might inhibit platelet aggregation by decreased availability and function of membrane receptors and channels or by modifying the signal transducing enzymes (25).

The characteristic features of the antiplatelet activity of ß-adrenergic blockers is the modification of the microenvironment of the platelets membrane. It has been documented that the inhibition of platelet function may be related to the lipophilic character of these drugs (12, 13).

In comparison to propranolol, the antiplatelet effect of carvedilol was found to be more pronounced; this may result from the different structure and more suitable physico-chemical parameters of the carvedilol molecule. Compared to propranolol and atenolol, its higher lipophility and lower dipole moment enable a faster and more extensive incorporation into platelet membranes; the more massive molecule of carvedilol can further intensify the resulting membrane disorganisation (26). Finally, the higher index of molar refractivity indicated a more expressive carvedilol affinity to membrane macromolecules. The most pronounced effect on PMA stimulated aggregation and on reduced formation of thromboxane B_2, observed in our experiments, is indicative of carvedilol interference with proteinkinase C activity and with arachidonic acid cascade, respectively. Particularly the inhibition of arachidonate liberation can be assumed. Like other cationic amphiphilic drugs, carvedilol may decrease the activity of phospholipase A_2 by direct interaction (27), by formation of complexes with phopholipids, which are resistant to phospholipase A_2 hydrolysis (28), or via displacement of calcium ions (6).

Atenolol in all concentrations used was practically without effect on stimulated platelet aggregation and thromboxane B_2 formation.

The obtained data showed that the degree of the inhibitory effect of the drugs tested depended to a great extent on their physico-chemical parameters.

The favourable antiplatelet profile of carvedilol belonging to the group of ß-blockers with an intense antiaggregatory activity in vitro, may participate in its beneficial cardioprotective effect.

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