Biochemical and Biophysical Research Communications 435 (2013) 323–326

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Stimulation of platelet apoptosis by balhimycin

Syeda T. Towhid [a], Alexander Tolios [a,b], Patrick Münzer [a], Eva-Maria Schmidt [a], Oliver Borst [a,b], Meinrad Gawaz [b], Evi Stegmann [c], Florian Lang [a,*]

[a] Department of Physiology, Eberhard-Karls-University Tuebingen, Gmelinstr. 5, 72076 Tuebingen, Germany
[b] Department of Cardiology and Cardiovascular Medicine, University Hospital Tuebingen, Otfried-Mueller-Strasse 10, 72076 Tuebingen, Germany
[c] Department of Microbiology/Biotechnology, Eberhard-Karls-University Tuebingen, Auf der Morgenstelle 28, 72076 Tuebingen, Germany

ARTICLE INFO

Article history:
Received 29 January 2013
Available online 9 February 2013

Keywords:
Platelets
Phosphatidylserine
Caspase-3
Apoptosis
Cell membrane scambling
Mitochondrial potential
Ca^{2+}

ABSTRACT

Glycopeptides, such as vancomycin, are powerful antibiotics against methicillin-resistant *Staphylococcus aureus*. Balhimycin, a glycopeptide antibiotic isolated from *Amycolatopsis balhimycina*, is similarly effective as vancomycin. Side effects of vancomycin include triggering of platelet apoptosis, which is characterized by cell shrinkage and by cell membrane scrambling with phosphatidylserine exposure at the cell surface. Stimulation of apoptosis may involve increase of cytosolic Ca^{2+} activity, ceramide formation, mitochondrial depolarization and/or caspase activation. An effect of balhimycin on apoptosis has, however, never been reported. The present study thus tested whether balhimycin triggers platelet apoptosis. Human blood platelets were treated with balhimycin and cell volume was estimated from forward scatter, phosphatidylserine exposure from annexin V-binding, cytosolic Ca^{2+} activity from fluo-3AM fluorescence, ceramide formation utilizing antibodies, mitochondrial potential from $DiOC_6$ fluorescence, and caspase-3 activity utilizing antibodies. As a result, a 30 min exposure to balhimycin significantly decreased cell volume ($\geqslant$1 μg/ml), triggered annexin V binding ($\geqslant$1 μg/ml), increased cytosolic Ca^{2+} activity ($\geqslant$1 μg/ml), stimulated ceramide formation ($\geqslant$10 μg/ml), depolarized mitochondria ($\geqslant$1 μg/ml) and activated caspase-3 ($\geqslant$1 μg/ml). Cell membrane scrambling and caspase-3 activation were virtually abrogated by removal of extracellular Ca^{2+}. Cell membrane scrambling was not significantly blunted by pancaspase inhibition with zVAD-FMK (1 μM). In conclusion, balhimycin triggers cell membrane scrambling of platelets, an effect dependent on Ca^{2+}, but not on activation of caspases.

1. Introduction

Glycopeptides are valuable drugs in the treatment of infections with methicillin-resistant *Staphylococcus aureus* [1]. The most widely used glycopeptide is vancomycin [2–4]. Balhimycin is a vancomycin-like glycopeptide antibiotic isolated from *Amycolatopsis balhimycina* with *in vitro* and *in vivo* activity similar to that of vancomycin differs from vancomycin in the glycosylation pattern.[5–10]. Little is known about effects of balhimycin on host cells. Vancomyin has most recently been shown to trigger platelet apoptosis [11], which is characterized by cell shrinkage and cell membrane scrambling [12–14]. Cellular mechanisms contributing to the stimulation of platelet apoptosis include increase of cytosolic Ca^{2+} activity [15], ceramide formation [13], mitochondrial depolarization [12] and caspase-3 activation [12–14].

The present study has been performed to elucidate the effect of balhimycin on platelet apoptosis. It is shown that balhimycin indeed stimulates cell shrinkage and cell membrane scrambling, effects paralleled by increase of cytosolic Ca^{2+} activity, ceramide formation, mitochondrial depolarization and caspase-3 activation. The cell membrane scrambling was significantly blunted by removal of extracellular Ca^{2+}, but not by pancaspase inhibitor zVAD.

2. Materials and methods

2.1. Isolation and stimulation of human platelets

Fresh ACD-anticoagulated blood was obtained from healthy volunteers between the age of 22–50 years with informed consent according to the Ethics Committee of the Eberhard Karls University Tuebingen, Germany (184/2003V). The blood was centrifuged at 200*g* for 20 min at 25 °C. The platelet rich plasma was separated, added with Tyrode buffer (137 mM NaCl, 2.8 mM KCl, 12 mM $NaHCO_3$, 5 mM glucose, 0.4 mM Na_2HPO_4, 10 mM HEPES, 0.1% BSA), pH 6.5 in 1:7 volumetric ratio and centrifuged at 900*g* and 25 °C for 10 min. The platelet pellet was resuspended in 500 μl of Tyrode buffer (pH 7.0). Care was taken not to expose platelets to excess shear or oxygen.

* Corresponding author. Address: Department of Physiology, University of Tübingen, Gmelinstr. 5, 72076 Tübingen, Germany. Fax: +49 7071 29 5618.
E-mail address: florian.lang@uni-tuebingen.de (F. Lang).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.01.120

The platelets were stimulated in Tyrode buffer (pH 7.4) with 2 mM $CaCl_2$ containing 1 million platelets in each reaction in a total volume of 1 ml. Where indicated, balhimycin (synthesized by the department of Microbiology and Biotechnology, Eberhard Karls University Tuebingen) was added at the indicated concentrations (1, 5, 10, and 15 μg/ml) for 30 min at 37 °C. A negative control without balhimycin and a positive control with ionomycin (1 μM) was analyzed simultaneously with each set of experiments.

2.2. Phosphatidylserine exposure

Phosphatidylserine exposure was measured following stimulation with balhimycin, centrifuging the cells at 1000*g* for 2 min followed by washing once with Tyrode buffer (pH 7.4) with 2 mM $CaCl_2$, staining with 1:20 dilution of annexin V Fluos (Immunotools, Germany) in Tyrode buffer (pH 7.4) with 2 mM $CaCl_2$ and incubating at 37 °C for 30 min. The fluorescence was measured in FL-1 of a BD FacsCalibur (BD Biosciences, CA, USA).

2.3. Calcium measurements

Intracellular calcium concentration was measured following stimulation with balhimycin as indicated, washing once in Tyrode buffer (pH 7.4) with 2 mM $CaCl_2$, staining with 5 μM Fluo-3AM (Biotium, USA) in the same buffer and incubating at 37 °C for 30 min. The fluorescence was measured in FL-1 of a BD FacsCalibur (BD Biosciences, CA, USA).

2.4. Caspase-3 activity

Caspase-3 formation produced in the cells was determined utilizing CaspGlow Fluorescein Active Caspase-3 Staining kit from BioVision (CA, USA) as per the manufacturer's instruction. The fluorescence intensity was measured in FL-1 in BD FACS Calibur (BD Biosciences, USA).

2.5. Mitochondrial membrane potential

For determination of mitochondrial potential platelets were centrifuged and resuspended in phosphate buffered saline (PBS) (Invitrogen, CA, USA) supplemented with 1 mM $MgCl_2$, 5.6 mM glucose, 0.1% BSA and 10 mM HEPES (pH 7.4) in a total volume of 1 ml and stained with 10 nM $DiOC_6$ (Invitrogen, CA, USA) for 10 min. The stained cells were centrifuged at 1000*g* for 2 min at 25 °C, resuspended in PBS and measured in FL-1 of FACS [12].

2.6. Ceramide formation

For detection of ceramide formation, 10^8 platelets were stimulated for 30 min as described before and centrifuged at 1000*g* for 2 min and the pellet was incubated with 50 μl of 1:5 dilution of mice antibody to human ceramide (Alexis, USA) in PBS with 1% BSA for 1 h at 37 °C and 5% CO_2. Then primary stained cells were centrifuged at 1000*g* for 2 min and the pellet was stained with 50 μl of 1:50 dilution secondary goat anti-mouse IgG (BD Pharmingen, Hamburg, Germany) for 20 min. The reaction was stopped with 200 μl PBS and measured immediately in FACS analysis. The mean fluorescence of the FITC-labeled secondary Ig was measured.

2.7. Statistical analysis

Data are provided as arithmetic means ± SEM, statistical analysis was made by one-way ANOVA or student's paired *t*-test, where applicable.

3. Results

Hallmarks of apoptosis include cell shrinkage and cell membrane scrambling with subsequent exposure of phosphatidylserine at the cell surface. In order to identify phosphatidylserine exposing platelets, phosphatidylserine at the platelet surface was detected in flow cytometry from binding of fluorescent annexin V. As shown in Fig. 1(A), a 30 min exposure to balhimycin resulted in annexin V binding to the surface of human platelets. The effect of balhimycin reached statistical significance at 1 μg/ml substance concentration. Forward scatter was employed to estimate cell volume. As illustrated in Fig. 1(B), balhimycin exposure was followed by a decrease of forward scatter reflecting cell shrinkage.

Further experiments were performed to identify mechanisms possibly involved in the triggering of cell membrane scrambling by balhimycin. In a first series of experiments, Fluo-3AM fluorescence was employed to determine cytosolic Ca^{2+} activity. As shown in Fig. 2(A), a 30 min exposure of human platelets to balhimycin increased Fluo-3AM fluorescence, an effect reaching statistical significance at >1 μg/ml balhimycin concentration.

In a second series of experiments, antibodies were used to determine ceramide formation. As illustrated in Fig. 2(B), balhimycin-treatment was followed by ceramide formation, an effect reaching statistical significance at ⩾10 μg/ml balhimycin concentration.

In a third series of experiments $DiOC_6$ fluorescence was utilized to determine the effect of balhimycin on mitochondrial membrane potential. As illustrated in Fig. 3(A), a 30 min exposure to

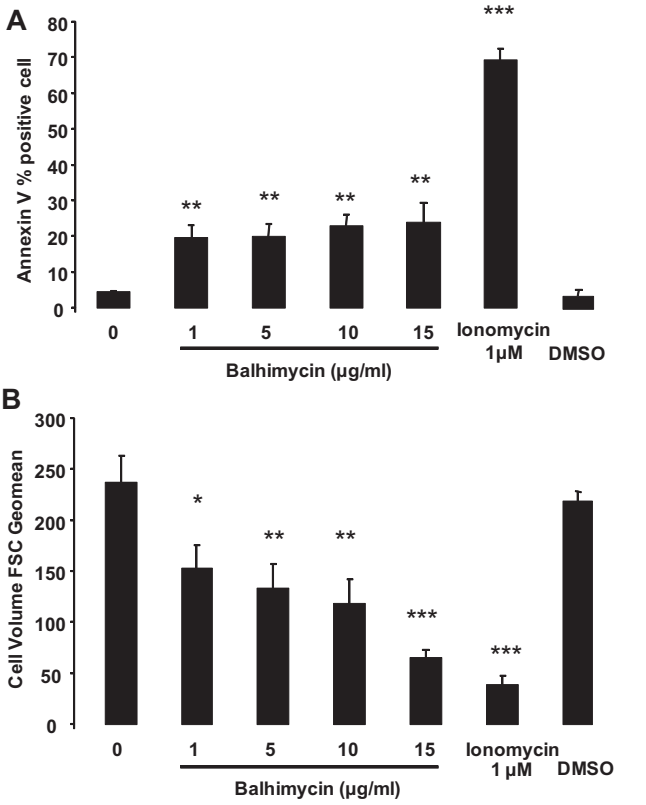

Fig. 1. Effect of balhimycin on phosphatidylserine exposure and forward scatter of human platelets. (A) Arithmetic means ± SEM ($n = 6$) of the percentage of human platelets binding annexin V Flous following a 30 min exposure to Tyrode buffer (2 mM $CaCl_2$) in the absence (control) and presence of 1–15 μg/ml balhimycin. $^{**}p < 0.01$ and $^{***}p < 0.001$ indicates statistically significant difference to value in the absence of balhimycin. (B) Arithmetic means ± SEM ($n = 5$) of the forward scatter of human platelets following a 30 min exposure to Tyrode buffer in the absence (control) and presence of 1–15 μg/ml balhimycin. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ indicates statistically significant difference to value in the absence of balhimycin.

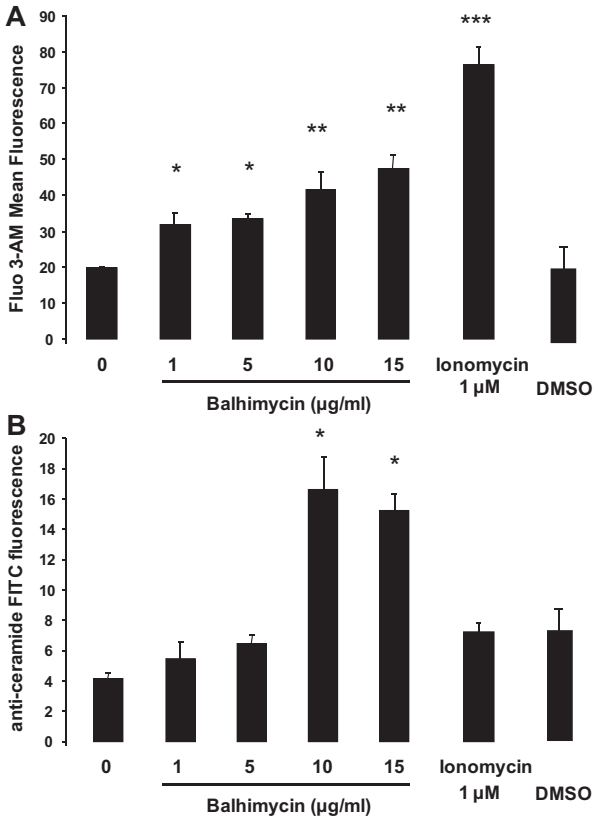

Fig. 2. Effect of balhimycin on cytosolic Ca^{2+} activity and ceramide formation in human platelets. (A) Arithmetic means ± SEM (n = 10) of Fluo3AM fluorescence in FACS analysis reflecting calcium mobilisation of platelets following a 30 min exposure to Tyrode buffer (2 mM $CaCl_2$) in the absence (control) and presence of balhimycin (1–15 μg/ml). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ indicates statistically significant difference compared to control. (B) Arithmetic means ± SEM (n = 11) of IgG-FITC attached to anti-ceramide monoclonal Ig in FACS analysis reflecting ceramide formation in platelets following a 30 min exposure to Tyrode buffer in the absence (control) and presence of balhimycin (1–15 μg/ml). $^{*}p < 0.05$ shows significant increase in ceramide (one-way ANOVA).

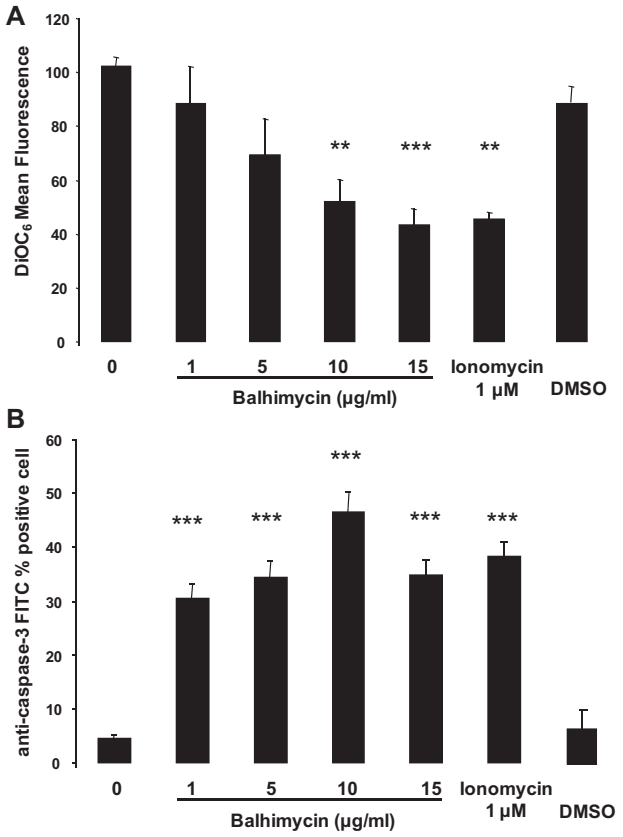

Fig. 3. Effect of balhimycin on mitochondrial potential and caspase activation in human platelets. (A) Arithmetic means ± SEM (n = 7) of $DiOC_6$ fluorescence in FACS analysis reflecting mitochondrial membrane potential of platelets following a 30 min exposure to Tyrode buffer in the absence (control) and presence of balhimycin (1–15 μg/ml). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ shows significant decrease in mitochondrial membrane potential (one-way ANOVA). (B) Arithmetic means ± SEM (n = 11) of the % human platelets expressing active caspase-3 following a 30 min exposure to Tyrode buffer (pH 7.4) 2 mM $CaCl_2$ in the absence (white bar) and presence (black bars) of balhimycin (1–15 μg/ml). $^{***}p < 0.001$ indicate statistically significant difference to value in the absence of balhimycin (one-way ANOVA).

balhimycin resulted in a decline of the mitochondrial potential, an effect reaching statistical significance at 1 μg/ml balhimycin concentration.

In a fourth series of experiments caspase-3 activity was estimated with FACS. As illustrated in Fig. 3(B), a 30 min exposure to balhimycin increased caspase-3 activity, an effect reaching statistical significance at $\geqslant$1 μg/ml balhimycin concentration.

Further experiments were performed to test, whether the observed alterations were critically important for the triggering of cell membrane scrambling by balhimycin.

To define the importance of caspase activation, platelets were exposed to balhimycin in the presence and nominal absence of the pancaspase inhibitor zVAD-FMK (1 μM). As illustrated in Fig. 4(A), zVAD-FMK did not significantly blunt the increase of annexin V binding following balhimycin exposure.

To define the role of Ca^{2+} entry, cell membrane scrambling was determined following balhimycin treatment in the presence and absence (0.5 mM EGTA) of extracellular $CaCl_2$. As illustrated in Fig. 4(B) and (C), removal of Ca^{2+} virtually abolished the cell membrane scrambling (4B) and caspase-3 activation (4C) following treatment of the platelets with 1, 5, 10 or 15 μg/ml balhimycin.

4. Discussion

The present study reveals that the glycopeptide balhimycin from *A. balhimycina* triggers platelet apoptosis, which is characterized by cell shrinkage and cell membrane scrambling. Balhimycin is similarly effective as vancomycin [11], the most widely used glycopeptide antibiotic [5–8,10].

The balhimycin induced platelet apoptosis was paralleled by increase of cytosolic Ca^{2+} activity, formation of ceramide, depolarization of the mitochondria and activation of caspase-3. The balhimycin-induced platelet apoptosis was abrogated in the absence of extracellular Ca^{2+}. Accordingly, the presence of Ca^{2+} is a prerequisite for the stimulation of caspase 3 and of cell membrane scrambling. Ceramide has previously been shown to sensitize erythrocytes to the scrambling effect of cytosolic Ca^{2+} [16,17]. Thus, Ca^{2+} and ceramide cooperate in the stimulation of caspase activity and cell membrane scrambling following balhimycin treatment. Cellular Ca^{2+} overload is a well known trigger of apoptosis in a wide variety of cells [18–20].

Surprisingly, balhimycin induced platelet apoptosis was not significantly blunted by the pancaspase inhibitor zVAD. Thus, Ca^{2+} triggers cell membrane scrambling in platelets at least in part by mechanisms other than caspase-3 activation.

Consequences of platelet apoptosis may include thrombocytopenia, as phosphatidylserine exposing platelets are engulfed by phagocytosing cells and are thus cleared from circulating blood. Thrombocytopenia is a well known side effect of vancomycin [21–23]. Whether or not balhimycin similarly decreases the

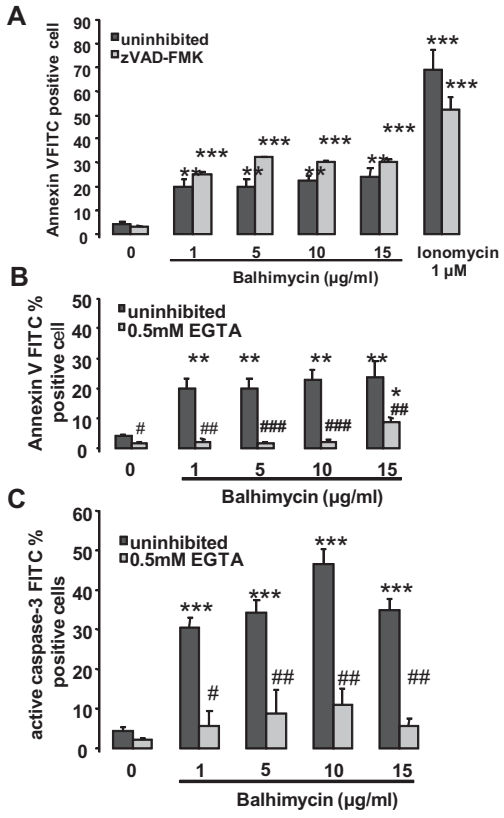

Fig. 4. Influence of caspase inhibitor zVAD-FMK and Ca^{2+} removal on balhimycin-induced phosphatidylserine exposure in human platelets. (A) Arithmetic means ± SEM ($n = 5$) of the percentage of human platelets binding annexin V Flous following a 30 min exposure to Tyrode buffer (pH 7.4) in the absence (0, white bar) and presence of 10–15 µg/ml balhimycin in the absence (black bars) and presence (light grey bars) of 1 µM zVAD-FMK. $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate statistically significant difference to value of respective controls (ANOVA). (B) Arithmetic means ± SEM ($n = 4$) of the percentage of human platelets binding annexin V Fluos following 30 min treatment with balhimycin in the presence (black bar) and nominal absence (grey bar) of extracellular Ca^{2+}. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate statistically significant difference to value of respective controls (ANOVA) and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ indicate statistically significant difference to the presence of 2 mM $CaCl_2$ (paired T test). (C) Arithmetic means ± SEM ($n = 4$) of the percentage of human platelets with activated caspase-3 following 30 min treatment with balhimycin in the presence (black bar) and absence (grey bar) of extra-cellular Ca^{2+}. $^{***}p < 0.001$ indicate statistically significant difference to value of respective control (ANOVA) and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ denote statistically significant difference to the value of caspase-3 in 2 mM $CaCl_2$ (paired t-test).

number of circulating platelets remains to be shown. Platelet apoptosis is further expected to foster adhesion of platelets to endothelial cells of the vascular wall, which may trigger hemostasis and thrombosis [24,25].

In conclusion, balhimycin treatment of human blood platelets increases cytosolic Ca^{2+} activity, stimulates ceramide formation, activates caspase-3, depolarizes mitochondria and eventually leads to cell shrinkage and cell membrane scrambling. The balhimycin induced cell membrane scrambling requires the presence of Ca^{2+}.

Acknowledgments

The authors acknowledge the meticulous preparation of the manuscript by Lejla Subasic. This study was supported by The Deutsche Forschungsgemeinschaft (SFB766 and KFO 274), and The Tuebingen Platelet Investigative Consortium (*TuePIC*).

References

[1] R.G. Finch, G.M. Eliopoulos, Safety and efficacy of glycopeptide antibiotics, J. Antimicrob. Chemother. 55 (Suppl. 2) (2005) ii5–ii13.
[2] S. Deresinski, Vancomycin in combination with other antibiotics for the treatment of serious methicillin-resistant *Staphylococcus aureus* infections, Clin. Infect. Dis. 49 (2009) 1072–1079.
[3] C. Giuliano, K.K. Haase, R. Hall, Use of vancomycin pharmacokinetic–pharmacodynamic properties in the treatment of MRSA infections, Expert Rev. Anti Infect. Ther. 8 (2010) 95–106.
[4] A. Marsot, A. Boulamery, B. Bruguerolle, N. Simon, Vancomycin: a review of population pharmacokinetic analyses, Clin. Pharmacokinet. 51 (2012) 1–13.
[5] D.K. Chatterjee, P. Sarma, R.H. Jani, N. Klesel, D. Isert, Comparative chemotherapeutic efficacy of balhimycin, desgluco-balhimycin against experimental MSSA and MRSA infection in mice, Indian J. Exp. Biol. 38 (2000) 681–686.
[6] S.R. Nadkarni, M.V. Patel, S. Chatterjee, E.K. Vijayakumar, K.R. Desikan, J. Blumbach, B.N. Ganguli, M. Limbert, Balhimycin, a new glycopeptide antibiotic produced by *Amycolatopsis* sp. Y-86,21022. Taxonomy, production, isolation and biological activity, J. Antibiot. (Tokyo) 47 (1994) 334–341.
[7] T.F. Schaberle, W. Vollmer, H.J. Frasch, S. Huttel, A. Kulik, M. Rottgen, A.K. von Thaler, W. Wohlleben, E. Stegmann, Self-resistance and cell wall composition in the glycopeptide producer *Amycolatopsis balhimycina*, Antimicrob. Agents Chemother. 55 (2011) 4283–4289.
[8] L. Vertesy, H.W. Fehlhaber, H. Kogler, M. Limbert, New 4-oxovancosamine-containing glycopeptide antibiotics from *Amycolatopsis* sp. Y-86,21022, J. Antibiot. (Tokyo) 49 (1996) 115–118.
[9] W. Vongsangnak, L.F. Figueiredo, J. Forster, T. Weber, J. Thykaer, E. Stegmann, W. Wohlleben, J. Nielsen, Genome-scale metabolic representation of *Amycolatopsis balhimycina*, Biotechnol. Bioeng. (2012).
[10] E. Stegmann, H.J. Frasch, W. Wohlleben, Glycopeptide biosynthesis in the context of basic cellular functions, Curr. Opin. Microbiol. 13 (2010) 595–602.
[11] S.T. Towhid, P. Münzer, E.M. Schmidt, O. Bors, E. Stegmann, F. Lang, Stimulation of platelet apoptosis by vancomycin, Cell Physiol Biochem. 31 (2013) 102–112.
[12] V. Leytin, D.J. Allen, S. Mykhaylov, E. Lyubimov, J. Freedman, Thrombin-triggered platelet apoptosis, J. Thromb. Haemost. 4 (2006) 2656–2663.
[13] S.T. Towhid, E.M. Schmidt, E. Schmid, P. Munzer, S.M. Qadri, O. Borst, F. Lang, Thymoquinone-induced platelet apoptosis, J. Cell. Biochem. 112 (2011) 3112–3121.
[14] W. Zhang, J. Liu, R. Sun, L. Zhao, J. Du, C. Ruan, K. Dai, Calpain activator dibucaine induces platelet apoptosis, Int. J. Mol. Sci. 12 (2011) 2125–2137.
[15] D. Varga-Szabo, A. Braun, B. Nieswandt, STIM and Orai in platelet function, Cell Calcium 50 (2011) 270–278.
[16] F. Lang, E. Gulbins, P.A. Lang, D. Zappulla, M. Foller, Ceramide in suicidal death of erythrocytes, Cell. Physiol. Biochem. 26 (2010) 21–28.
[17] K.S. Lang, S. Myssina, V. Brand, C. Sandu, P.A. Lang, S. Berchtold, S.M. Huber, F. Lang, T. Wieder, Involvement of ceramide in hyperosmotic shock-induced death of erythrocytes, Cell Death Differ. 11 (2004) 231–243.
[18] P. Nicotera, S. Orrenius, The role of calcium in apoptosis, Cell Calcium 23 (1998) 173–180.
[19] S. Orrenius, B. Zhivotovsky, P. Nicotera, Regulation of cell death: the calcium-apoptosis link, Nat. Rev. Mol. Cell Biol. 4 (2003) 552–565.
[20] P. Pinton, C. Giorgi, R. Siviero, E. Zecchini, R. Rizzuto, Calcium and apoptosis: ER-mitochondria Ca2+ transfer in the control of apoptosis, Oncogene 27 (2008) 6407–6418.
[21] A. Anand, H.K. Chauhan, Piperacillin and vancomycin induced severe thrombocytopenia in a hospitalized patient, Platelets 22 (2011) 294–301.
[22] B. Kenney, C.A. Tormey, Acute vancomycin-dependent immune thrombocytopenia as an anamnestic response, Platelets 19 (2008) 379–383.
[23] S.A. Nasraway, A.F. Shorr, D.J. Kuter, N. O'Grady, V.H. Le, S.K. Cammarata, Linezolid does not increase the risk of thrombocytopenia in patients with nosocomial pneumonia: comparative analysis of linezolid and vancomycin use, Clin. Infect. Dis. 37 (2003) 1609–1616.
[24] B.T. Kile, The role of the intrinsic apoptosis pathway in platelet life and death, J. Thromb. Haemost. 7 (Suppl. 1) (2009) 214–217.
[25] M.J. White, B.T. Kile, Apoptotic processes in megakaryocytes and platelets, Semin. Hematol. 47 (2010) 227–234.