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# Clinically relevant interactions of anti-apoptotic Bcl-2 protein inhibitors with ABC transporters

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In this work we studied clinically relevant interactions between the BH3 mimetics and the ABCB1 and ABCG2 transporters. We observed that the intracellular levels of ABT-263 and ABT-199, but not ABT-737, might be reduced by ABCB1 or ABCG2. Importantly, this effect was proportional to the transporter expression level. High transporter expression levels decreased the intracellular levels of ABT-263 and ABT-199 substantially. Low transporter expression levels, which are clinically relevant, affected the intracellular level of ABT-263 slightly but significantly, however, they failed to decrease the intracellular level of ABT-199 below the control level in parental cells. Our results further revealed that ABT-263 did not inhibit the ABCB1 mediated transport, however, it partially inhibited the ABCG2 mediated transport at clinically relevant concentrations. In contrast, ABT-199 inhibited partially the ABCB1 mediated transport and it fully inhibited the ABCG2 mediated transport at clinically relevant concentrations. Importantly, cells expressing higher drug transporters levels required higher concentrations of ABT-263 or ABT-199 to achieve certain inhibition of substrate efflux. Conclusions: Antiproliferative effects of ABT-263 and ABT-199 might be reduced by ABCB1 or ABCG2, however, this effect depends on transporter expression levels. Since the expression levels of ABCB1 and ABCG2 are rarely high in clinical samples, their contribution to the overall resistance to ABT-263 or ABT-199 is probably low. Inhibition study revealed that ABT-199, but not ABT-263, fully inhibited low expression level of ABCG2. Our data suggest that ABT-199 should be evaluated beyond its original application as an inhibitor of the ABCG2 transporter in clinical settings.

## 1. Introduction

The Bcl-2 family proteins are key regulators of the apoptotic cell death pathway. This multigene family of cell death regulators is usually divided into pro-apoptotic members, including Bax, Bak, Bim, Bad, Bmf, Bid, Noxa, Bik, Puma, and Hrk, which enhance rates of apoptosis, and anti-apoptotic members, including Bcl-2, Bcl-XL, Mcl-1, Bcl-W, and A1, which inhibit apoptosis. Upregulation of anti-apoptotic members may lead to malignant transformation and resistance of cancer cells to chemotherapy (Czabotar et al. 2014; Gross et al. 1999). Advances in the knowledge of the structure and function of anti-apoptotic and pro-apoptotic members of the Bcl-2 family have enabled the development of small molecules that block the function of anti-apoptotic members. These compounds known as "BH3 mimetics" bind the BH3 domain of anti-apoptotic proteins, which inactivates these proteins. Synthetic BH3 mimetics ABT-737 and its orally bioavailable derivative ABT-263 (navitoclax) selectively target the three pro-survival proteins Bcl-2, Bcl-XL, and Bcl-W, but not Mcl-1 or A1 (Oltersdorf et al. 2005; Tse et al. 2008; Zhai et al. 2006). ABT-263 exhibited antitumor activity in patients with relapsed or refractory chronic lymphocytic leukemia and non-Hodgkin lymphoma (Roberts et al. 2012). However, dose-dependent thrombocytopenia due to the inhibition of Bcl-XL limits application of this agent. This prompted the development of ABT-199 (venetoclax) a selective Bcl-2 inhibitor sparing BCL-XL and platelets (Souers et al. 2013). Despite promising results both *in vitro* and in clinical trials recent studies suggested that the efficiency of BH3 mimetics might be significantly reduced due to the overexpression of ABCB1 (P-glycoprotein, MDR1), a member of the ATP-binding cassette (ABC) transporter family. Thus, Vogler et al. (2011) reported that ABT-737 and ABT-263 are substrates for ABCB1. In addition, cells with increased ABCB1 expression were more resistant to apoptosis induced by either ABT-737 or ABT-263 (Vogler et al. 2011). An ABCB1 mediated resistance to ABT-737 in medulloblastoma cells was published recently (Othman et al. 2014). Furthermore, Weiss et al. (2016) observed that overexpression of ABCB1 reduced antiproliferative effects of ABT-199.

However, interactions between many drugs and ABC transporters seems to be more complex. For example, some tyrosine kinase inhibitors, including imatinib (Glivec), nilotinib (Tasigna), and dasatinib (Sprycel) may serve as substrates of ABCB1 or ABCG2 (BCRP) at low concentrations, and as their inhibitors at high concentrations (Dohse et al. 2010; Eadie et al. 2014). Not surprisingly, similar results were observed also for BH3 mimetics. Indeed, recent suggested that ABT-199 moderately inhibited ABCB1 and ABCG2. In addition, ABT-199 supressed expression of ABCB1 at mRNA level (Weiss et al. 2016).

ABC transporters comprise one of the largest and functionally diverse family of membrane transporters. ABC transporters utilize ATP hydrolysis as a driving force to translocate a wide variety of substrates from the cytoplasm to the outside of the cell or into intracellular compartments (Sharom 2008). Three members of ABC transporters, namely ABCB1, ABCC1 (MRP1), and ABCG2, are thought to be involved in multidrug resistance (MDR), at least in *in vitro* experiments (Sharom 2008). However, the causal relationship between overexpression of the above mentioned drug transporters and clinical drug resistance is difficult to establish (Amiri-Kordestani et al. 2012; Robey et al. 2010; Tamaki et al. 2011). Explanation of the existing controversy between laboratory and clinical results is difficult to explain as many factors may have an impact on the results. We believe that contradictory results might be due to oversimplification of evaluation of the MDR phenotype in cancer cells. The approach to this issue must be more relevant to clinics at

least in two aspects. First, the transporter expression level must be similar to that found in clinical samples. Indeed, many laboratories use drug selected cells or transfected cells which enhanced expression of the studied transporter. Such cells express huge levels of transporters which are usually not present in real samples. Indeed, tumor ABCB1 and/or ABCG2 levels are in general lower than in normal cells, as judged from The Cancer Genome Atlas (TCGA; https://cancergenome.nih.gov). Even in the earliest studies it was observed that while a few tumor types – renal cancer, pheochromocytoma, and renal caner reach the high levels readily obtained in drug selected cell lines, most tumor types – breast, ovary, lung, and prostate – have much lower levels (Fojo et al. 1987; Goldstein et al. 1989). Because the drug transporter expression level is a crucial factor that affects the results its consideration may help to explain existing controversy (Kosztyu et al. 2013, 2014; Mlejnek et al. 2017a, Mlejnek et al. 2017b). Second, drug concentration must be used in a clinically relevant range. As mentioned above, the drug concentration may critically affect whether it acts as a substrate or inhibitor (Dohse et al. 2010; Eadie et al. 2014).

Here we addressed the question whether ABCB1 and/or ABCG2 overexpression might affect the intracellular levels of ABT-737, ABT-263, and ABT-199 in human leukemia cells. We observed that ABT-263 or ABT-199, in contrast to ABT-737, might be significantly decreased by overexpression of ABCB1 and/or ABCG2. However, the real effect of ABCB1 and/or ABCG2 on intracellular level of ABT-263 and ABT-199 and thus cell sensitivity will be probably low for low transporter expression levels, which are relevant to clinical samples. Our results further indicated that ABT-263 inhibited partially the ABCG2 mediated transport but it failed to inhibit the ABCB1 mediated transport at clinically relevant concentrations. In contrast, ABT-199 fully inhibited the ABCG2 mediated transport and only partially inhibited the ABCB1 mediated transport at clinically relevant concentrations.

## 2. Investigations and results

### *2.1. Isolation and characterization of cells expressing ABCB1 and ABCG2*

In our study, human leukemia cells with high and low expression levels of ABCB1 or ABCG2 were used. Expression levels of ABCB1 transporter in K562/Dox and K562/DoxDR1 cells are given in Fig. 1a, b. Cells overexpressing ABCG2, K562/ABCGCL10 and K562/ABCGCL1, are characterized in Fig. 1c,

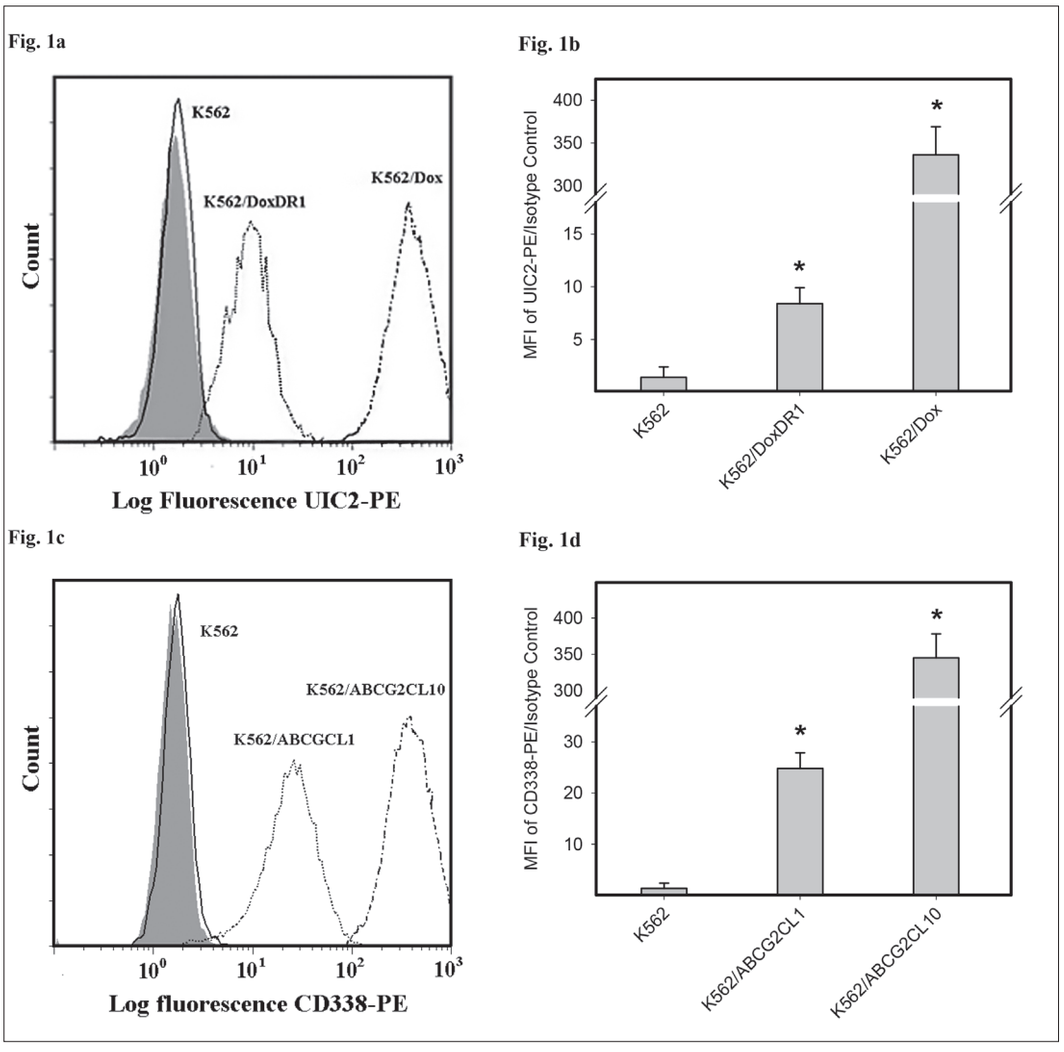



Fig. 1: Analysis of ABCB1 and ABCG2 expression: (a) Flow cytometric analysis of ABCB1 expression. Isotype control (grey histogram); K562 parental cell line (solid line); K562/Dox cells (dash-dot line); K562/DoxDR1 cells (dot line). (b) Quantitative analysis of ABCB1 expression. ABCB1 expression was quantified as the mean fluorescence intensity (MFI) shift (ratio of MFI of UIC2-PE antibody and isotype control). The experimental points represent mean values from three replicate experiments, with standard deviations. * denotes significant change in ABCB1 expression ($P<0.05$) between K562 cells and cells expressing various levels of ABCB1 (K562/Dox, K562/DoxDR1). (c) Flow cytometric analysis of ABCG2 expression. Isotype control (grey histogram); K562 parental cell line (solid line); K562/ABCG2CL10 cells (dash-dot line); K562/ABCGCL1 cells (dot line). (d) Quantitative analysis of ABCG2 expression. ABCG2 expression was quantified as the mean fluorescence intensity (MFI) shift (ratio of MFI of CD338-PE antibody and isotype control). The experimental points represent mean values from three replicate experiments, with standard deviations. * denotes significant change in ABCG2 expression ($P<0.05$) between K562 cells and cells expressing various levels of ABCG2 (K562/ABCGCL10, K562/ABCGCL1).

d. Parental K562 cells, which do not express either of transporters, were used as a control. Analysis of ABCB1 and ABCG2 function was done using calcein accumulation assay (Holló et al. 1994) and pheophorbide A accumulation assay, respectively (data not shown; Robey et al. 2004).

### *2.2. The effect of ABCB1 and ABCG2 expression on intracellular levels of BH3 mimetics*

We observed that K562/Dox and K562/DoxDR1 cells expressing high and low level of ABCB1, respectively, or K562/ABCG2CL10 and K562/ABCG2CL1 cells expressing high and low level of ABCG2 transporter, respectively, did not contain significantly reduced intracellular levels of ABT-737 when compared to their parental K562 cells, which do not express either of transporters (Fig. 2a, b). In addition, ZSQ and Ko143, specific inhibitors of ABCB1 and ABCG2, respectively, did not changed significantly intracellular levels of ABT-737 (Fig. 2a, b).

In contrast to ABT-737, intracellular levels of ABT-263 were significantly reduced in ABCB1 and ABCG2 overexpressing cells (Fig. 2c, d). Importantly, this effect strongly depended on transporter expression levels (Fig 2c, d). Intracellular level of ABT-263 was inversely related to the ABCB1expression (Fig. 2c). Exactly the same effect was found in K562/ABCG2CL10 and K562/ABCG2CL1 cells with high and low expression level of ABCG2 (Fig. 2d). Transporter inhibitors, ZSQ and Ko143, significantly increased intracellular levels of ABT-263 in cells expressing ABCB1 and ABCG2, respectively (Fig. 2c, d).

Analysis of cell extracts clearly indicated a significantly reduced accumulation of ABT-199 in K562/Dox and K562/ABCG2CL10 cells expressing high levels of ABCB1 and ABCG2, respectively (Fig. 2e, f). However, intracellular level of ABT-199 in K562/DoxDR1 and K562/ABCG2CL1 cells expressing low levels of ABCB1 and ABCG2, respectively, was comparable to that found in parental K562 cells (Fig. 2e, f).

### *2.3. The effect of BH3 mimetics on fluorescence probe accumulation in cells expressing various levels of ABCB1 or ABCG2*

To study the efficiency of ABCB1 inhibitors, ABT-263 and ABT-199, the calcein accumulation assay was used (Hegedus et al.

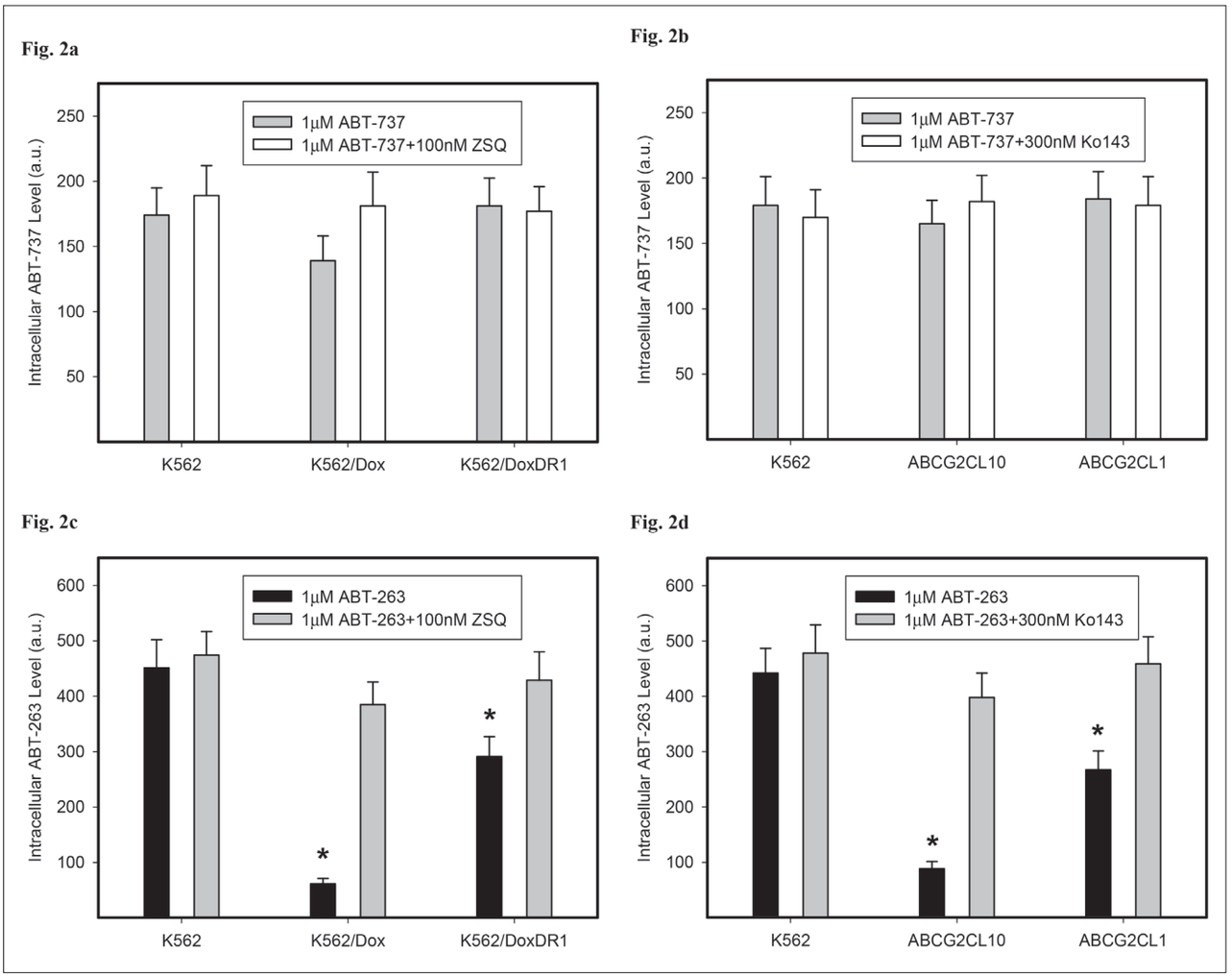
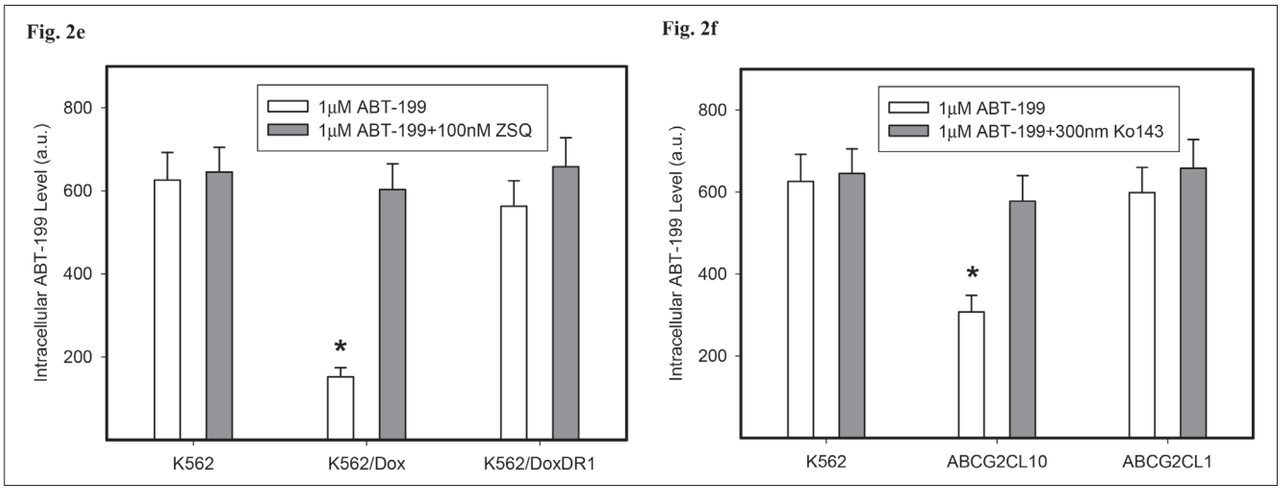
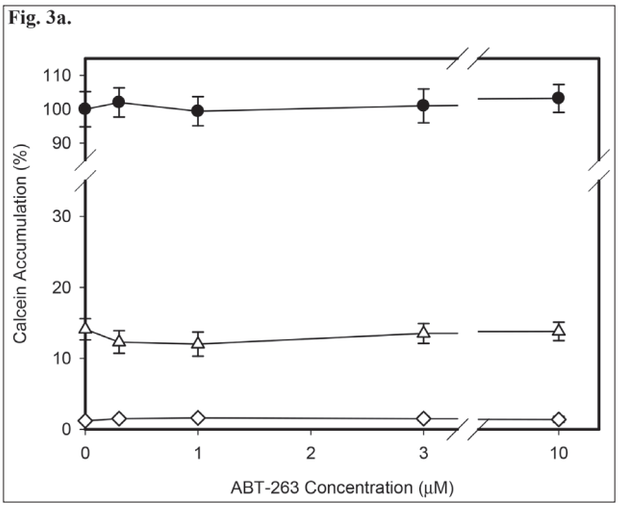


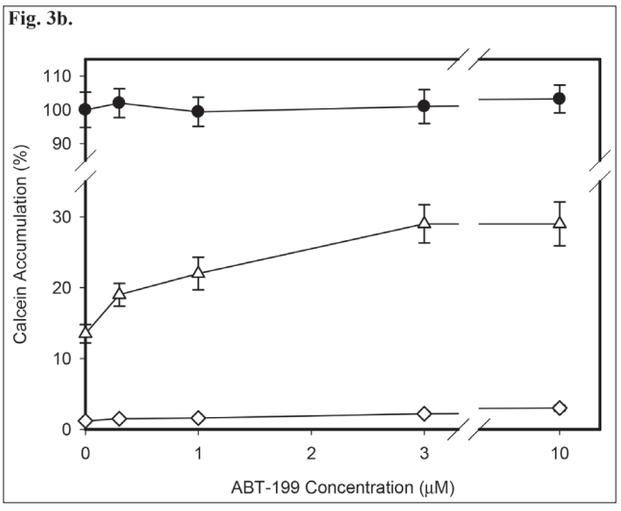

Fig. 2: Intracellular levels of BH3 mimetics in cells expressing ABCB1 or ABCG2. Parental cell line K562, which does not express any of transporter was used as a control. Cells were incubated with the studied BH3 mimetic or with the studied BH3 mimetic + appropriate inhibitor, as indicated. Intracellular level of the studied BH3 mimetic was determined after 3h incubation at 37°C. The experimental points represent mean values from three replicate experiments, with standard deviations. (a) A relative intracellular levels of ABT-737 in cells overexpressing ABCB1 transporter. (b) A relative intracellular levels of ABT-737 in cells overexpressing ABCG2 transporter. (c) A relative intracellular levels of ABT-263 in cells overexpressing ABCB1 transporter. * denotes significant change in intracellular level of ABT-263 ($P<0.05$) between K562 cells and cells expressing various levels of ABCB1, K562/Dox and K562/DoxDR1. (d) A relative intracellular levels of ABT-263 in cells overexpressing ABCG2 transporter. * denotes significant change in intracellular level of ABT-263 ($P<0.05$) between K562 cells and cells expressing various levels of ABCG2, K562/ABCG2CL10 and K562/ABCG2CL1. (e) A relative intracellular levels of ABT-199 in cells overexpressing ABCB1 transporter. * denotes significant change in intracellular level of ABT-199 ($P<0.05$) between K562 cells and cells expressing various levels of ABCB1, K562/Dox and K562/DoxDR1. (f) A relative intracellular levels of ABT-199 in cells overexpressing ABCG2 transporter. * denotes significant change in intracellular level of ABT-199 ($P<0.05$) between K562 cells and cells expressing various levels of ABCG2, K562/ABCG2CL10 and K562/ABCG2CL1. For the simplicty ABCG2CL10 stands for K562/ABCG2CL10 and ABCG2CL1 stands for K562/ABCG2CL1 in the graph captions.




2002). For comparison, CsA was used as well characterised inhibitor of ABCB1. We observed that ABT-263 did not exhibit inhibition of calcein AM extrusion (Fig. 3a) within clinically relevant drug concentrations which were reported to be 3-6µM (Gandhi et al. 2011). In contrast, ABT-199 exhibited a concentration-dependent inhibition of dye extrusion (Fig. 3b). Unfortunately, ABT-199 failed to reach full inhibition of dye extrusion within clinically relevant drug concentrations (Fig. 3b). Data mined from the literature indicated a peak level of ABT-199 around 3µM (Roberts et al. 2016). Importantly, we observed that inhibition efficiency of ABT-199 similarly to CsA depended on ABCB1 expression level (Fig. 3b). Cells with the lower expression levels of ABCB1 required lower concentrations of ABT-199 or CsA to achieve a certain inhibitory effect (Fig. 3b, c).

Accordingly, pheophorbide A accumulation can be used to study the efficiency of ABCG2 inhibitors (Weidner et al. 2015). Our results indicated that ABT-263 exhibited a concentration-dependent inhibition of dye extrusion, however, inhibition efficacy reached only approximately 50% within clinically relevant drug concentrations in cells with lower transporter expression level (Fig. 4a). Similarly, ABT-199 exhibited a concentration-dependent inhibition of dye extrusion (Fig. 4b). Importantly, ABT-199 reached full inhibition of dye extrusion within clinically relevant drug concentrations in cells expressing low level of ABCG2 (Fig. 4b). Also here, the inhibition efficiency of ABT-263, ABT-199, and reference inhibitor Ko143 was inversely related to the ABCG2 expression level. Cells with higher expression levels of ABCG2 required higher inhibitor concentration to achieve certain inhibition efficacy (Fig. 4).



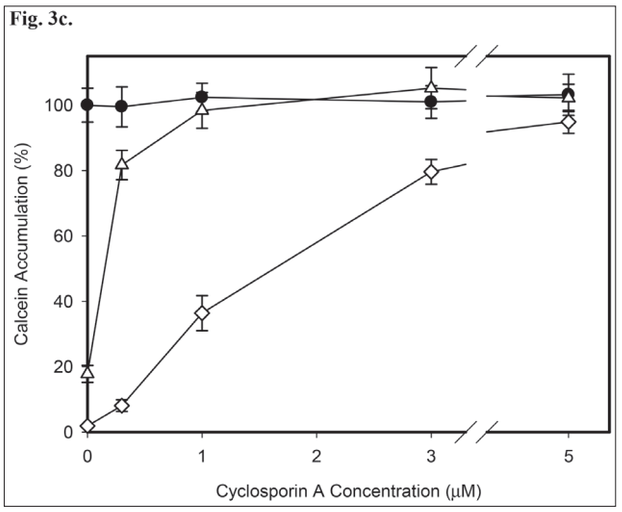




Fig. 3: The effect of ABCB1 expression on calcein accumulation in the presence of transporter inhibitor. Cells with various expression of ABCB1 were incubated with 0.25µM calcein AM in the presence of transporter inhibitor, as indicated. (a) Effect of ABT-263. (b) Effect of ABT-199. (c) Effect of CsA. Calcein uptake, expressed as the mean fluorescence intensity (MFI), was analysed using flow cytometry. K562/Dox cells (open diamond), K562/DoxDR1 cells (open triangle). Calcein fluorescence in K562 cells, which do not express ABCB1, was used as control (100%; filled circles). The experimental points represent mean values from three replicate experiments, with standard deviations.

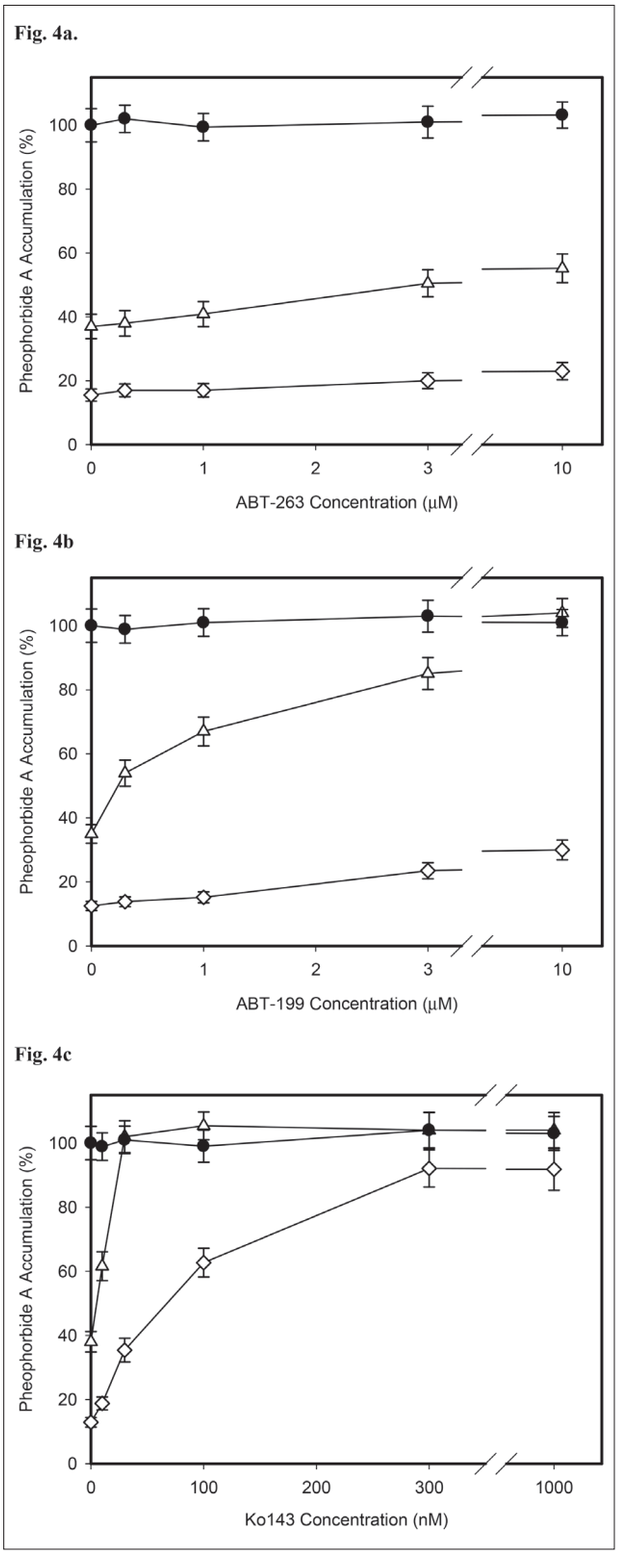



Fig. 4: The effect of ABCG2 expression on pheophorbide A accumulation in the presence of transporter inhibitor. Cells with various expression of ABCG2 were incubated with 1.0μM pheophorbide A in the presence of transporter inhibitor, as indicated. (a) Effect of ABT-263. (b) Effect of ABT-199. (c) Effect of Ko143. Pheophorbide A uptake, expressed as the mean fluorescence intensity (MFI), was analysed using flow cytometry. K562/ABCG2CL10 cells (open diamonds), K562/ABCG2CL1 cells (open triangles). Pheophorbide A fluorescence in K562 cells, which do not express ABCG2, was used as control (100%; filled circles). The experimental points represent mean values from three replicate experiments, with standard deviations.

ABT-737 was not tested since its autofluorescence interfered with both calcein and pheophorbide A fluorescence assays using flow cytometry (not shown).

## 3. Discussion

In the last decades considerable attention has been dedicated to the role of ABC transporters in the development of MDR to chemotherapy in cancer cells. In vitro studies on cultures of cancer cells usually consistently show a clear association between ABCB1, ABCC1, or ABCG2 expression and resistance to chemotherapy. However, the correlation between above mentioned ABC transporters and clinical drug resistance is difficult to establish (Amiri-Kordestani et al. 2012; Tamaki et al. 2011).

Controversy in results is a common feature of studies evaluating the expression and prognostic role of these proteins and is mostly attributed to methodical factors, largely caused by insufficient reliability and accuracy of methods used for the assessment of the ABC transporters expression and function (Beck et al. 1996; Chevillard et al. 1994). Indeed, there is no doubt that methods for evaluation of ABC transporters expression and function are important and may fundamentally affect the results. In addition, not always the assessment of ABCB1 expression can predict its function even in well-defined in vitro experiments (Kosztyu et al. 2015).

We believe that discrepancies between laboratory and clinics may be due to the use of clinically irrelevant transporter expression levels and clinically irrelevant drug concentrations in laboratory experiments. Therefore, we used cells with high K562/Dox and K562/ABCG2CL10, and low K562/DoxDR1 and K562/ABCG2CL1 expression levels of ABCB1 and ABCG2, respectively (Fig. 1). We observed that neither ABCB1 nor ABCG2 overexpression reduced significantly the intracellular level of ABT-737 (Fig. 2a, b). These results suggested that neither ABCB1 nor ABCG2 would compromise the antiproliferative effects of ABT-737. Our results are different from those published by other authors (Othman et al. 2014; Vogler et al. 2011). However, these authors did not determine the intracellular level of ABT-737 (Othman et al. 2014; Vogler et al. 2011). Both research groups just compared the sensitivity to apoptosis between cells overexpressing ABCB1 and parental cells in the absence and in the presence of transporter inhibitor (Othman et al. 2014; Vogler et al. 2011). Such an approach omits to prove that the observed resistance was due to the decreased intracellular drug level.

Our results indicated that both drug transporters, ABCB1 and ABCG2, are able to reduce intracellular levels of ABT-263 and thus mediated resistance to this drug. However, this effect strongly depends on their expression levels (Figs. 1 and 2c, d). Generally, our observation well corresponds to the results reported by Vogler et al. (2011) who demonstrated that observed ABCB1 mediated resistance was due to the decreased intracellular level of ABT-263. Unfortunately, they did not consider the possible effect of transporter expression level on observed resistance (Vogler at al. 2011). Therefore, our interpretation of results with respect to clinics might be somewhat different. Owing to the fact that expression levels of ABCB1 and ABCG2 are usually higher in normal tissue than in cancer tissue, as suggested by early works (Fojo et al. 1989; Goldstein al. 1989) and by The Cancer Genome Atlas (TCGA) (http://cancergenome.nih.gov), we assume that the resistance mediated by these transporters to ABT-263 is probably low.

The observed decrease in intracellular level of ABT-199 depends on the ABCB1 and ABCG2 expression levels (Fig. 2e, f). Cells with high expression levels of ABCB1 or ABCG2 significantly decreased the intracellular level of ABT-199, however, cells with low expression level of ABCB1 or ABCG2 exhibited intracellular level of ABT-199 which was indistinguishable from parental K562 cells (Fig. 2e, f). Therefore, we assume that expression of ABCB1 or ABCG2 in cancer tissue will probably mediate only low or undetectable resistance to ABT-199. Our results are partially in accordance with the findings of Weiss et al. (2016), who demonstrated a reduced antiproliferative effect of ABT-199 in cells overexpressing ABCB1 but not in cells overexpressing ABCG2. Unfortunately,

neither expression levels of ABCB1 and ABCG2 nor intracellular levels of ABT-199 are mentioned in this work (Weiss et al. 2016). We believe that our approach has a high potential to estimate the real contribution of a particular ABC transporter to drug resistance. Recently, we demonstrated that the expression level of drug transporters, including ABCB1 and ABCG2 is a key factor which always affects the drug resistance (Kosztyu et al. 2014, 2013; Mlejnek et al. 2017).

Importantly, many anticancer drugs may serve as ABC transporter inhibitors at high concentrations. Tyrosine kinase inhibitors can be used as a typical example of the above mentioned interaction between drugs and ABC transporters (Dohse et al. 2010; Hegedűs et al. 2009). Recent in vitro studies suggested a dual function of ABT-199. Indeed, Weiss and al. (2016) demonstrated that ABT-199 may act as a substrate for ABCB1 but not for ABCG2 and as an inhibitor for both transporters. However, whether ABT-199 may increase intracellular drug levels of other anticancer drugs and thereby increase their therapeutic efficacy in resistant tumor cells, which resistance is mediated by ABCB1 or ABCG2 is unclear.

Therefore, we further addressed the question whether ABT-263 and ABT-199 may serve as efficient inhibitors of ABCB1 or ABCG2 and whether such an effect is potentially applicable in clinical settings. Here we define an efficient inhibitor as a compound (drug) that achieves the full (90-100%) reversal of intracellular probe accumulation at concentration that does not exceed the clinically relevant concentrations. We believe that only the full inhibition found in vitro might provide profit in clinics because of the following reasons: i) the partial inhibitory effect in cancer cells, despite positive transient results, still opens the door for the resistance development; ii) we can hardly expect the same efficiency of inhibitors in clinics as in in vitro experiments.

We observed that ABT-263 affected the calcein accumulation neither in cells with high nor with low expression level of ABCB1 at clinically relevant concentrations (Fig. 3a). In contrast, ABT-199 achieved only partial inhibition of calcein accumulation at clinically relevant concentrations (Fig. 3b). Importantly, the inhibitor concentration was not the only parameter that affected their efficiency. The efficacy of ABCB1 inhibitors, including ABT-199 and CsA, depended also on the transporter expression level. The higher transporter expression level requires the higher concentration of inhibitor to achieve a certain inhibition efficacy (Fig. 3b, c).

Similarly, the efficacy of ABCG2 inhibitors, including ABT-263, ABT-199, and Ko143 depended also on the transporter expression level. The higher transporter expression level requires a higher concentration of inhibitor to achieve a certain inhibition efficacy (Fig. 4). ABT-263 achieved only partial inhibition of pheophorbide A accumulation at clinically relevant concentrations (Fig. 4a). However, ABT-199 fully inhibited the ABCG2 mediated efflux of pheophorbide A in cells with low transporter expression level at clinically relevant concentrations (Fig. 4b). These results are in a good agreement with our previous work where we demonstrated that inhibition efficiency of imatinib, nilotinib, and CsA depends on the ABCB1 expression level (Mlejnek et al. 2017b).

Our results clearly indicate that clinically relevant ABT-263 concentrations, which are used in clinical trials (Gandhi et al. 2011), either failed to inhibit ABCB1 or caused only a partial effect even in the K562/ABCG2CL1 cells with the low ABCG2 expression (Figs. 3a and 4a). In contrast, ABT-199 is a much stronger inhibitor than ABT-263 and exhibits 90-100% inhibitory efficacy in cells expressing low level of ABCG2 (Fig. 4b) at clinically relevant concentrations (Weidner et al. 2015). ABT-199 exhibited only a partial inhibitory effect only in the K562/ABCG2CL1 cells with the low ABCG2 expression (Fig. 4a).

Our results suggest that ABT-199 but not ABT-263 is a promising candidate to be studied beyond its original application, as efficient inhibitor of the drug efflux pump ABCG2. We hypothesize that patients whose tumors express low levels of ABCG2 could benefit from ABT-199 in addition to its anti Bcl-2 effect. As judged from the pioneer works (Fojo et al. 1987; Goldstein et. al. 1989) and from The Cancer Genome Atlaas (TCGA), the tumor ABCG2 levels are in general lower than in normal cells, and potentially amenable to inhibition while leaving the higher level ABCG2 in normal bone marrow and other tissues ubinhibited or only partially inhibited. In contrast, patients with a high expression level of ABCG2 may benefit from inhibitors with higher potency. However, patients with tumors with very high ABCG2 levels appear to be rare, based on the TCGA data.

In conclusion, we observed that antiproliferative effects of ABT-263 and ABT-199 might be reduced by ABCB1 or ABCG2, however, this effect depends on transporter expression levels. Since the expression levels of ABCB1 and ABCG2 are hardly high in clinical samples, their contribution to the overall resistance to ABT-263 or ABT-199 is probably low. Neither ABCB1 nor ABCG2 provide significantly reduced intracellular levels of ABT-737. Inhibition study revealed that ABT-263 partially inhibited the ABCG2 mediated transport but it failed to inhibit the ABCB1 mediated transport at clinically relevant concentrations. In contrast, ABT-199 fully inhibited the ABCG2 mediated transport and only partially inhibited the ABCB1 mediated transport at clinically relevant concentrations. Our data suggest that ABT-199 should be evaluated for its potential to inhibit ABCG2 in the clinical settings.

## 4. Experimental

### *4.1. Chemicals*

ABT-737 4-[4-[[2-(4-chlorophenyl)phenyl]methyl]piperazin-1-yl]-N-[4-[[(2R)-4-(dimethylamino) -1-phenylsulfanylbutan-2-yl]amino]-3-nitrophenyl] sulfonylbenzamide, ABT-263 4-(4-{[2-(4-Chlorophenyl)-5,5-dimethyl-1-cyclohexen-1-yl]methyl}-1-piperazinyl)-*N*-[(4-{[(2*R*)-4-(4-morpholinyl)-1-(phenylsulfanyl)-2-butanyl] amino}-3-[(trifluoromethyl)sulfonyl]phenyl)sulfonyl] benzamide, and ABT-199 4-[4-[[2-(4-chlorophenyl)-4,4-dimethyl-1-cyclohexen-1-yl]methyl]-1-piperazinyl]-N-[[3-nitro-4-[[(tetrahydro-2H-pyran-4-yl)methyl]amino]phenyl]sulfonyl]-2-(1H-pyrrolo[2,3-b]pyridin-5-yloxy)-benzamide (Cayman Chemical, Michigan, USA). Cyclosporin A (CsA) and Ko143 (3S,6S,12aS)-1,2,3,4,6,7,12,12a-Octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxopyrazino-[1',2':1,6] pyrido [3,4-b]indole-3-propanoic acid 1,1-dimethylethyl ester were obtained from Enzo Life Sciences AG (Lausen, Switzerland).

### *4.2. Cell culture*

Human chronic myelogenous leukemia K562 cells, obtained from ECACC, were cultured in the RPMI-1640 medium supplemented with a 10% calf foetal serum and antibiotics in 5% $CO_2$ atmosphere at 37 °C. K562/Dox cells, which overexpress P-gp (ABCB1, MDR1), were kindly provided by Prof J.P. Marie (University of Paris 6, France). K562/Dox cells were cultured under the same conditions. More detailed characterisation of K562/Dox cell line is given elsewhere (Tang et al. 2008).

K562/DoxDR1 cells with downregulated expression of P-gp were established by stable transfection of K562/Dox cells with a plasmid vector expressing shRNA targeting the *ABCB1* gene (Kosztyu et al. 2013; Mlejnek et al. 2012).

K562/ABCG2 cells, which overexpress wild type ABCG2 (BCRP1), were kindly provided by Prof B. Sarkadi (National Blood Center and Semmelweis University, Budapest, Hungary). K562/ABCG2 cells were cultured under the same conditions. Detailed characterisation of K562/ABCG2 cell line is given elsewhere (Elkind et al. 2005; Hegedűs et al. 2009).

Cells expressing different levels of ABCG2 were established by a single cell cloning by limiting dilution of K562/ABCG2 cells (Kosztyu et al. 2014). In this study we used two subclones, K562/ABCG2CL10 and K562/ABCG2CL1, with high and low expression level of ABCG2, respectively. The detailed characteristics of the obtained subclones are given elsewhere (Kosztyu et al. 2014).

### *4.3. ABCB1 and ABCG2 expression analysis using flow cytometry*

ABCB1 expression was studied by using UIC2 (Beckman Coulter, USA) monoclonal antibody conjugated with phycoerythrin (UIC2-PE) according to the manufacturer's instruction. Phycoerythrin conjugated isotype IgG2a was used as a control. ABCG2 expression was studied by using mouse anti-human CD 338 (BD Biosciences, USA) monoclonal antibody conjugated with phycoerythrin (CD338-PE) according to the manufacturer's instruction. Phycoerythrin conjugated isotype IgG2b was used as a control. The fluorescence of the cells was analysed by flow cytometry (Cytomics FC500, Beckman Coulter, USA). ABCB1 expression was determined by the ratio of the mean fluorescence intensity (MFI) shift of UIC2-PE antibody to isotype control (UIC2-PE/IgG2a-PE). Similarly, ABCG2 expression was determined by the ratio of the MFI shift of CD338-PE antibody to isotype control (CD338- PE/IgG2b-PE). For each sample 10 000 events were collected. All the experiments were performed in triplicate.

### *4.4. Functional assay of ABCB1 and ABCG2*

Calcein acetoxymethyl ester (calcein AM) accumulation was used as functional assay of ABCB1 (Holló et al. 1994). Cells were loaded with calcein AM (Molecular Probes, Eugene, OR, USA) and then analysed by flow cytometry (Cytomics FC500, Beckman

Coulter, USA), as described elsewhere (Kosztyu et al. 2013). Dye uptake was expressed as the mean fluorescence intensity (MFI) in the presence and absence of ABCB1 inhibitor. For each sample 10 000 events were collected. All the experiments were performed in triplicate. Pheophorbide A accumulation was used as functional assay of ABCG2 (Robey et al. 2004). Cells ($2.5x10^5$ cells/ml) were incubated in a growth medium containing 1 μM pheophorbide A (Sigma, Saint Louis, Missouri, USA) with or without 0.3mM Ko143 for 30 min at 37 °C. Cells were immediately analysed by flow cytometry (Cytomics FC500, Beckman Coulter, USA) at excitation and emission wavelength of 635 and 675 mm, respectively. Dye uptake was expressed as the mean fluorescence intensity (MFI) in the presence and absence of ABCG2 inhibitor. For each sample 10 000 events were collected. All the experiments were performed in triplicate.

### *4.5. Preparation of cell extracts*

The method is based on an optimised extraction of cells with formic acid after their separation from the growth medium by centrifugation through a layer of silicone oil (Mlejnek et al. 2011) with a slight modification. Briefly, cells at the density of $7x10^5$/ml and $5x10^5$/ml were incubated in the growth medium with ABT-737, ABT-263, and ABT-199, respectively, for 3hrs in 5% $CO_2$ atmosphere at 37 °C. The cells were then centrifuged through silicone oil and cell pellets were extracted using ice cold 3% (w/v) formic acid + 50% (v/v) ethanol in water. Cell extracts were clarified by centrifugation (40 000 g x 10 min at 4 °C) and either analysed using spectrophotometry (for ABT-737) or diluted with extraction solution and analysed by liquid chromatography coupled with a low-energy collision tandem mass spectrometer (LC/MS/MS; for ABT-263 or ABT-199).

### *4.6. Assay for determination of intracellular ABT-737 level*

ABT-737 is quantified in cell extracts spectrophotometrically at 533nm using external standards diluted in extraction solution.

### *4.7. Assay for determination of intracellular ABT-263 and ABT-199 levels*

The HPLC system consisted of UltiMate 3000 RS pump, degasser, autosampler and column compartment (Dionex, Germering, Germany). Separations were performed at ambient temperature on a Polaris C18-A 250 x 2.0mm (i.d.), 5 μm particle size column (Varian Inc., Lake Forest, CA, USA) connected with a guard $C_{18}$ 4.0 x 2.0 mm (i.d.) precolumn (Phenomenex, Torrance, CA, USA). Solvents used for separation were A (95% acetonitrile in 0.5% FA, v/v) and B (0.5% FA, v/v). The flow rate was 300 μl/min with linear gradient elution from 0 to 3 min (45 to 95% of solvent A), from 3 to 4 min (95% of solvent A), from 4 to 5 min (95 to 45% of solvent A) and from 5 to 8 min (45% of solvent A). Sample injection volume was set at 10 μl. The effluent was introduced into the API 3200 triple quadrupole mass spectrometer (MDS SCIEX, Ontario, Canada) and electrospray ionization in positive ion mode was used for detection. The mass spectrometer was operated in the multiple-reaction monitoring (MRM) mode. ABT263 and ABT199 was monitored by MRM transition 974 > 233 (dwell-time = 150 ms) and 868 > 321 (dwell-time = 150 ms), respectively. Ion spray probe parameters for both compounds were set to the following values: needle voltage 5500 V, temperature 400 ºC, curtain gas (nitrogen) 1.38 bar, nebulizer gas (zero air quality) 3.45 bar, turbo V-gas (zero air quality) 3.45 bar. The nitrogen pressure in the second quadrupole was measured at 4.0 $x10^{-8}$ bar, declastering potential 96 V for ABT-263 and 76 V for ABT-199. The collision energy and entrance potential for ABT-263 was set at 47.0 V and 9.5 V, respectively, and for ABT-199 at 51.0 V and 7.5 V, respectively. The instrument was operated in unit resolution.

### *4.8. Statistical analysis*

Data are reported as the mean ± S.D. Statistical significance of differences was determined by Student's *t*-test. Only the P values less than 0.05 were considered significant.

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