Tipifarnib and tanespimycin show synergic proapoptotic activity in U937 cells

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Abstract

Background Farnesyltransferase inhibitor tipifarnib (R115777) has been used for treatment of hematological malignancies; however, its observed anticancer effect was limited. This prompted us to search for inhibitors that would show synergic, proapoptotic effect when combined with R115777. We decided to study LY294002, which inhibits PI-3 kinase, and tanespimycin (17AAG), which inhibits Hsp90—a chaperone for a number of proteins, including Akt kinase.

Methods The effect of drugs, used alone or in combination, was tested in U937 cells (human leukemic monocyte lymphoma), which are often used as a model for liquid tumor. The number of viable cells was evaluated with trypan blue staining, while apoptosis was assessed by presence of active caspase-3 and terminal dUTP nick-end labeling of DNA (TUNEL).

Results At concentrations in which R115777, LY294002 and 17AAG were only slowing down the proliferation rate, when used separately, the combination of R115777 + LY294002 and R115777 + 17AAG significantly reduced the number of cells and induced cellular apoptosis.

Conclusions Our results suggest that the combination of R115777 + 17AAG could be useful in treating some of the hematological malignancies.

Keywords Tipifarnib · R115777 · Tanespimycin · 17AAG · Hsp90 · Apoptosis
the intrinsic pathway (Le Gouill et al. 2002; Rolland et al. 2008). Such concentrations are very difficult to reach in humans, because when R115777 is administered orally, at the typical dose of 300–600 mg bid, its maximum plasma level reaches only ∼2.5 μM (Zujewski et al. 2000; Karp et al. 2001). Thus, insufficient concentration of the drug can partially explain the rather disappointing effects of FTI monotherapy. Much better anticancer activity was achieved with combinatorial approaches, when R115777 was used together with other classical or modern chemotherapeutics (Zhu et al. 2005; Lancet et al. 2011; Jabbour et al. 2011).

Hsp90 is the most abundant protein in the cell and acts as chaperone to maintain client proteins in their active conformation and to protect them against degradation by proteasome. Hsp90 concentration is further increased in tumor cells, in response to stress induced by elevated concentration of oncogenic proteins, which are often thermodynamically unstable (Mosser and Morimoto 2004; Whitesell and Lindquist 2005). The list of Hsp90 clients contains a few hundred proteins that participate in all crucial aspects of cancer biology, such as uncontrolled proliferation, protection against apoptosis, angiogenesis and increased metastasis (Zhao et al. 2005). Examples of Hsp90 client proteins include growth factor and steroid hormone receptors, tyrosine and serine/threonine kinases (e.g. Akt kinase), telomerase, P53 and survivin. Hsp90 has long represented an attractive target for chemotherapy, since its inhibition is influencing simultaneously a whole range of processes important for cancer development (Isaacs et al. 2003; Bagatell and Whitesell 2004).

Geldanamycin and its derivative 17-allylamino-17-demethoxygeldanamycin (tanespimycin; 17AAG) are competitive inhibitors of ATP-binding pocket of Hsp90 that block its ATPase-dependent chaperone activity (Schulte and Neckers 1998). 17AAG was used in several clinical trials as anticancer agent to treat different types of tumors (Solit et al. 2007; Richardson et al. 2010a). These tests showed that 17AAG can be safely administered to humans, but unfortunately, its anticancer activity in monotherapy was very limited. Much better antitumor effects were obtained when 17AAG was combined with other modern chemotherapeutics (Ramalingam et al. 2008; Richardson et al. 2010b).

It is now generally accepted that multidrug, multitargeted strategies are likely to be more successful in fighting cancer comparing with single drug, single target approaches. In our previous study, we have shown that combination of drugs that inhibit different signaling pathways can result in an unexpectedly strong synergistic proapoptotic effect (Małecki et al. 2010). The above rationale convinced us to search for drugs that would show synergism in inducing apoptosis in combination with R115777. Since high concentrations of R115777 induce apoptosis by stimulating the release of cytochrome c from mitochondria to cytosol, we decided to test drugs that inhibit the PI-3 kinase/Akt kinase pathway, which suppresses induction of apoptosis through the intrinsic pathway. First, we tested the combined effect of R115777 and LY294002, which is a direct inhibitor of the PI-3 kinase. Next, we used 17AAG that inhibits Hsp90, and among many other molecular targets, it indirectly down-regulates the activity of Akt kinase. Our results show that the proapoptotic activity of R115777 can be increased by combining it with LY294002 or 17AAG. This is an important finding, because both R115777 and 17AAG are registered anticancer drugs, and therefore, their combination could be implemented in the near future as part of the strategy to fight hematologic malignancies.

Materials and methods

Materials

Fluorogenic substrate for caspase-3 (Ac-DEVD-AFC) was purchased from MP Biomedicals (Solon, OH, USA), TitertACS In Situ Apoptosis Detection Kit (Colorimetric) was from R&D Systems (Minneapolis, MN, USA), and R115777 was from Johnson & Johnson Pharmaceutical Research and Development (Raritan, NJ, USA). Anti-cleaved caspase-9 (No. 9505), anti-phospho-ERK1/2 (No. 9101) and anti-phospho-Akt (No. 9271) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA), and anti-Hsp90 (No. 610418) antibody was from BD Biosciences (Mississauga, ON, Canada). Caspase-3 inhibitor (Ac-DEVD-CHO), 17AAG, LY294002, anti-β-actin (No. A1978) antibody and other chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

Cell treatment and quantitation

U937 cells were provided by Polish National Cell Bank (Wroclaw, Poland). Cells were propagated in standard conditions (37°C, 5% CO2) in RPMI-1640 medium supplemented with 10% fetal bovine serum. Cells were seeded (2–5 × 105 cells/ml), treated with inhibitors or vehicle (0.2% dimethyl sulfoxide (DMSO)) for desired time and harvested by centrifugation. After washing with PBS, cells were labeled with trypan blue to visualize dead cells (blue), and the amount of viable cells (white) were counted. In proliferation assays, the data were normalized to the initial viable cell number (100%), that is, number of cells present at the moment of inhibitor administration. Each experiment was run at least three times in duplicates. The obtained data were averaged, and the standard deviation is represented in graphs by bars.
The inhibitory concentration 50% (IC$_{50}$) was determined based on relation between the number of viable cells and the inhibitor concentration, using the following formula (Eq. 1):

$$ V = \frac{V_{\text{max}} \cdot \text{IC}_{50}}{\text{IC}_{50} + [S]} $$

where $V$ is the normalized cells viability, $V_{\text{max}}$ is the maximum normalized cells viability, and $[S]$ is the inhibitor concentration. The standard error of estimated IC$_{50}$ is given with 95% confidence limit.

Results

Tipifarnib (R115777) has been used previously to treat some hematological malignancies but with limited success. We decided to test its effect on U937 cells (human leukemic monocyte lymphoma cell line), which are frequently used as a model for liquid tumor and antitumor testing. Cells were seeded, treated with increasing concentrations of R115777 for 24 h and stained with trypan blue, and the number of viable cells was counted. The results were normalized to the initial (100%) viable cell number. Results in Fig. 1a show decrease in the number of viable cells with increasing concentration of R115777. The number of viable cells was reduced below their initial level when R115777 was present at concentration higher than $\sim 5 \mu M$. Same data were also plotted as: number of viable cells versus inhibitor concentration (Fig. 1b) and used to estimate the IC$_{50}$ value, which was $3.3 \pm 0.3 \mu M$.

Reduction in cell number could result from apoptotic death, and so we measured the activity of caspase-3 in cells exposed to increasing concentrations of R115777 (Fig. 2a). For concentrations lower than IC$_{50}$, the activity of caspase-3 was only slightly elevated, while it increased considerably at higher inhibitor concentrations. This indicates that at lower concentrations, R115777 acted mainly by slowing down the proliferation rate, while at higher concentrations, the inhibitor very likely induced apoptosis. Further experiments showed that incubation with 10 $\mu M$ R115777 induced cleavage of caspase-9 (Fig. 2b) and at the same time reduced the level of phosphorylation of Akt and ERK 1/2. The apoptosis was confirmed with results obtained from TUNEL (Fig. 2c). Treatment of U937 cells with 10 $\mu M$ R115777 for 48 h increased the amount of DNA nick-ends over 10 times, with respect to control cells.

Although 10 $\mu M$ R115777 induces apoptotic death in U937 cells, it is unlikely that this drug can reach such a concentration in human plasma, since its oral administration at typical doses gives a maximum plasma concentration of up to $\sim 2.5 \mu M$ (Zujewski et al. 2000; Karp et al. 2001). On the other hand, R115777 at concentrations below IC$_{50}$ (e.g. $2.5 \mu M$) was not inducing apoptosis to large extent (Fig. 2a; see also Figs. 3c, 4c later in the text). This suggests that at low concentrations, R115777 is just slowing down the proliferation rate, which can partly explain its limited success in clinical trials. Such observation prompted us to test R115777 in combination with other inhibitors in hope to find a combination that would synergize in inducing apoptosis.
Since R115777 reduced the level of Akt phosphorylation (Fig. 2b), the first choice was to use LY294002 to inhibit the PI3-kinase/Akt kinase pathway, which protects cells against apoptosis initiated by the intrinsic pathway. Initially, the effect of increasing concentration of LY294002 on the proliferation rate was tested. Results in Fig. 3a show the decrease in the number of viable cells with increasing concentration of LY294002. The number of viable cells was reduced below their initial amount when LY294002 was present at concentration higher than ~10 μM. The estimated IC50 was 6.3 ± 0.8 μM.

Next, cells were treated with the combination of R115777 and LY294002. Both inhibitors were used at concentrations below their IC50 values, so that when used individually, they were mainly slowing down the proliferation without actually reducing the number of viable cells below their initial amount (Fig. 3b). Interestingly, 24-h incubation with the combination of 2.5 μM R115777 + 5 μM LY294002 was able to reduce the number of viable cells to ~50% of their initial amount.

Parallel experiments showed that after 48-h incubation with the combination of 2.5 μM R115777 + 5 μM
LY294002, the amount of DNA nick-ends were increased over 10 times with respect to control (Fig. 3c), while incubation with individual inhibitors did not cause substantial increase in TUNEL signal. This demonstrates that R115777 and LY294002 exhibit synergism in inducing apoptosis in U937 cells, while the individual inhibitors, when used at concentrations below IC$_{50}$, do not cause apoptosis, but act mainly by decreasing the proliferation rate.

In U937 cells, 5 µM LY294002 reduced the phosphorylation of Akt, and combined action of R115777 + LY294002 reduced the phosphorylation of Akt even more, while the phosphorylation of ERK1/2 remained basically unchanged (Fig. 3d).

The increased proapoptotic activity of the combination of R115777 + LY294002 is an interesting phenomenon, which is, however, of limited importance to clinical practice, since LY294002 is known to induce severe liver toxicity. Therefore, we searched for other inhibitors that would synergize with R115777 in inducing apoptosis. We decided to test tanespimycin (17AAG) that is a registered anticancer drug, and its use was shown to be safe for humans. Since 17AAG inhibits the chaperone activity of Hsp90, and Akt is one of the client proteins for Hsp90, one could expect that 17AAG would influence the phosphorylation status of Akt.

When U937 cells were treated with increasing concentration of 17AAG for 24 h, they responded by decreasing their apparent proliferation rate (Fig. 4a). At higher concentrations, 17AAG was able to reduce viable cell number below their initial amount. The estimated IC$_{50}$ value was 54 ± 8 nM.

Next, cells were treated with the combination of R115777 + 17AAG. Also in this case, the inhibitors were used at concentrations that were slightly below their determined IC$_{50}$, so that when used individually, they were...
only slowing down proliferation without reducing the number of viable cells below their initial amount (Fig. 4b). After 24-h incubation of cells with the combination of 2.5 μM R115777 + 50 nM 17AAG, the number of viable cells was reduced to ~65% comparing with their initial amount.

The combination of R115777 + 17AAG was also tested for induction of apoptosis using TUNEL-based assay. The results in Fig. 4c show that the amount of DNA nick-ends were considerably increased after incubation with the combination of R115777 + 17AAG, but not after incubation with the individual inhibitors. This shows that at tested concentrations, the individual inhibitors do not cause apoptosis but rather act by decreasing the proliferation rate. Thus, R115777 and 17AAG exhibit synergy in inducing apoptosis in U937 cells.

In U937 cells, 50 nM 17AAG was able to reduce the phosphorylation of both Akt and ERK1/2, while the combination of R115777 + 17AAG almost completely blocked the phosphorylation of both Akt and ERK1/2 (Fig. 3d).

Discussion

In this paper, we provide evidence that 10 μM R115777 reduces viable cell number and induces apoptosis in U937 cells. The induction of apoptosis is shown by increased activity of caspase-3 and increased amount of DNA nick-ends (TUNEL). The increased cleavage of caspase-9 suggests that 10 μM R115777 induces apoptosis by the intrinsic pathway, which is in agreement with results published earlier by other groups (Le Gouill et al. 2002; Rolland et al. 2008). High concentrations of R115777 also reduced the phosphorylation of Akt and ERK1/2, suggesting the possible mechanistic explanation for efficient induction of apoptosis.

The main finding of our study is that the proapoptotic activity of R115777 can be increased by using it in combination with either LY294002 or 17AAG. The synergic proapoptotic activity was demonstrated using individual inhibitors at concentrations at which they acted mainly as cytostatic agents, slowing down proliferation rate. Yet in combination, they were able to exert cytotoxic effect and truly diminish cell number. We have shown that cytotoxicity of combinations R115777 + LY294002 and R115777 + 17AAG was due to induction of apoptosis, as seen by using TUNEL. Most importantly, the combination of R115777 + 17AAG showed synergic proapoptotic activity at concentrations of individual inhibitors that are certainly possible to achieve in human plasma after oral administration at typical dosage (Zujewski et al. 2000; Karp et al. 2001). This finding is of great importance, as both compounds are registered anticancer drugs, which passed clinical trials on humans, and hence, their combination could be used in the near future to fight certain hematological malignancies and possibly other types of cancer.

We observed that 10 μM R115777 was inducing apoptosis in U937 cells and, at the same time, was reducing the level of phosphorylated Akt and ERK1/2. Moreover, the combination of R115777 + LY294002 reduced phosphorylation of Akt, while the combination of R115777 + 17AAG strongly decreased the phosphorylation of both Akt and ERK1/2. It seems therefore that in U937 cells, the ability of drugs and
their combinations to simultaneously inhibit both the Akt kinase and the MAP kinase signaling pathways contributes to increased efficiency of apoptosis induction. The synergy between R115777 and LY294002 can be logically explained. Since R115777 induces apoptosis via the release of cytochrome c, the direct inhibition of PI3 kinase with LY294002 should decrease the activity of Akt kinase and consequently block the whole prosurvival pathway and help to unblock the apoptosis. The reasons for the synergy between R115777 and 17AAG are probably more complex. Since Akt is an important client protein for Hsp90, inhibition of Hsp90 by 17AAG should decrease the activity of Akt, and the results in Fig. 3d seem to support this rationale. However, there are a few hundred client proteins for Hsp90, and it is actually difficult to assign which target is the most important. For example, survivin, which inhibits the activity of caspases, is another client of Hsp90 (Zhao et al. 2005), and its decreased activity would also help in unblocking apoptosis. Definitely, further research is required to better explain the observed synergy between tipifarnib and tanespimycin.

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Conflict of interest We declare that no actual or potential conflict of interest in relation to this article exists.

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