Original Paper

Lapatinib Induced Suicidal Death of Human Erythrocytes

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Key Words

Phosphatidylserine • Cell volume • Eryptosis • Calcium • Lapatinib

Abstract

Background/Aims: The human epidermal growth factor receptors tyrosine kinase inhibitor lapatinib has been shown to trigger suicidal death or apoptosis of tumor cells and is thus used for the treatment of malignancy. Side effects of lapatinib include anemia, which could, at least in theory, result from stimulation of eryptosis, the suicidal death of erythrocytes which is characterized by cell shrinkage and phospholipid scrambling of the cell membrane leading to phosphatidylserine translocation to the erythrocyte surface. Mechanisms involved in the triggering of eryptosis include oxidative stress, increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), and ceramide. The present study explored, whether lapatinib induces eryptosis. **Methods:** Phosphatidylserine exposure at the cell surface was estimated from annexin-V-binding, cell volume from forward scatter, $[\text{Ca}^{2+}]_i$ from Fluo3-fluorescence, abundance of reactive oxygen species (ROS) from DCFDA dependent fluorescence, and ceramide abundance utilizing labelled specific antibodies. **Results:** A 48 hours exposure of human erythrocytes to lapatinib ($\geq 1 \mu\text{g/ml}$) significantly increased the percentage of annexin-V-binding cells, and significantly decreased forward scatter. Lapatinib ($7.5 \mu\text{g/ml}$) did not significantly modify DCFDA fluorescence and ceramide abundance. Lapatinib slightly, but significantly decreased Fluo3-fluorescence ($\geq 5 \mu\text{g/ml}$). Lapatinib ($7.5 \mu\text{g/ml}$) enhanced the annexin-V-binding in the presence of the Ca^{2+} ionophore ionomycin ($1 \mu\text{M}$) without significantly modifying Fluo3 fluorescence in the presence of ionomycin. The effect of lapatinib on forward scatter but not on annexin-V-binding was significantly blunted by removal of extracellular Ca^{2+} . **Conclusion:** Lapatinib triggers cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect occurring despite decrease of cytosolic Ca^{2+} activity.

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Introduction

Lapatinib, an inhibitor of human epidermal growth factor receptor (HER) tyrosine kinases [1-7], is utilized in the treatment of malignancy [1, 8-12], such as breast cancer [2-7, 13-45]. The drug is at least partially effective by triggering suicidal death or apoptosis

of tumor cells [46-57]. On the other hand, lapatinib could counteract apoptosis induced by excessive glucose concentrations [58]. Side effects of lapatinib include skin rash, hand foot skin reaction and pruritus [8, 9, 11, 16, 23, 25, 26, 30, 59], alopecia [9, 23], leukopenia [9, 16, 25, 30], diarrhea, nausea and vomiting [9, 11, 16, 23, 25, 26, 30, 38, 59, 60], fatigue [11, 23, 30], peripheral neuropathy [9, 30], and anemia [9, 11, 25, 26, 61, 62].

The anemia could, at least in theory, result from eryptosis [63], the suicidal death of erythrocytes characterized by cell shrinkage [64] and phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the cell surface [63]. Mechanisms stimulating eryptosis include increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), oxidative stress [63], ceramide [65], energy depletion [63], caspases [63, 66, 67], casein kinase 1 α , Janus-activated kinase JAK3, protein kinase C, and p38 kinase [63]. Eryptosis is inhibited by AMP activated kinase AMPK, cGMP-dependent protein kinase, sorafenib/sunitinib sensitive kinases, and PAK2 kinase [63].

The present study explored whether lapatinib triggers eryptosis. To this end, human erythrocytes from healthy volunteers were treated with lapatinib and phosphatidylserine surface abundance, cell volume, $[\text{Ca}^{2+}]_i$, reactive oxygen species and ceramide abundance determined by flow cytometry.

Materials and Methods

Erythrocytes, solutions and chemicals

Fresh Li-Heparin-anticoagulated blood samples were kindly provided by the blood bank of the University of Tübingen. The study is approved by the ethics committee of the University of Tübingen (184/2003 V). The blood was centrifuged at 120 x g for 20 min at 21°C and the platelets and leukocytes-containing supernatant was disposed. Erythrocytes were incubated *in vitro* at a hematocrit of 0.4% in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO_4 , 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES; pH 7.4), 5 glucose, 1 CaCl_2 , at 37°C for 48 hours. Where indicated, erythrocytes were exposed to lapatinib (MedChem Express, Princeton, NJ, USA) at the indicated concentrations.

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, a 150 μl cell suspension was washed in Ringer solution containing 5 mM CaCl_2 and then stained with Annexin-V-FITC (1:200 dilution; ImmunoTools, Friesoythe, Germany) in this solution at 37°C for 20 min under protection from light. The annexin V abundance at the erythrocyte surface was subsequently determined on a FACS Calibur (BD, Heidelberg, Germany). Annexin-V-binding was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. A marker (M1) was placed to set an arbitrary threshold between annexin-V-binding cells and control cells. The same threshold was used for untreated and lapatinib treated erythrocytes. A dot plot of forward scatter (FSC) vs. side scatter (SSC) was set to linear scale for both parameters. The threshold of forward scatter was set at the default value of "52".

Intracellular Ca^{2+}

After incubation, erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 mM CaCl_2 and 5 μM Fluo-3/AM. The cells were incubated at 37°C for 30 min. Ca^{2+} -dependent fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur.

Reactive oxidant species (ROS)

Oxidative stress was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). After incubation, a 150 μl suspension of erythrocytes was washed in Ringer solution and then stained with DCFDA (Sigma, Schnellendorf, Germany) in Ringer solution containing DCFDA at a final concentration of 10 μM . Erythrocytes were incubated at 37°C for 30 min in the dark and then washed two times in Ringer solution. The DCFDA-loaded erythrocytes were resuspended in 200 μl Ringer solution, and ROS-dependent fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD).

Ceramide abundance

To determine the ceramide abundance at the erythrocyte surface, a monoclonal antibody was used. After incubation, cells were stained for 1 h at 37°C with 1 µg/ml anti-ceramide antibody (clone MID 15B4; Alexis, Grünberg, Germany) in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) at a dilution of 1:10. After two washing steps with PBS-BSA, cells were stained for 30 min with polyclonal fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG and IgM specific antibody (BD Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA. Unbound secondary antibody was removed by repeated washing with PBS-BSA. Samples were then analyzed by flow cytometric analysis at an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Statistics

Data are expressed as arithmetic means ± SEM. As indicated in the figure legends, statistical analysis was made using ANOVA with Tukey's test as post-test and *t* test as appropriate. *n* denotes the number of different erythrocyte specimens studied. Since different erythrocyte specimens used in distinct experiments are differently susceptible to triggers of eryptosis, the same erythrocyte specimens have been used for control and experimental conditions.

Results

The present study explored, whether lapatinib stimulates suicidal erythrocyte death or eryptosis, which is characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the cell surface. Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding, as determined by flow cytometry. The erythrocytes were analysed following incubation for 48 hours in Ringer solution without or with lapatinib (1 - 7.5 µg/ml). As shown in Fig. 1, a 48 hours exposure to lapatinib increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 1 µg/ml lapatinib.

The second hallmark of eryptosis is cell shrinkage. In order to estimate erythrocyte volume, forward scatter was determined utilizing flow cytometry following a 48 hours incubation in Ringer solution without or with lapatinib (1 - 7.5 µg/ml). As illustrated in Fig. 2, lapatinib decreased erythrocyte forward scatter, an effect reaching statistical significance at 1 µg/ml lapatinib concentration. Fluo3 fluorescence was taken as measure of cytosolic Ca²⁺ activity ([Ca²⁺]_i). As shown in Fig. 3, a 48 hours exposure to lapatinib slightly decreased the Fluo3 fluorescence, an effect reaching statistical significance at 5 µg/ml lapatinib.

In order to test whether lapatinib interfered with the measurement of cytosolic Ca²⁺ activity, additional experiments were performed in the absence and presence of the Ca²⁺ ionophore ionomycin (1 µM). As illustrated in Fig. 4, ionomycin increased the Fluo3 fluorescence to similarly high levels in the absence and presence of lapatinib (7.5 µg/ml). Despite the similar increase of [Ca²⁺]_i in the absence and presence of lapatinib, ionomycin increased annexin-V-binding to significantly higher levels in the presence than in the absence of 7.5 µg/ml lapatinib (Fig. 5). Ionomycin treatment was further followed by marked cell shrinkage, an effect slightly, but significantly blunted in the presence of 7.5 µg/ml lapatinib (Fig. 6).

A next series of experiments explored whether the lapatinib-induced translocation of phosphatidylserine or erythrocyte shrinkage required entry of extracellular Ca²⁺. To this end, erythrocytes were incubated for 48 hours in the absence or presence of 7.5 µg/ml lapatinib in the presence or nominal absence of extracellular Ca²⁺. As illustrated in Fig. 7, removal of extracellular Ca²⁺ did not significantly modify the effect of lapatinib on annexin-V-binding and even in the absence of extracellular Ca²⁺, lapatinib significantly increased the percentage of annexin-V-binding erythrocytes. Thus, lapatinib-induced cell membrane scrambling did not depend on entry of extracellular Ca²⁺. Removal of extracellular Ca²⁺ significantly blunted the effect of lapatinib on forward scatter (Fig. 8). However, even in the absence of extracellular Ca²⁺, lapatinib significantly decreased the erythrocyte forward scatter. Thus, lapatinib-induced cell shrinkage was in part sensitive to the presence of extracellular Ca²⁺.

Fig. 1. Effect of lapatinib on phosphatidylserine exposure. (A) Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 7.5 µg/ml lapatinib. (B) Arithmetic means $\pm$ SEM (n = 20) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) presence of lapatinib (1 - 7.5 µg/ml). For comparison, the effect of the solvent DMSO is shown (grey bar). ** (p < 0.01), *** (p < 0.001) indicates significant difference from the absence of lapatinib (ANOVA).

Fig. 2. Effect of lapatinib on erythrocyte forward scatter. (A) Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 7.5 µg/ml lapatinib. (B) Arithmetic means $\pm$ SEM (n = 20) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) presence of lapatinib (1 - 7.5 µg/ml). For comparison, the effect of the solvent DMSO is shown (grey bar). *** (p < 0.001) indicates significant difference from the absence of lapatinib (ANOVA).

Fig. 3. Effect of lapatinib on erythrocyte Ca²⁺ activity. (A) Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of lapatinib (7.5 µg/ml). (B) Arithmetic means $\pm$ SEM (n = 17) of the Fluo3 fluorescence (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bars) presence of lapatinib (1 - 7.5 µg/ml). For comparison, the effect of the solvent DMSO is shown (grey bar). ** (p < 0.01) indicates significant difference from the absence of lapatinib (ANOVA).

Reactive oxygen species (ROS) was quantified utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). A 48 hours exposure to lapatinib (7.5 µg/ml) did not significantly

Fig. 4. Effect of the Ca^{2+} ionophore ionomycin on Fluo3 fluorescence in the absence and presence of lapatinib. (A, B) Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 1 h to Ringer solution without (grey area) and with (black line) presence of lapatinib (7.5 $\mu\text{g/ml}$) in the absence (A) and presence (B) of ionomycin (1 μM).

(C) Arithmetic means $\pm$ SEM ($n = 10$) of Fluo3 fluorescence in erythrocytes after a 1 h treatment with Ringer solution without (left bars) or with (right bars) ionomycin (1 μM) in the absence (white bars) and presence (black bars) of lapatinib (7.5 $\mu\text{g/ml}$). ###($p < 0.001$) indicates significant difference from the absence of ionomycin (ANOVA).

Fig. 5. Effect of the Ca^{2+} ionophore ionomycin on phosphatidylserine exposure in the absence and presence of lapatinib. (A, B) Original histogram of annexin-V-binding of erythrocytes following exposure for 1 h to Ringer solution without (grey area) and with (black line) presence of lapatinib (7.5 $\mu\text{g/ml}$) in the absence (A) and presence (B) of ionomycin (1 μM). (C) Arithmetic means $\pm$ SEM ($n = 7$) of annexin-V-binding of erythrocytes after a 1 h treatment with Ringer solution without (left bars) or with (right bars) ionomycin (1 μM) in the absence (white bars) and presence (black bars) of lapatinib (7.5 $\mu\text{g/ml}$). ***($p < 0.001$) indicates significant difference from the absence of lapatinib, ###($p < 0.001$) indicates significant difference from the absence of ionomycin (ANOVA).

(C) Arithmetic means $\pm$ SEM ($n = 7$) of annexin-V-binding of erythrocytes after a 1 h treatment with Ringer solution without (left bars) or with (right bars) ionomycin (1 μM) in the absence (white bars) and presence (black bars) of lapatinib (7.5 $\mu\text{g/ml}$). ***($p < 0.001$) indicates significant difference from the absence of lapatinib, ###($p < 0.001$) indicates significant difference from the absence of ionomycin (ANOVA).

increase the DCFDA fluorescence, which was similar in the presence (15.57 ± 0.38 a.u., $n = 4$) and in the absence (17.57 ± 0.99 a.u., $n = 4$) of lapatinib.

Fig. 6. Effect of the Ca^{2+} ionophore ionomycin on forward scatter in the absence and presence of lapatinib. (A, B) Original histogram of erythrocyte forward scatter following exposure for 1 h to Ringer solution without (grey area) and with (black line) presence of lapatinib (7.5 $\mu\text{g}/\text{ml}$) in the absence (A) and presence (B) of ionomycin (1 μM).

(C) Arithmetic means $\pm$ SEM ($n = 7$) of erythrocyte forward scatter after a 1 h treatment with Ringer solution without (left bars) or with (right bars) ionomycin (1 μM) in the absence (white bars) and presence (black bars) of lapatinib (7.5 $\mu\text{g}/\text{ml}$). * ($p < 0.05$) indicates significant difference from the absence of lapatinib, ### ($p < 0.001$) indicates significant difference from the absence of ionomycin (ANOVA).

Fig. 7. Ca^{2+} sensitivity of lapatinib-induced phosphatidylserine exposure. (A, B) Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of lapatinib (7.5 $\mu\text{g}/\text{ml}$) in the presence (A) and absence (B) of extracellular Ca^{2+} . (C) Arithmetic means $\pm$ SEM ($n = 23$) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) lapatinib (7.5 $\mu\text{g}/\text{ml}$) in the presence (left bars, $+\text{Ca}^{2+}$) and absence (right bars, $-\text{Ca}^{2+}$) of Ca^{2+} . *** ($p < 0.001$) indicates significant difference from the absence of lapatinib (ANOVA).

annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) lapatinib (7.5 $\mu\text{g}/\text{ml}$) in the presence (left bars, $+\text{Ca}^{2+}$) and absence (right bars, $-\text{Ca}^{2+}$) of Ca^{2+} . *** ($p < 0.001$) indicates significant difference from the absence of lapatinib (ANOVA).

Additional experiments addressed the impact of lapatinib on ceramide abundance at the erythrocyte surface. The ceramide abundance was quantified utilizing specific antibodies. As a result, following a 48 hours incubation, the ceramide abundance was similar following

Fig. 8. Ca^{2+} sensitivity of lapatinib-induced erythrocyte shrinkage. (A, B) Original histogram of erythrocyte forward scatter following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of lapatinib (7.5 $\mu\text{g/ml}$) in the presence (A) and absence (B) of extracellular Ca^{2+} . (C) Arithmetic means $\pm$ SEM ($n = 23$) of erythrocyte

forward scatter after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) lapatinib (7.5 $\mu\text{g/ml}$) in the presence (left bars, $+\text{Ca}^{2+}$) and absence (right bars, $-\text{Ca}^{2+}$) of Ca^{2+} . ***($p < 0.001$) indicates significant difference from the absence of lapatinib, ##($p < 0.01$) indicates significant difference from the presence of Ca^{2+} (ANOVA).

incubation with 7.5 $\mu\text{g/ml}$ lapatinib (13.9 ± 0.5 a.u., $n = 4$) and in the absence of lapatinib (14.2 ± 0.3 a.u., $n = 4$).

Discussion

The present observations reveal a novel effect of lapatinib, i.e. the triggering of suicidal erythrocyte death or eryptosis. Exposure of human erythrocytes to lapatinib results in cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. The concentrations required for those effects are well in the range of lapatinib concentrations determined in patients [61]. Thus, the observed effect of lapatinib on eryptosis may well contribute to or even account for its effect on anemia, a major side effect of the drug [9, 11, 25, 26, 61, 62]. The sensitivity to lapatinib may be enhanced by clinical conditions known to enhance the susceptibility to triggers of eryptosis, such as dehydration [68], hyperphosphatemia [69], chronic kidney disease (CKD) [70-73], hemolytic-uremic syndrome [74], diabetes [75], hepatic failure [76], malignancy [63], sepsis [77], sickle-cell disease [63], beta-thalassemia [63], Hb-C and G6PD-deficiency [63], as well as Wilsons disease [78]. Moreover, eryptosis is stimulated by a wide variety of xenobiotics, which could, at least in theory, potentiate the effect of lapatinib on eryptosis [63, 68, 69, 71, 79-102].

Besides its effect on anemia [63], eryptosis fosters adherence of phosphatidylserine exposing erythrocytes to the vascular wall [103], stimulates blood clotting and triggers thrombosis [104-106]. Stimulation of eryptosis may thus impair microcirculation [65, 104, 107-110].

The effect of lapatinib on cell membrane scrambling and cell shrinkage was not due to increase of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), which was actually decreased by lapatinib treatment. Moreover, the effect of lapatinib on cell membrane scrambling was insensitive to removal of extracellular Ca^{2+} . Instead, lapatinib augmented the effect of the Ca^{2+} ionophore ionomycin on cell membrane scrambling, an observation pointing to an effect of lapatinib downstream of $[\text{Ca}^{2+}]_i$.

Removal of extracellular Ca^{2+} slightly but significantly blunted the effect of lapatinib on erythrocyte shrinkage, which may partially depend on activity of Ca^{2+} sensitive K^+ channels accomplishing K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water [64]. Increase of $[\text{Ca}^{2+}]_i$ by the Ca^{2+} ionophore ionomycin was followed by the expected sharp decrease of forward scatter. The ionomycin induced cell shrinkage was slightly, but significantly blunted in the presence of lapatinib, an observation contrasting the shrinking effect of lapatinib in the absence of ionomycin. The present observations do not allow safe conclusions on the underlying mechanism. Possibly, lapatinib compromises Na^+/K^+ ATPase activity, which would cause cellular K^+ loss, decrease cytosolic K^+ concentration and thus blunt the hyperpolarization following activation of Ca^{2+} sensitive K^+ channels.

The mechanism stimulating eryptosis following lapatinib treatment remains illdefined. According to DCFDA fluorescence, lapatinib treatment does not increase reactive oxygen species, a well known stimulator of eryptosis [63]. Moreover, Ca^{2+} entry is apparently not required for the stimulation of eryptosis by lapatinib. The effect of lapatinib is further not dependent on ceramide formation.

In conclusion, lapatinib triggers eryptosis with cell shrinkage and cell membrane scrambling, an effect not requiring increase of cytosolic Ca^{2+} activity.

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Disclosure Statement

The authors declare no conflict of interest.

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