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Modulation of Regorafenib effects on HCC cell lines by epidermal growth factor

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Abstract

Purpose—Blood platelet numbers are correlated to growth and aggressiveness of several tumor types, including hepatocellular carcinoma (HCC). We previously found that platelet lysates (hPLs) also stimulated growth and migration, and antagonized the growth-inhibitory and apoptotic effects of both Sorafenib and Regorafenib, two multikinase inhibitors, on three HCC cell lines. In this study, in vitro function of human epidermal growth factor (EGF) with and without Sorafenib or Regorafenib was investigated.

Methods—An ELISA kit was used to evaluate the EGF concentrations in hPLs. In vitro function of EGF was assessed with proliferation MTT test. Apoptosis assay, scratch assays, and Transwell assays were performed for apoptosis, invasion, and migration, respectively. MAPK Activation Kit was used to explore MAPK phosphorylation.

Results—EGF antagonized the growth inhibition of Regorafenib on three HCC cell lines. Regorafenib-mediated growth inhibition was blocked by 70 % when the cells were pre-treated with EGF. EGF also blocked Regorafenib-induced apoptosis, as well as Regorafenib-induced decreases in cell migration and invasion. The EGF effects were in turn antagonized by concomitant addition to the cultures of EGF receptor antagonist Erlotinib, showing that the EGF receptor was involved in the mechanisms of EGF-mediated blocking of Regorafenib effects. Erlotinib also partially blocked the effects of hPLs in antagonizing Regorafenib-mediated growth inhibition, showing that EGF was an important component of hPL actions.

Conclusions—All these results show that EGF antagonized Regorafenib-mediated growth and migration inhibition and apoptosis induction in HCC cells and reinforce the idea that microenvironment can influence cancer drug actions.

Keywords

Platelets; Epidermal growth factor; Regorafenib; HCC cells

Introduction

Many tumors are associated with thromboembolism and thrombocytosis [1]. Several findings have demonstrated the interaction between blood platelets and the aggressive biology in many human cancers, including ovarian, breast, and colon cancer [2–5]. Platelets have been reported to be involved in liver growth, and their products likely contribute to the development of human hepatocellular carcinoma (HCC) [6, 7]. HCC typically arises on the basis of cirrhosis, and the consequent fibrosis is associated with portal hypertension and splenomegaly that causes thrombocytopenia, recently associated with small-size HCCs [8], whereas large-size HCCs often have normal platelet counts [9]. Very large tumors are often correlated with thrombocytosis [7]. We recently found that human platelet lysates (hPLs) could stimulate the growth of several human HCC cell lines in vitro and decreased their baseline apoptosis levels as well as apoptosis markers [10]. We also showed that hPLs can antagonize the growth-inhibitory actions on human HCC cells mediated by Regorafenib and Sorafenib, both multi-kinase inhibitors, as well as by doxorubicin, a commonly used HCC chemotherapy [11, 12].

Platelets are the source of multiple growth factors, cytokines and inflammatory mediators, including epidermal growth factor (EGF), insulin-like growth factor I (IGF-I), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and serotonin [13]. The modulation of these factors has been shown to alter cancer therapy sensitivity or resistance [14–20]. Our preliminary results showed that EGF and to a lesser extent IGF-I were able to antagonize Sorafenib actions in a proliferation assay [11]. EGF has been implicated in the progression of cirrhosis and development of HCC, although the details of the mechanisms remain to be worked out [21]. We report here an examination of likely mechanisms involved in EGF-mediated antagonism of Regorafenib-mediated growth inhibition and provide evidence that EGF effects may explain some platelet lysate growth effects.

Materials and methods

Cells and drugs

Regorafenib and Sorafenib were gifts from the Bayer Corp (West Haven, CT, USA). Recombinant human EGF was purchased from Pepro-Tech (Rocky Hill, NJ, USA). Erlotinib HCl and GSK1838705A were purchased from Selleckchem (Houston, TX, USA). Hep3B, HepG2, and PLC/PRF/5 human HCC cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The culture medium was Dulbecco's modified eagle's medium (DMEM). All cell culture components were purchased from Sigma-Aldrich (Milan, Italy).

Cell culture

Cells were cultured in DMEM in monolayer culture, supplemented with 10 % fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin, and incubated at 37 °C in a humidified atmosphere containing 5 % CO₂ in air.

EGF concentration in platelet lysates

Human platelet lysates (hPLs), obtained as previously described [11], was used for the in vitro quantitative determination of human EGF by the Human EGF ELISA kit (Invitrogen Corporation, Camarillo, CA, USA). Serial dilutions of hPL or FBS, used as control, were prepared and processed according to the manufactures' instruction.

Growth assay

The cells were cultured in 1 % FBS medium containing EGF 2 ng/ml, and the concentration was derived from the EGF ELISA dosage in hPLs, corresponding to 3.75×10^7 platelets/ml, or equivalent percentage of FBS, in the presence of 1 µM (HepG2 cell line) or 5 µM (Hep3B and PLC/RFP/5) of Regorafenib. In the same growth condition, HCC cell lines were cultured in the presence of EGFR inhibitor Erlotinib 1.25 µM or IGFR inhibitor GSK1838705A 1 µM. After a defined time of incubation, the proliferative response was estimated by colorimetric 3-(4,5 di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. The trypan blue exclusion assay was used to evaluate cell viability. Each experiment was performed in triplicate and repeated three times.

Apoptosis assay

Annexin V—The Muse Annexin V/Dead Cell Assay Kit (Millipore, Darmstadt, Germany) for quantitative analysis of live, early/late apoptotic and dead cells was used with a Muse Cell Analyzer (Millipore). Briefly, the assay utilizes Annexin V to detect PS on the external membrane of apoptotic cells. A dead cell marker (7-AAD) is also used. HepG2 cell line, including positive and negative controls, was cultured in 1 % FBS medium in the presence of Regorafenib 1 µM alone (control cells) or in combination with EGF 2 ng/ml or EGF 2 ng/ml and Erlotinib 1.25 µM for 48 h. The cells were then processed as described in the user's guide.

Caspase-3/7 quantitative measurements—The Muse Caspase-3/7 kit (Millipore) permits simultaneous evaluation of apoptotic status based on Caspase-3 and Caspase-7 activation and cellular plasma membrane permeabilization (cell death). The assay provides relative percentage of cells that are live, early/late apoptotic, or dead. Cells were cultured as described above and processed according to the manufacture's instruction.

Migration assay

A scratch assay was performed as previously described [22, 23]. Briefly, a wound was generated with a pipette tip. After rinsing, medium containing EGF 2 ng/ml or 1 % FBS (control) alone or in combination with Regorafenib 1 µM and/or Erlotinib 1.25 µM. Photographs were taken of each well immediately (T0) and after 24 h (T1), 48 h (T2), and 72 h (T3). The values were expressed as percentage of migration, with 100 % being when

the wound was completely closed. The results were representative of three independent experiments.

Invasion assay

Cell invasion assays were performed using Matrigel (BD Transduction, San Jose, CA, USA)-coated Transwells (8 μ m pore PET membrane, Millipore, Billerica, MA, USA) as previously described [23]. Briefly, Regorafenib 1 μ M and/or Erlotinib 1.25 μ M-treated cells were suspended in low serum medium. Medium containing EGF 2 ng/ml or FBS was added to the bottom wells. After incubating for 24 h, the invading cells were fixed and stained. The images were acquired and analyzed counting the cells with ImageJ Software (National Institute of Health, USA). Values obtained were expressed as percentage of invading cells, setting the cell counts of control cells as 100 %. Results were representative of three independent experiments.

EGFR Activation

The Muse EGFR-RTK Activation Dual Detection Kit (Millipore, Darmstadt, Germany), including a phospho-specific anti-phospho-EGFR (Tyr1173)-Alexa Fluor555 and an anti-EGFR-PECy5-conjugated antibody, was used to measure total level of EGFR expression. This two-color kit is designed to detect the extent of EGFR pathway activation by measuring EGFR phosphorylation relative to the total EGFR expression in the cell population processed as described in the user's guide. HepG2 cell line was cultured in 1 % FBS medium in the presence of Regorafenib 1 μ M and EGF 2 ng/ml alone or in combination for 15, 60, and 180 min. Results were representative of three independent experiments.

MAPK activation

The Muse MAPK Activation Dual Detection Kit (Millipore, Darmstadt, Germany), including a phospho-specific anti-phospho-ERK1/2 (Thr202/Tyr204, Thr185/Tyr187)-Phycoerythrin and an anti-ERK1/2-PECy5-conjugated antibody, was used to measure total levels of ERK. This two-color kit is designed to measure the extent of MAPK phosphorylation relative to the total MAPK expression in the cell population processed as described in the user's guide. HepG2 cell line was cultured in 1 % FBS medium in the presence of Regorafenib 1 μ M and EGF 2 ng/ml alone or in combination for 10, 20, and 40 min. Results were representative of three independent experiments.

Statistical analysis

GraphPad Prism 5.0 software (La Jolla, CA, USA) was used for all statistical analysis. Mann–Whitney nonparametric test was employed to assess the statistical significance of differences between two groups. For multiple comparisons, oneway ANOVA test was used followed by appropriate posttest. $P < 0.05$ was considered statistically significant. All experiments were done in triplicate, and data are presented as mean $\pm$ standard deviation (SD).

Results

Antagonism by EGF of Regorafenib-mediated inhibition of HCC cell growth

hPLs were previously examined for their ability to antagonize Regorafenib-mediated inhibition of human HCC cell line growth [10]. Preliminary data revealed that EGF and, to some extent, IGF-I could antagonize Sorafenib in a proliferation assay [11]. To further investigate the role of EGF in counteracting Regorafenib-mediated inhibition of HCC cell growth, the amounts of this mitogen were measured in hPL as described in methods. The results indicated that 1.7 ± 0.3 ng/ml of EGF was present in hPL corresponding to 3.75×10^7 platelets/ml. This EGF concentration range was used in all the subsequent experiments.

Hep3B, PLC/PRF/5, and HepG2 human HCC cells were treated in log phase growth in culture dishes with Regorafenib 1–5 μ M or EGF 2 ng/ml alone or in combination, with appropriate solvent controls, and proliferation was evaluated by MTT assay. We found that EGF significantly antagonized the growth-inhibitory actions of Regorafenib. This effect was blocked by Erlotinib, a potent inhibitor of the HER1/EGFR autophosphorylation, used at a nontoxic concentration (1.25 μ M) that did not affect the proliferation by itself. GSK1838705A, known to inhibit IGF-1 receptor, had not effects on EGF action (Fig. 1a).

We next investigate whether the timing of the EGF addition to the cell cultures might affect Regorafenib-mediated growth inhibition. Two different culture conditions were used: In the first condition, cells that had been pre-treated for 24 or 48 h with Regorafenib were subsequently cultured for the next 24 or 48 h, respectively, in the presence of EGF 2 ng/ml or equivalent percentage of FBS (controls). In the second condition, cells that had been previously cultured for 24 or 48 h with EGF were then treated with Regorafenib for the next 24 or 48 h, respectively. We found that in the first culture condition, the Regorafenib-mediated inhibition of cell growth was only partially rescued by subsequent addition of EGF. In the second culture condition, the Regorafenib-mediated growth inhibition was blocked by 40 % when the cells received EGF pre-treatment for the first 24 h (Fig. 1b).

The antagonism exerted by EGF on Regorafenib-mediated growth-inhibitory actions was also observed on cell cycle progression. Regorafenib caused an inhibition in the progression from S phase of the cell cycle to G2/M phase. As shown in Fig. 1c, after 6 h (T1) from block release, Regorafenib-treated cells in G2/M phase were similar to the control cells at T0, while the number of control cells that proceeded through the cell cycle doubled with respect to the number of cells at T0. The Regorafenib effect was partially blocked by the addition of EGF, but not when EGF and Erlotinib were added in combination.

Antagonism by EGF of Regorafenib-mediated induction of apoptosis

The effects of EGF on Regorafenib-mediated apoptosis, a major factor in its growth-inhibitory actions, were then examined. Regorafenib induced both an increase in cellular Annexin V and activation of Caspase-3/7, two different apoptosis markers. When EGF was also added to the cell medium together with Regorafenib a significant inhibition in apoptosis induction was found. This effect was abrogated by the EGFR inhibitor Erlotinib 1.25 μ M (Fig. 2).

Antagonism by EGF of Regorafenib-mediated inhibition of cell migration and invasion

Regorafenib inhibits both HCC cell migration and invasion through Matrigel membranes [23]. Furthermore, EGF has been shown to stimulate cell motility [24, 25]. We therefore added EGF 2 ng/ml to Regorafenib 1 μ M, a concentration that can inhibit both migration and invasion in HCC cells. We found that EGF antagonized the inhibition by Regorafenib of both migration and invasion, and this effect was abrogated by Erlotinib 1.25 μ M (Fig. 3, panel a–c).

EGF as a major contributor to hPL actions

Our results using Erlotinib showed that EGF receptors were involved in the mechanisms of EGF-mediated blocking of Regorafenib effects. HepG2 cells were treated with Regorafenib 1 μ M or Erlotinib 1.25 μ M alone or in combination, and proliferation was evaluated by MTT assay. We found that the growth inhibition observed with both drugs was significantly higher when they were used in combination (Fig. 4a). In addition, we saw that Erlotinib 1.25 μ M also partially blocked the effects of hPLs in antagonizing Regorafenib-mediated cell growth inhibition (Fig. 4b).

EGF effects on EGFR and MAPK activation

To elucidate the relationship between the Regorafenib and EGF actions, we investigate the extent of EGFR pathway activation, by measuring EGFR phosphorylation relative to the total EGFR expression in the cell population processed using the Muse EGFR-RTK Activation Dual Detection Kit. Cells were cultured in 1 % FBS medium in the presence of Regorafenib 1 μ M or EGF 2 ng/ml alone or in combination for 15, 60, and 180 min. The percentage of cells with activated EGFR was significantly increased in EGF-treated cells after 15 min of treatment. Furthermore, EGF-mediated activation was minimally affected by the presence of Regorafenib (Fig. 5a).

We next examined the phosphorylation of ERK1/2, which is a key step in the MAPK signaling cascade and known to regulate a number of cellular processes, including proliferation, survival, and apoptosis, and is involved in both Regorafenib and EGF actions. Cells were cultured in 1 % FBS medium in the presence of Regorafenib 1 μ M or EGF 2 ng/ml alone or in combination for 10, 20, and 40 min and analyzed with the Muse MAPK Activation Dual Detection Kit. The percentage of cells with activated ERK was significantly increased after EGF treatment and decreased after Regorafenib treatment (Fig. 5b). This effect was almost completely blocked by simultaneous addition of EGF and Regorafenib, showing that EGF and Regorafenib interactions are downstream of the EGFR.

Discussion

Large HCCs, unlike small HCCs, are often associated with thrombocytosis [7–9]. There are many reports of an increased number of platelets occurring in the blood of patients with a variety of malignancies and likely contributing to thromboembolism, which is considered a negative prognostic factor [1–7].

The presence of many mitogens in platelets is thought to be important in stimulating the progression and development of HCC and in enhancing several biological tumor processes including growth, migration, and tumor invasiveness [26–29]. Included among the mitogens are EGF, IGFI, FGF, PDGF, VEGF, and serotonin, several of which may also contribute to chemotherapy drug resistance [13–20, 30–32].

We previously showed that hPL stimulates the growth of several human HCC cell lines in vitro and induces the levels of several kinases involved in cell proliferation, such as ERK, Akt, JNK, and its targets p38 and STAT3, as well as inhibiting apoptotic processes [10]. We also observed that hPLs can antagonize the growth-inhibitory effects of both Regorafenib and Sorafenib, multikinase inhibitors, and by doxorubicin, a commonly used HCC chemotherapy [11, 12], on several human HCC cell lines. Our preliminary data also showed that EGF and IGF-I were able to antagonize Sorafenib in a proliferation assay [11], and several studies provide evidence that EGF plays an initial role in the development of cancer inflammation, promoting enhanced metastatic potential [33]. In HCC, EGF pathways are associated with progression of cirrhosis to mortality and also have a role in hepatocellular transformation [21]. However, Regorafenib, a close analog of Sorafenib, is currently used in clinical trials to assess the safety and efficacy of this agent in patients with HCC (and was recently FDA-approved for colorectal cancer). Molecules affecting signaling pathways that cross talk with those involved in Regorafenib action have the potential for use in the combination therapies [23, 34, 35]. The observations included in the present work point to the importance of the simultaneous control of molecular pathways involved in both Regorafenib and EGF activity in the treatment of HCC.

We found that EGF, used in the same concentration as was measured in hPLs, significantly antagonized the growth-inhibitory actions of Regorafenib in Hep3B, PLC/PRF/5, and HepG2 human HCC cells. This effect was blocked by Erlotinib, a potent inhibitor of HER1/EGFR autophosphorylation. Erlotinib was used at nontoxic concentrations that do not affect proliferation. The inhibition exerted by Erlotinib was likely EGFR-specific, since other growth factor inhibitors such as GSK1838705A, known to inhibit IGF-1 receptor, did not counteract EGF action.

We also observed that the Regorafenib pre-treatment could modify the stimulatory action of the EGF. Regorafenib-mediated inhibition of cell growth was only partially rescued by EGF post-treatment, showing that the Regorafenib treatment, even if reversible, modified the cells [35]. However, EGF pre-treatment protected the cells from Regorafenib-mediated growth inhibition, which was reduced by 40 %, suggesting that EGF signaling is implicated in the observed Regorafenib resistance or that a major mechanism of Regorafenib-mediated growth control is through interference in the EGF pathway, which is then counteracted by the addition of EGF. A corollary is that EGFR inhibitors might enhance the growth-inhibitory actions of Regorafenib. Our results on cell cycle progression support the idea of antagonism exerted by EGF on Regorafenib-mediated growth inhibition. Erlotinib reversed the EGF actions, showing that the antagonistic effects on Regorafenib growth inhibition are exerted through the EGFR. The antagonism exerted by EGF on Regorafenib effect was shown for drug-mediated apoptosis, as a decrease in Annexin V levels and in the Caspase-3/7 activation.

Recent findings suggest that EGF/EGFR signaling pathway plays a crucial role in mechanisms of HCC cell movement. EGF could increase cell motility through EGFR binding and PI3 K and ERK signaling pathway activation, transforming cells from low-to-high metastatic capacity [33]. Our data showed that this stimulatory effect of EGF was still evident in HCC cells treated with Regorafenib, which is a potent inhibitor of cell motility, even at low concentrations [23].

The cell growth experiments reported here showed that Erlotinib consistently abrogated the effects of hPL in antagonizing Regorafenib-mediated growth inhibition, thus indicating that EGF is one of the major components of hPL in these phenomena.

The cross talk between the Regorafenib and EGF was initially investigated by measuring EGFR phosphorylation in the treated cell population. Since EGF-mediated activation was minimally affected by the presence of Regorafenib, the downstream signaling molecules were then explored by measuring the phosphorylation of ERK1/2. The percentage of cells with activated ERK significantly decreased in Regorafenib-treated cells, and this effect was partially counteracted by concomitant presence in the culture medium of EGF. These results suggest that the efficacy of Regorafenib will be likely increased when EGFR is inhibited, as was demonstrated using Erlotinib and culturing the HCC cells in normal serum conditions (10 % FBS). A combination of Erlotinib and Regorafenib will thus likely result in greater HCC cell growth inhibition, probably through enhanced inhibition of RAF kinases. A combination therapy in which EGFR inhibitor is combined with Regorafenib might thus be potentially useful in HCC therapy. These experiments also emphasize the importance of tumor microenvironment, not only for tumor growth, but also in the modulation of drug actions. Many cell types are part of the tumor microenvironment, including platelets, and their particles contain multiple growth and inflammation-modifying factors. These considerations add another layer of complexity in evaluating and modifying drug action. In the example of Regorafenib, this could be effected either in combination with inhibitors of EGF, EGFR or its downstream pathway, or via antagonists of platelet numbers or activity (aspirin) in patients who are not already thrombocytopenic.

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Fig. 1.

Antagonism by EGF of Regorafenib-mediated growth inhibition of HCC cell lines. **a** Hep3B, PLC/PRF/5, and HepG2 cells were cultured in the presence of Regorafenib 1–5 μ M, EGF 2 ng/ml, Erlotinib 1.25 μ M, and GSK 1 μ M using the conditions indicated in the graphs. MTT assay was assessed after 48 h. **b** HepG2 cells were cultured in the presence of Regorafenib 1 μ M and EGF 2 ng/ml in different time conditions. In the first group (*dark gray*), the cells, previously treated with Regorafenib for 24 or 48 h, received EGF for the following 24 or 48 h. In the second group (*light gray*), the cells previously treated with EGF for 48 or 24 h received Regorafenib for the following 24 or 48 h. MTT assay was assessed after 72 h. **c** HepG2 cells were synchronized in the S phase of the cell cycle using thymidine 0.2 M (T0), and after 6 h from block release (T1), the cells were processed with the cell cycle kit and analyzed with Muse Cell Analyzer to evaluate the percentage of cells in G0/G1, S, and G2/M phases. The *panels* represent an example of DNA content profile in different treatment conditions. The mean of three independent experiments was plotted in the relative graph, where the values are calculated as fold increase in the cells in G2/M at T1 with respect to control cells at T0. Data are presented as mean $\pm$ SD of three independent experiments. Differences between two groups were determined by Mann–Whitney nonparametric test (**a** and **b**). Multiple comparisons were performed by one-way ANOVA test and Dunn's multiple comparison posttest (**c**). * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$

Fig. 2.

Antagonism by EGF of multikinase inhibitor-mediated induction of apoptosis. HepG2 cell line cultured in 1 % FBS medium was treated with 2 ng/ml EGF alone or in combination with 1 μ M Regorafenib (**b** and **c**) or Sorafenib (**b**) and 1.25 μ M Erlotinib. The Muse Annexin V kit (upper panels) or Caspase-3/7 kit (*lower panel*) was used to evaluate the percentage of apoptotic cells. The mean $\pm$ SD of three independent experiments is plotted in the relative graph. Data are presented as mean $\pm$ SD of three independent experiments. Multiple comparisons were performed by one-way ANOVA test and Dunn's multiple comparison posttest (**a**, **b**, and **c**). ** $P < 0.001$; *** $P < 0.0001$

Fig. 3.

Antagonism by EGF of regorafenib-mediated inhibition of migration and invasion. HepG2 cell line cultured in 1 % FBS medium was treated with 2 ng/ml EGF alone or in combination with 1 μ M Regorafenib and/or 1.25 μ M Erlotinib. **a** Migration assay was performed as described in Methods section, and the microscopic analysis was assessed at the time of the scratch (T0) and after 48 h (T2). The values were expressed as percentage of migration, where 100 % represents the scratch completely closed. **b** The percentage of invasion was calculated comparing the invading drug-treated cells to drug-untreated control cells (100 %). Data are presented as mean $\pm$ SD of three independent experiments. Differences between two groups were determined by Mann–Whitney nonparametric test (**a** and **b**). *** $P < 0.0001$

Fig. 4.

Effects of EGFR inhibitor in counteracting the antagonistic effect of platelets lysate and serum on Regorafenib-mediated growth inhibition in HepG2 cells. HepG2 cells were cultured in 10 % FBS medium (**a**) or in 1 % FBS medium in the presence of EGF 2 ng/ml, platelet lysate corresponding to 3.75×10^7 platelets/ml (**b**), in both experiments were added Regorafenib 1 μ M and Erlotinib 1.25 μ M, using the conditions indicated in the graph. MTT assay was assessed after 48 and 72 h, respectively. Data are presented as mean $\pm$ SD of three independent experiments. Multiple comparisons were performed by one-way ANOVA test and Dunn's multiple comparison posttest (**a**). Differences between two groups were determined by Mann–Whitney nonparametric test (**b**). ** $P < 0.001$; *** $P < 0.0001$

Fig. 5. EGF effects on EGFR and MAPK activation. HepG2 cells were cultured in the presence of Regorafenib 1 μ M and EGF 2 ng/ml alone or in combination. **a** The Muse EGFR-RTK Activation Kit was used to evaluate the extent of EGFR pathway activation after 15 min. The percentage of activated and inactivated cells are indicated in the graph. **b** The Muse MAPK Activation Kit was used to evaluate the MAPK phosphorylation relative to the total MAPK expression after 10 min. Data are presented as mean $\pm$ SD of three independent experiments. One-way ANOVA test and Dunn's multiple comparison post-test were employed (**a** and **b**). * P < 0.05; ** P < 0.001; *** P < 0.0001