Biochemical Pharmacology 98 (2015) 639–648

Contents lists available at ScienceDirect

Biochemical Pharmacology

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

Inhibition of epithelial growth factor receptor can play an important role in reducing cell growth and survival in adrenocortical tumors

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ARTICLE INFO

Article history:
Received 17 September 2015
Accepted 15 October 2015
Available online 17 October 2015

Keywords:
EGFR
ACC
Sunitinib
Erlotinib

ABSTRACT

Medical treatment of adrenocortical carcinoma (ACC) is still far from optimal, since even molecular targeted therapy failed to demonstrate striking results. Clinical trials enrolling ACC patients with high tissue vascular endothelial growth factor receptor (VEGFR) expression levels showed controversial results after treatment with Sunitinib, possibly due to variability in the expression of drug targets, which include epidermal growth factor receptor (EGFR). To better clarify this issue, we evaluated whether VEGFR may play a crucial role in ACC responsiveness to Sunitinib and whether EGFR may represent an alternative target in ACC medical treatment, by employing two ACC cell lines, the NCI-H295 and SW13 cells lines, and adrenocortical tissues primary cultures. Our data show that VEGF/VEGFR system may not be crucial in modulating ACC proliferation and responsiveness to Sunitinib. In addition, by cell viability, proliferation and caspase activation assays we found that Sunitinib inhibits adrenocortical cell viability acting, at least in part, through EGFR, that, in turn, is crucial for EGF proliferative effect on adrenocortical cells. The latter depends, at least in part, on ERK 1/2 activation. An EGFR selective inhibitor was highly effective in reducing cell viability in an adrenocortical tumor primary culture and in the SW13 cells, which express high EGFR levels. Our results suggest that EGFR inhibitors could represent effective therapeutic tools in ACC patients whose tumors express high EGFR levels, that, in turn, may be considered a predictive factor of response. Accurate molecular tumor profiling is crucial to predict drug efficacy and to tailor ACC patients therapeutic approach.

1. Introduction

Adrenocortical carcinoma (ACC) is a rare malignancy with a general poor prognosis, despite extensive surgical approaches are employed. In addition, the disease is often metastatic at diagnosis and relapses very frequently, with consequent very low survival rates at 5 years [1,2]. The high ACC recurrence rates have prompted the use of adjuvant therapy by treatment with mitotane, an adrenolytic drug, that since late 60s has been employed in ACC medical treatment, demonstrating a significant increase in recurrence free survival rates [3]. Multiple cytotoxic approaches have also been attempted, with limited results when used alone [2]. On the other hand, the combination of three chemotherapeutic agents (etoposide, doxorubicin and cisplatin) with mitotane provided important results on clinical grounds, with a significant improvement in 5-year survival rates [4]. However, this drug regimen has a high impact on patients' quality of life, with a high burden in terms of side effects [2]. Therefore, it is mandatory to characterize novel factors regulating adrenocortical cell proliferation and transformation leading to the employment of new therapeutic agents capable of inhibiting tumor growth.

Sunitinib is a receptor tyrosine kinase (RTK) inhibitor that targets multiple receptors, including vascular endothelial growth factor receptor (VEGFR) 1 and 2, as well as epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor and

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http://dx.doi.org/10.1016/j.bcp.2015.10.012
0006-2952/

others [5]. Sunitinib has been employed in clinical studies involving ACC [6], where patients were selected for treatment on the basis of high VEGFR expression levels [7–10]. The results of these studies are not clear-cut, since some patients displayed stable disease under treatment with Sunitinib, while others showed progressive disease. Therefore, treatment failure may be due to variability in drug target expression, indicating the need to better characterize the role of Sunitinib targets in ACC treatment [6]. Among others, EGFR plays a pivotal role in neoplastic transformation [11] and is over-expressed in the majority of ACC [12,13], suggesting a possible role for EGFR pathway in regulating adrenocortical cell proliferation and transformation.

The aim of our study is to clarify whether VEGFR plays a crucial role in ACC responsiveness to Sunitinib and whether EGFR may represent an alternative target in ACC medical treatment.

2. Material and methods

2.1. Compounds

Sunitinib malate was kindly provided by Pfizer Inc. (New York, NY, USA). Erlotinib and SCH772984 were purchased from Selleckchem (Boston, MA, USA). All other reagents, including EGF and VEGF, if not otherwise specified, were purchased from Sigma.

2.2. Cell culture and tissues

The NCI-H295 and the SW-13 human ACC cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). NCI-H295 and SW-13 cells were maintained as previously described [14,15]. Primary cultures from human adrenocortical tissues were obtained as previously described, with minor modifications [16]. Briefly, tissue samples were collected in accordance with the guidelines of the local committee on human research, obtained under sterile conditions, and immediately minced in RPMI 1640 medium. Tissues were dissociated using 0.35% collagenase and 1% trypsin at 37 °C for 60 min. Cell suspensions were washed twice with serum-free RPMI (Euroclone Ltd., Wetherby, UK), and then passed through 18 gauge and then 20 gauge syringe needles. Tumor cells were resuspended in F-12 medium with 10% FBS and antibiotics (Euroclone Ltd., Wetherby, UK), seeded in 96-well culture plates (2×10^4 cells/well) and incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air, as previously described [17]. After ~18 h, cells were treated with test substances, with further evaluation of cell viability and/or caspase activity.

2.3. Viable cell number assessment

Variations in viable cell number were assessed by the ATPlite kit (PerkinElmer Life Sciences, MA, USA), seeding 2×10^4 cells/well in 96-well white plates, as previously described [18], and treated with the indicated compounds for 120 h. Control cells were treated with vehicle alone (0.1% DMSO). After incubation, the revealing solution was added and the luminescent output (relative luminescence units, RLU) was recorded using the Envision Multilabel Reader (PerkinElmer, Monza, Italy). Results are expressed as mean value ± standard error of the mean (SEM) percent relative light units (RLU) vs. vehicle-treated control cells from 3 independent experiments in 6 replicates.

2.4. Caspase activity evaluation

Caspase activity was measured using Caspase-Glo 3/7 assay (Promega, Milano, Italy), as previously described [19]. Results are expressed as mean value ± SEM percent RLU vs. vehicle-treated control cells from three independent experiments in six replicates.

2.5. Measurement of DNA synthesis

DNA synthesis was assed by evaluating the incorporation of 5-bromo-2-deoxyuridine (BrdU) into DNA using the BrdU Cell Proliferation Assay kit (Cell Signaling Technology, Danvers, MA) as previously described [20].

2.6. Western blot analysis

For immunoblotting, cells and tissues were dissolved in RIPA Buffer (Thermolab Inc., Waltham, MA, USA), as previously described [18]. Protein concentration was measured by BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA), as previously described [21]. Proteins were fractionated on 7.5% SDS-PAGE, as previously described [22], and transferred by electrophoresis to Nitrocellulose Transfer Membrane (PROTRAN, Dassel, Germany). Membranes were incubated with anti-p44/42 MAPK (ERK 1/2), anti-phospho-p44/42 MAPK (ERK 1/2) (Thr202/Thy 204), anti-EGFR, (Cell Signaling, Danvers, MA, USA). Horseradish peroxidase-conjugated antibody IgG (Dako, Cernusco sul Naviglio, Milan, Italy) was used to detect immunoreactive bands and binding was revealed using enhanced chemiluminescence (Pierce, Rockford, IL, USA). The blots were then stripped and used for further blotting with anti-GAPDH antibody (Cell Signaling, Danvers, MA, USA). Quantification of the bands was performed by the Quantity One software (Bio-Rad, Milano, Italy).

2.7. Kinase activity assay

Phosphorylated levels of EGFR (Tyr1068) were measured using AlphaScreen SureFire assays (PerkinElmer). Briefly, cells were seeded at 2×10^4 cells/well in 96-well plates and, after overnight attachment, were incubated for 1 h with or without Sunitinib;

Table 1
PCR primers and conditions for EGFR exons 18–21 amplification.

Exon	Primers	Amplicon	Cycles	Denaturation	Annealing	Extension
18	For: 5′-GTGAGGGCTGAGGTGACCCTTGTCTC-3′	368 bp	35	94 °C	58 °C	72 °C
	Rev: 5′-CAGTGGTCCTGTGAGACCAA-3′			30″	1′	30″
19	For: 5′-GCAGCATGTGGCACCATCTCACAATTGCC-3′	265 bp	35	94 °C	57 °C	72 °C
	Rev: 5′-TCTAGACCCTGCTCATCTCCACATCC-3′			30″	1′	30″
20	For: 5′-GTCTTCACCTGGAAGGGGT-3′	314 bp	35	94 °C	56 °C	72 °C
	Rev: 5′-GGAGGGGAGATAAGGAGCCAGGA-3′			30″	1′	30″
21	For: 5′-CTCAGA GCCTGGCATGAA-3′	255 bp	35	94 °C	56 °C	72 °C
	Rev: 5′-GGCAAAGTAAGGAGGTGGCT-3′			30″	1′	30″