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Chapter X

The Role of Membrane Transporters in Cellular Resistance of Pancreatic Carcinoma to Gemcitabine and Erlotinib

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Abstract

Background: Pancreatic cancer remains an important health problem and belongs to one of the most difficult conditions to treat. The etiology and molecular pathogenesis of the disease is still weakly understood. Gemcitabine is a cytotoxic nucleoside analog, which is nowadays the standard chemotherapeutic drug for patients with advanced pancreatic cancer. Inter-individual differences in gemcitabine pharmacokinetics and pharmacodynamics have been demonstrated. Resistance of tumor cells against the nucleoside analogs limits their clinical use and it may seriously contribute to the fact that 5-years survival of the pancreatic carcinoma is about 5%. The first results of targeted therapies have shown promising results in various cancers. However, until now just erlotinib (an epidermal growth-factor receptor tyrosine kinase inhibitor) has demonstrated modest survival benefit in phase III clinical trial on pancreatic cancer when combined with gemcitabine. Despite this fact, its clinical significance has been criticized and its cost-effectiveness is low.

Aim: This review provides survey of available data concerning the molecular factors contributing to the resistance of pancreatic carcinoma against gemcitabine and erlotinib. Mechanisms of transport of these drugs into and outside of the tumor cells and perspectives of their use in personalized medicine will also be discussed.

Introduction

Pancreatic carcinoma (ICD-10: C.25; OMIM: 260350) is one of the most severe forms of malignant disease with high mortality. It is the fourth leading cause of cancer-related deaths, with only about 5% of patients surviving by 5 years. This figure has remained relatively unchanged over the past 25 years [1]. The etiology and molecular pathogenesis of the disease is still weakly understood. Known risk factors for the disease include age, sex, late-onset diabetes, chronic or hereditary pancreatitis, familial cancer syndromes, cigarette smoking, dietary habits and infection by *Helicobacter pylori* [2,3]. Furthermore, low-penetrance genes, e.g. those coding xenobiotic-metabolizing enzymes, DNA repair and cell-cycle regulating machinery may be relevant factors modifying an individual susceptibility [4, 5].

The prognosis of patients with pancreatic cancer remains very poor. Operative resection is the only therapeutic option with curative potential, but just 10-15% of patients have potentially operable tumors, and many of them experience recurrence of the disease despite the radical surgery [6]. Although many studies have evaluated the prognostic factors of pancreatic cancer, their results are mostly inconclusive. The prognosis of operable pancreatic cancer is dependent rather on tumor-related factors, while the prognosis of patients with more advanced pancreatic cancer is dependent on patient-related factors. The onset of symptoms as abdominal pain, back pain, jaundice, general fatigue and nausea closely correlates with prognosis [7]. Tumor markers such as CEA, CA 19-9, CA 72-4, hCG beta and CA 242, C-reactive protein and albumin have limited prognostic value [8-10]. Multivariate analysis showed that CA 19-9, poor tumor differentiation, large tumor size, regional lymph node involvement and venous and/or neural invasion were poor prognostic factors for survival in the group of operable patients [11-13]. Interestingly, an extent of blood loss during the surgery and/or a serum albumin level monitored during the first postoperative month seemed to be significant poor prognostic factors as well [13]. The role of positive resection margin is still discussed and remains controversial [14]. The strongest currently recognized prognostic factors are the extent of the spread of the disease and performance status.

Patients who received chemotherapy or chemoradiotherapy showed better survival than those who received only the best supportive care [15]. Several studies have been performed to determine factors that could predict survival time in gemcitabine-treated patients with advanced pancreatic cancer. The chemotherapy has had poor results, mostly because of the advanced stage of the disease and low chemosensitivity of the pancreatic tumor cells. Gemcitabine, a deoxycytidine analogue, is now the standard drug for treatment of the advanced pancreatic cancer [16]. The first results of targeted therapies have shown promising results in various cancers. However, until now just erlotinib (an epidermal growth-factor receptor tyrosine kinase inhibitor) when combined with gemcitabine has demonstrated modest survival benefit in phase III clinical trial on pancreatic cancer [17]. Despite this fact, its clinical significance has been criticized and its cost-effectiveness is low. Resistance developed against anticancer drugs significantly limits their clinical use and influences the overall survival. Inter-individual differences in gemcitabine pharmacokinetics and pharmacodynamics have been demonstrated, which may be due to the variability in cellular transport systems enabling the drug to enter the cancer cell (drug uptake) or pumping the drug outside the cell (drug efflux).

This review provides survey of available data concerning the importance of drug transporting enzymes for the resistance of pancreatic carcinoma against gemcitabine and erlotinib as major anticancer drugs used in pancreatic cancer treatment.

Gemcitabine

Gemcitabine (2´,2´-difluorodeoxycytidine, Gemzar, Eli-Lilly, Indianapolis, IN, Figure 1) belongs to the second generation of nucleoside analogues [18].

A pivotal randomized trial found a significant improvement of the one-year median survival rate in patients with advanced pancreatic cancer treated with gemcitabine over those who were treated with 5-fluorouracil (18 vs. 2%; p=0.0001) [19]. Despite the fact that gemcitabine became the gold standard of first-line therapy for advanced pancreatic cancer [20], the response rate of pancreatic tumors to gemcitabine remains less than 10% [21,22]. Acquired resistance against gemcitabine demonstrated by inter-individual differences in its pharmacokinetics and pharmacodynamics contributes to this fact.

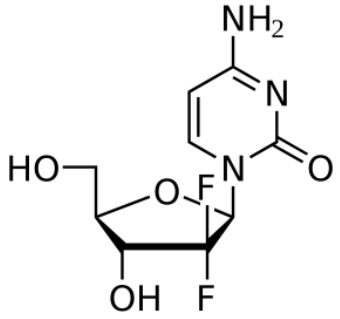

Figure 1. Structure of gemcitabine.

Drug resistance may be attributed to different, unrelated mechanisms, including abnormal membrane transport, ineffective metabolic drug conversion, enhanced metabolite inactivation, increased DNA repair and alterations in the apoptotic pathways (Figure 2, [23,24]).

Gemcitabine is transported into the cell via nucleoside (SLC) transporters, whereas several members of the ATP-binding cassette (ABC) transporter superfamily cause its efflux. Gemcitabine (dFdC) is phosphorylated to its monophosphate mainly by deoxycytidine kinase (DCK, OMIM:125450). It may also be phosphorylated by thymidine kinase 2 (TK2, OMIM:188250) but the substrate specificity of TK2 for gemcitabine is, just 5–10% of that for deoxycytidine [25]. Gemcitabine monophosphate is then converted to diphosphate and triphosphate (dFdCTP) responsible for the major cytostatic effect [26,27]. The gemcitabine-triphosphate incorporates into DNA and causes chain termination [28] and inhibits CTP synthetase (CTPS, OMIM:123860) and dCMP deaminase (DCTD, OMIM:607638) [24]. Gemcitabine is also known to incorporate to RNA [29]. The active gemcitabine-diphosphate also inhibits DNA synthesis indirectly through the inhibition of ribonucleotide reductase (subunits RRM1 and RRM2, OMIM:180410 and 180390). This inhibition causes a decrease of competing nucleoside pools as the RRM is an unique enzyme converting ribonucleotides to deoxyribonucleotides required for DNA polymerization and repair [30,31]. Gemcitabine is

inactivated mainly by cytidine deaminase (CDA, OMIM:123920) mediated conversion to difluorodeoxyuridine (dFdU) and partly by DCTD to difluorodeoxyuridine-monophosphate [24].

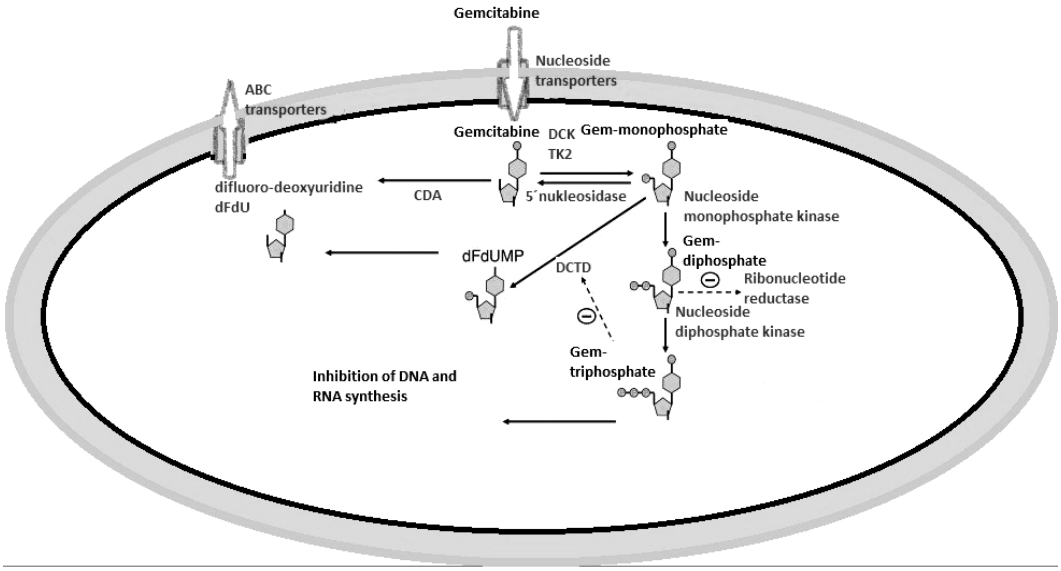

Figure 2. Cellular metabolism and mechanism of action of gemcitabine.

Research addressing the pharmacogenomics of gemcitabine so far focused mainly at inter-individual differences in gemcitabine pharmakokinetics and variation in genes coding transmembrane proteins such as ABC and SLC transporters and gemcitabine-metabolizing enzymes such as DCK, RRM, DCTD, CDA [32-37].

ATP-Binding Cassette (ABC) Transporters

One of the most important mechanisms of the drug resistance is low accumulation of the drug in cancer cells caused by increased efflux. The efflux is performed mainly by transmembrane ABC transporters.

The human family of ABC transporters has 49 members divided into 7 subfamilies (named ABCA - ABCG based on sequence similarities, ref. 38). Fourteen ABC transporters which may be divided into two groups have been shown to determine drug resistance in cancer cells (Table 1). First group contains proteins causing so called “multi drug resistance” (MDR) phenotype in both cell lines and patients with various types of cancers. ABCB1 (or MDR1, OMIM:171050), ABCC1 (MRP1, OMIM:158343) and ABCG2 (BCRP, OMIM: 603756) belong to this group. In the second group of transporters (i.e. ABCA2, OMIM: 600047; ABCB4, OMIM:171060; ABCB11, OMIM:603201; ABCC2, OMIM:601107; ABCC3, OMIM:604323, ABCC4, OMIM:605250; ABCC5, OMIM:605251;ABCC6, OMIM:603234; ABCC10, OMIM:612509; and ABCC11, OMIM:607040; ABCC12, OMIM:607041) the ability to confer drug resistance has been demonstrated just in limited number of experiments [39].

ABCA2 is highly expressed in the cells of the nervous and hematopoetic systems and is associated with lipid transport and drug resistance in cancer cells. Recently, a single nucleotide polymorphism (SNP) in *ABCA2* was linked to early onset of Alzheimer's disease (reviewed in 40). Data on ABCA2 expression and genetic variability in pancreatic tissue were not published so far.

ABCB1 (P-glycoprotein) is the first human ABC transporter cloned and characterized through its ability to confer a MDR phenotype in cancer cells. ABCB1 substrates are generally hydrophobic drugs with polyaromatic nucleus, such as adriamycin, etoposide or vinblastine [41]. SNPs in *ABCB1* have been shown to influence its phenotype (expression, protein function and drug response) in context with numerous diseases [42-44]. ABCB1 mRNA and P-glycoprotein levels were detected by reverse transcription-polymerase chain reaction (RT-PCR) and by immunohistochemistry (IHC) in 2 pancreatic cancer cell lines SW1990 and CAPAN-1 [45]. Jensen et al. 1997 found higher sensitivity to gemcitabine in small-cell lung cancer (SCLC) cells with overexpressed ABCB1 [46]. Similarly, non-small cell lung cancer (2R160), ovarian (2780ADd), and epidermoid (KB8-5e) cell lines with ABCB1 overexpression were also highly sensitive to gemcitabine [47]. High intensity of IHC staining for P-glycoprotein was observed in 73.2% of pancreatic ductal adenocarcinomas (PDAC, n=103) [48] and the staining inversely correlated with grade, tumor size, and retroperitoneal and portal invasion. *ABCB1* gene expression was significantly higher in pancreatic tumors compared to unaffected tissue and high expression levels seemed to be factor of better prognosis in patients with PDAC. Accordingly, northern blot analysis showed that ABCB1 mRNA levels were increased by 1.4-fold in the pancreatic cancer samples compared with the normal pancreas. Another study found IHC positivity of P-glycoprotein in 75% of pancreatic cancer samples (n=63). Patients with weak to moderate staining had longer overall survival than patients without P-glycoprotein expression. In contrast, strong staining was associated with shorter survival compared with weak to moderate staining. The authors suggested that overexpression of other drug resistance genes (such as ABCB2) could possibly exert a compensatory effect [49].

Thus, the ABCB1 (or P-glycoprotein) expression seems to be a quite common phenomenon in both tumor and adjacent non-tumor tissue and its clinical utility as prognostic or predictive factor remains unclear. However, from so far published promising results of functional and observational studies it seems worth to continue in study of complex relations between tumor phenotype and ABCB1 genotype and protein function.

The ABCC family contains 13 members, 9 of them are the multidrug resistance-associated proteins (ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCC6, ABCC10, ABCC11, ABCC12). ABCC7 (CFTR, OMIM:602421) is a chloride channel associated with cystic fibrosis. ABCC8 and ABCC9 (OMIM:600509 and 601439) are the sulfonylurea receptors and ABCC13 is clearly a pseudogene without transporting activity [50]. *In vitro*, ABCC transporters can collectively confer resistance to natural product-derived anticancer drugs and their conjugated metabolites, platinum compounds, folate antimetabolites, nucleoside and nucleotide analogues, arsenical and antimonial oxyanions, peptide-based agents, and (in concert with alterations in phase II conjugating or biosynthetic enzymes) also alkylating agents (reviewed in 51). The doxorubicin-resistant lung cancer cells 2R120 (overexpressing ABCC1) and 2R160 (overexpressing ABCB1) were nine- and 28-fold more sensitive to gemcitabine than their parental SW1573 line, respectively ($P<0.01$). In 2R120 and 2R160 cells, DCK activities were seven- and four-fold higher than in SW1573,

respectively. Thus, ABCB1 and ABCC1 overexpression possibly caused a cellular stress resulting in increased gemcitabine metabolism and sensitivity [47].

ABCC4 and ABCC5 transport nucleoside monophosphates [52,53]. Davidson et al. [34] found a substantial resistance to cytarabine, gemcitabine, and cladribine in ABCC5-overexpressing HEK293 cells. However, Reid et al. [54] observed no ABCC4- or ABCC5-mediated resistance against cytarabine, gemcitabine, or fludarabine in ABCC4- and ABCC5-overexpressing HEK293 cells. To clarify these contradictory results, Oguri et al. [35] examined the expression levels of *ABCC5* gene in non-small cell lung cancer cell lines. The ABCC5 expression levels inversely correlated with gemcitabine sensitivity. Furthermore, the treatment with the ABCC5 inhibitor zaprinast altered the sensitivity in ABCC5-expressing cells and it was confirmed by using siRNA as well [35]. Therefore, ABCC5 remains an intriguing target for future studies on drug resistance to gemcitabine.

ABCC10 gene expression assessed by quantitative PCR in different adult and fetal tissues and various tumors was found to be highest in pancreas. Moreover, ABCC10 mRNA levels were remarkably increased in doxorubicin-treated MCF7 cells. Conversely, in TP53-dominant-negative MCF7 cells the ABCC10 upregulation was suppressed. These results suggested that ABCC10 expression is regulated in a TP53-dependent manner and thus it may vary in concordance with DNA damage-induced apoptosis [55]. However, neither cAMP nor cGMP were found to be transported by ABCC10 [56].

ABCC11 is an amphipathic anion transporter able to efflux cAMP and cGMP and functions as a resistance factor for commonly used purine and pyrimidine nucleotide analogs. Analysis of the sensitivity of ABCC11-overexpressing cells (LLC-PK1) revealed that they are resistant to a range of clinically-relevant nucleotide analogs [57].

Konig et al. [58] quantified the mRNA expression of nine ABCC family members and of ABCG2 in normal human pancreas and in pancreatic carcinoma (n = 37). The expression of ABCC1, ABCC3, ABCC4 and ABCC5 mRNA in normal pancreatic tissue and in pancreatic carcinoma samples was found using real-time PCR. cDNA fragments of the length corresponding to ABCC2, ABCC6, ABCC10 and ABCC12 were also identified but the relative quantification to the respective beta-actin mRNA levels has shown that their amplification was very low. ABCC11 fragment was not amplified at all. The ABCC1 and ABCC4 mRNA levels did not significantly differ between the normal pancreas and the tumor tissues. On the other hand, the expression of ABCC3 mRNA was upregulated in pancreatic carcinoma samples and correlated with tumor grade. The ABCC5 expression was upregulated in carcinomas as well but did not correlate with tumor characteristics. The authors suggested that ABCC3 and ABCC5 are involved in drug resistance of pancreatic tumors and analysis of their expression may contribute to the prediction of the chemotherapy outcome. It is interesting that the ABCC12 was detected in normal pancreatic tissue only [58]. Although numerous SNPs in ABCC genes exist, their association with clinical phenotype mostly remains to be clarified [59].

The human ABCG2-transfected HEK293 cells are gemcitabine-resistant [60]. Five pancreatic carcinoma cell lines tested by real-time PCR expressed significantly higher levels of ABCG2 mRNA than non-malignant fibroblasts. Flow cytometry indicated the presence of ABCG2 on the cell surface of these cell lines, although ABCG2 protein levels did not correlate very well with the mRNA expression [61]. cDNA fragments of ABCG2 with the expected length were amplified in both pancreatic normal and carcinoma samples. However,

ABCG2 mRNA levels in the normal tissues did not significantly differ from those in tumors [58].

Table 1. Expression and function of ABCB1, ABCG2 and ABCC family in pancreatic cancer

Gene	Protein	OMIM	Locus	Activity towards nucleoside analogs	Expression in pancreas	Expression in tumor	Reference
ABCB1	MDR1	171050	7q21	Increased sensitivity	Yes	Upregulated	38,45-49
ABCC1	MRP1	158343	16p13	Increased sensitivity	Yes	Unchanged	38, 47, 58
ABCC2	MRP2	601107	10q24	Unknown	Yes	Yes	38, 58
ABCC3	MRP3	604323	17q22	Unknown	Yes	Upregulated	38, 58
ABCC4	MRP4	605250	13q32	None	Yes	Unchanged	38, 54, 58
ABCC5	MRP5	605251	3q27	Increased resistance	Yes	Upregulated	34, 35, 38, 58
ABCC6	MRP6	603234	16p13	Unknown	Yes	Yes	38, 58
ABCC10	MRP7	612509	6p12	Unknown	Yes	Yes	38, 58
ABCC11	MRP8	607040	16q12	Increased resistance	Yes	Yes	38, 57, 58
ABCC12	MRP9	607041	16q12	Unknown	Yes	No	38, 58
ABCG2	BCRP	603756	4q22	Increased resistance	Yes	Unchanged	38, 58, 60

Nucleoside Transporters

Gemcitabine is a hydrophilic substance and thus, it is not expected to permeate membranes by passive diffusion. Its cellular uptake is managed predominantly by specialized membrane nucleoside transporters [62]. Nucleoside transport occurs by sodium-dependent, inwardly directed concentrative process performed by hCNT (human Concentrative Nucleoside Transporter, belonging to SLC28 family) or by sodium-independent, bidirectional equilibrative process performed by hENT (human Equilibrative Nucleoside Transporters, belonging to SLC29 family). In humans, 7 nucleoside transporters divided into the above mentioned 2 families exist: SLC28A1 (hCNT1, OMIM:606207), SLC28A2 (hCNT2, OMIM:606208) and SLC28A3 (hCNT3, OMIM: 608269), SLC29A1 (hENT1, OMIM:602193), SLC29A2 (hENT2, OMIM:602110), SLC29A3 (hENT3, OMIM:612373), SLC29A4 (hENT4, OMIM:609149) [63,64].

Gemcitabine is routinely administered weekly as an i.v. bolus infusion lasting 30-60 minutes in doses ranging from 800 to 1200 mg/m^2 and the peak of serum concentration does not exceed 50 μM. From this fact it is obvious that tumor cells are *in vivo* exposed to effective gemcitabine concentration for quite short time period and therefore inefficient cellular uptake could be an underlaying mechanism for the observed resistance in some solid tumors [62]. Using the panel of 12 different cell lines it has been shown that functional nucleoside transporters are required for manifestation of gemcitabine toxicity *in vitro*. The efficiency of gemcitabine uptake varied markedly among the cell lines with expression of single transporters: SLC29A1 ≈ SLC28A1 > SLC29A2 > SLC28A3 >>> SLC28A2 [62]. Human pancreatic adenocarcinoma cells NP9, NP18, NP29, and NP31 overexpressed predominantly SLC29A1. The other transporters: SLC29A2, SLC28A1, SLC28A2 and SLC28A3 were expressed as well but their expression levels differed among the cell lines, with apparent selective loss or decrease of SLC28A mRNAs [36]. Exposure to 20 μM gemcitabine for 1h elicited upregulation of ABCC1, ABCC3, ABCC5 and SLC29A1 transporters *in vitro* suggesting a possible induction of drug resistance [65]. Cell lines lacking SLC29A1 are highly resistant to gemcitabine [62,66] and patients with PDAC with detectable SLC29A1 protein by IHC had a significantly longer survival after gemcitabine chemotherapy than patients whose tumors did not express SLC29A1 (13 vs. 4 months, p=0.01, n=21) [67]. This observation was supported by the study on 31 patients with inoperable biliary tract cancer treated by first-line gemcitabine-based regimen. SLC29A1 expression significantly correlated with time to progression (6.33 vs. 2.83 months, p=0.04) and patients with positive SLC29A1 IHC expression showed a longer but non-significant median survival (14 vs. 7 months, p=0.13). There were no significant associations between adverse drug toxicities, tumor response and SLC29A1 levels [37]. High SLC28A3 expression has also been associated with longer overall survival of patients with PDAC treated by gemcitabine-based chemoradiation after curative resection (n=45). Furthermore, the combined analysis has shown that, patients with both favorable prognostic factors (high SLC29A1 and SLC28A3 expression, n=15) had a longer overall survival (median survival, 94.8 months) than those having just one factor (18.7 months) or none (12.2 months) [68]. Therefore, expression of SLC29A1 and probably also other nucleoside transporters like SLC28A3 may become relevant predictive marker(s) of the outcome of cancer patients treated by gemcitabine-based regimen.

Erlotinib and ABC Transporters

Erlotinib hydrochloride (OSI-774, Tarceva, CP-358774) is an orally active small molecule that blocks downstream intracellular signaling of epidermal growth factor receptor (EGFR, OMIM:131550) by binding to its ATP binding site essential for tyrosine kinase activity (Figure 3). The signaling pathways of tyrosine kinases as EGFR are involved in cancer cell proliferation, apoptosis, angiogenesis and metastasis [69,70]. Erlotinib has been approved for the treatment of a locally advanced inoperable or metastatic pancreatic cancer in combination with gemcitabine [71].

Erlotinib is metabolized by several isoforms of the P450 enzymes (CYP), predominantly by CYP3A4 (OMIM:124010) and CYP3A5 (OMIM:605325), less by CYP1A1

(OMIM:108330), CYP1A2 (OMIM:124060), CYP1B1 (OMIM: 601771), CYP2D6 (OMIM:124030) and CYP2C8 (OMIM: 601129).

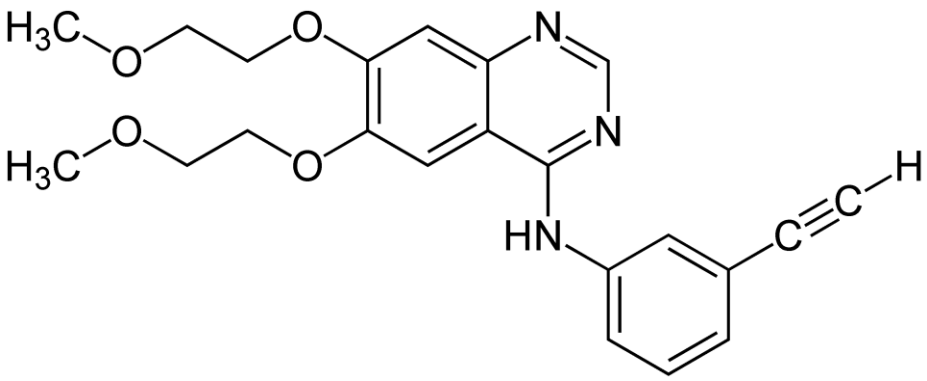

Figure 3. Structure of erlotinib.

The main circulating metabolites of erlotinib are products of the *O*-demethylation of the side chains (such as OSI-420 and OSI-413) [72-74]. Erlotinib is also a substrate for ABCB1 and ABCG2 but not ABCC2 *in vitro* [75]. The absence of ABCB1 and ABCG2 significantly affected the oral bioavailability of erlotinib in mice *in vivo*. Erlotinib also inhibited the ABCB1-, ABCG2- and ABCC10-mediated efflux at higher drug concentrations [76,77]. *ABCG2* SNP (421C>A, Q141K, dbSNP: rs2231142) was associated with increased accumulation of erlotinib and may be relevant to its toxicity and antitumor activity in patients. No significant associations were observed between *ABCB1* SNP 3435C>T (rs1045642) and pharmacokinetics of erlotinib [78]. A recently followed diplotype comprised of two polymorphic loci in the *ABCG2* promoter (-15622C>T, no rs number assigned yet and 1143C>T, rs2622604) was significantly associated with erlotinib pharmacokinetics parameters, including area under the curve and maximum plasma concentration. Variability in dermal and gastrointestinal toxicity following erlotinib treatment in association with the studied *ABCG2* SNPs has been demonstrated as well [79].

No data are available on the activity of erlotinib in relation to genotype or phenotype of the SLC transporter family.

In conclusion, erlotinib is doubtlessly a substrate and may act as inhibitor of ABCB1, ABCG2, and probably also of ABCC10. Such feature may be useful especially in combination with other anticancer drugs which are subject to efflux by these ABC transporters. Further studies on individual variation in genotype and phenotype of membrane transporters are needed to define the treatment modalities, e.g. erlotinib dose and personalized drug combination regimen in order to achieve better outcome of pancreatic cancer therapy.

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