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Original scientific paper

Tissue distribution of crizotinib and gemcitabine combination in a patient-derived orthotopic mouse model of pancreatic cancer

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Abstract

Pharmacokinetics focuses on the question whether a drug actually reaches its target in therapeutic concentrations or accumulates elsewhere, potentially causing toxicological or unpredictable side effects. We determined tissue distribution of gemcitabine, an antimetabolite, and crizotinib, a tyrosine-kinase inhibitor targeted against the anaplastic lymphoma kinase (ALK) and mesenchymal-epithelial transition factor (c-Met) receptors, in a validated orthotopic mouse model for pancreatic cancer. Mice with pancreatic cancer were treated with either oral crizotinib at 25 mg/kg, gemcitabine at 100 mg/kg or with their combination. Two hours after the last gemcitabine dose mice were sacrificed and all available blood/organs/tissues were sampled. Tissue was subsequently analyzed for drug concentrations using a validated liquid chromatography-mass spectrometry (LC-MS/MS) technique. In whole blood gemcitabine was about 1.0 µM and crizotinib 2.4 µM in the single treatment, whereas in the combination crizotinib increased the levels of gemcitabine. Crizotinib was found in all major tissues, being highest in the intestine. Comparison of crizotinib alone to the gemcitabine-crizotinib combination showed that crizotinib tissue concentrations were 3-6 fold lower in liver, lung, kidney and spleen, 30-fold lower in the skin, heart and pancreas and 200-fold lower in the brain. Tissue gemcitabine was highest in spleen and skin, being about 5-10 fold higher than in the other tissues, including brain, which still had a relatively high accumulation. In conclusion, both gemcitabine and crizotinib accumulate at clinically active but variable levels in tissues, possibly relating to the effects exerted by these drugs.

Keywords

Crizotinib; gemcitabine; tissue distribution

Introduction

Pharmacokinetics usually focuses on the behavior of a single drug in plasma in order to obtain information on its uptake, distribution and elimination [1]. In cancer patients this information can give insight in the mechanism of action, the relationship between dose, systemic concentration and biological effect (beneficial or otherwise) [2]. However, limited or insufficient attention is usually given to the question whether the drug actually reaches its target (the tumor) in therapeutic concentrations or accumulates in normal tissues potentially leading to toxicity. Usually drug interactions of combinations receive limited interest. Earlier we investigated the interaction between gemcitabine and crizotinib

(Figure 1) and demonstrated a synergistic effect between the drugs in in vitro and in vivo models of pancreatic ductal adenocarcinoma (PDAC) [3]. In the present study we aimed to analyze their interaction in normal tissues, in order to determine whether this information can give an explanation for toxic side effects seen with these drugs in patients.

Crizotinib is registered for the treatment of non-small cell lung cancer that expresses the abnormal EML4-ALK fusion gene, and has been evaluated for use with tumors expressing high c-Met [4-6]. In turn, gemcitabine is standard first line treatment in a wide range of carcinomas including non-small cell lung cancer, pancreatic cancer, bladder cancer and breast cancer [7]. In this paper we describe the tissue distribution of both crizotinib and gemcitabine, given alone or together in a patient-derived orthotopic mouse PDAC model.

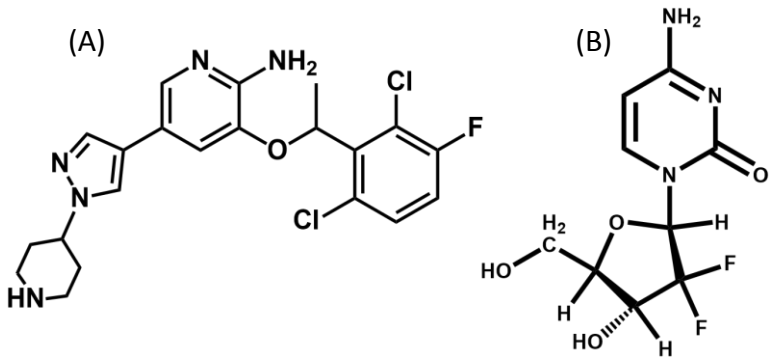

Figure 1. Structural formulae of crizotinib (A) and gemcitabine (B).

Model and methods

Mouse model

Orthotopic primary-PDAC mouse models were developed as reported by Avan et al. [3] by implantation of primary tumor cells into 6-8 week old female athymic nude mice (Harlan, Host, The Netherlands). Tumor growth was monitored via bioluminescence, as described previously [3]. The mice were treated with oral crizotinib at 25 mg/kg, or gemcitabine at 100 mg/kg intraperitoneally 4 times at 3-day intervals. The third group received a combination of both drugs. Blood was taken via the tail vein at 1 and 2 h after drug administration. Thereafter mice were sacrificed and all available organs/tissue was sampled. Sampled tissue was immediately frozen in liquid nitrogen and stored at -80 °C.

Drug analysis and equipment

Tissue and whole blood concentrations of gemcitabine and crizotinib were determined by adaptation of a validated liquid chromatography-mass spectrometry (LC-MS/MS) methodology previously reported [8,9] using a Sciex API3000 mass spectrometer coupled with a Dionex Ultimate 3000 capillary LC system. Precision, accuracy, robustness and stability were established for crizotinib and gemcitabine prior to tissue analysis in whole blood, plasma, serum and cell culture medium [8]. Parameters were determined with software “Analyst” version 6.2 (Sciex BV, Breda) and DMSlink ver 2.10 (Thermo Scientific - Dionex, Breda).

Whole blood, plasma and serum were extracted by adding 20 µl whole blood aliquots to 80 µl acetonitrile and sonicating for 15 seconds. After centrifugation at 2500 g for 10 minutes / 4 °C, the supernatant was taken and analyzed by LC-MS/MS. Tissue sample preparation was performed by taking triplicate portions of each tissue (5-30 mg), weighed into 1.5 ml cryogenic vials which were subsequently desiccated using the freeze drying processes for 48 h. Samples were then homogenized for 10 min with acetonitrile: water (5:1), sonicated and centrifuged. The subsequent supernatant was then analyzed by LC-MS/MS.

Crizotinib optimization

Crizotinib analysis was optimized for mass spectroscopic detection as described earlier for tyrosine kinase inhibitors. Briefly, 1 µg/ml solution was infused at 3 µl per min into the turbo ionisation source, the parent ion for crizotinib (MW-449) was established as 450, 451 and 452 amu with characteristic pattern of a chlorine containing molecule (Figure 2A). The major fragment of the 450 amu response was 259.9 (Figure 2A). Mass spectrometric parameters for the transition quadrupole pairing (Q1/Q3) of 450.0/259.1 are: declustering potential (DP), 61 V; focusing potential, 270 V; entrance potential, 10 V; collision energy, 31 V; cell exit potential, 18 V.

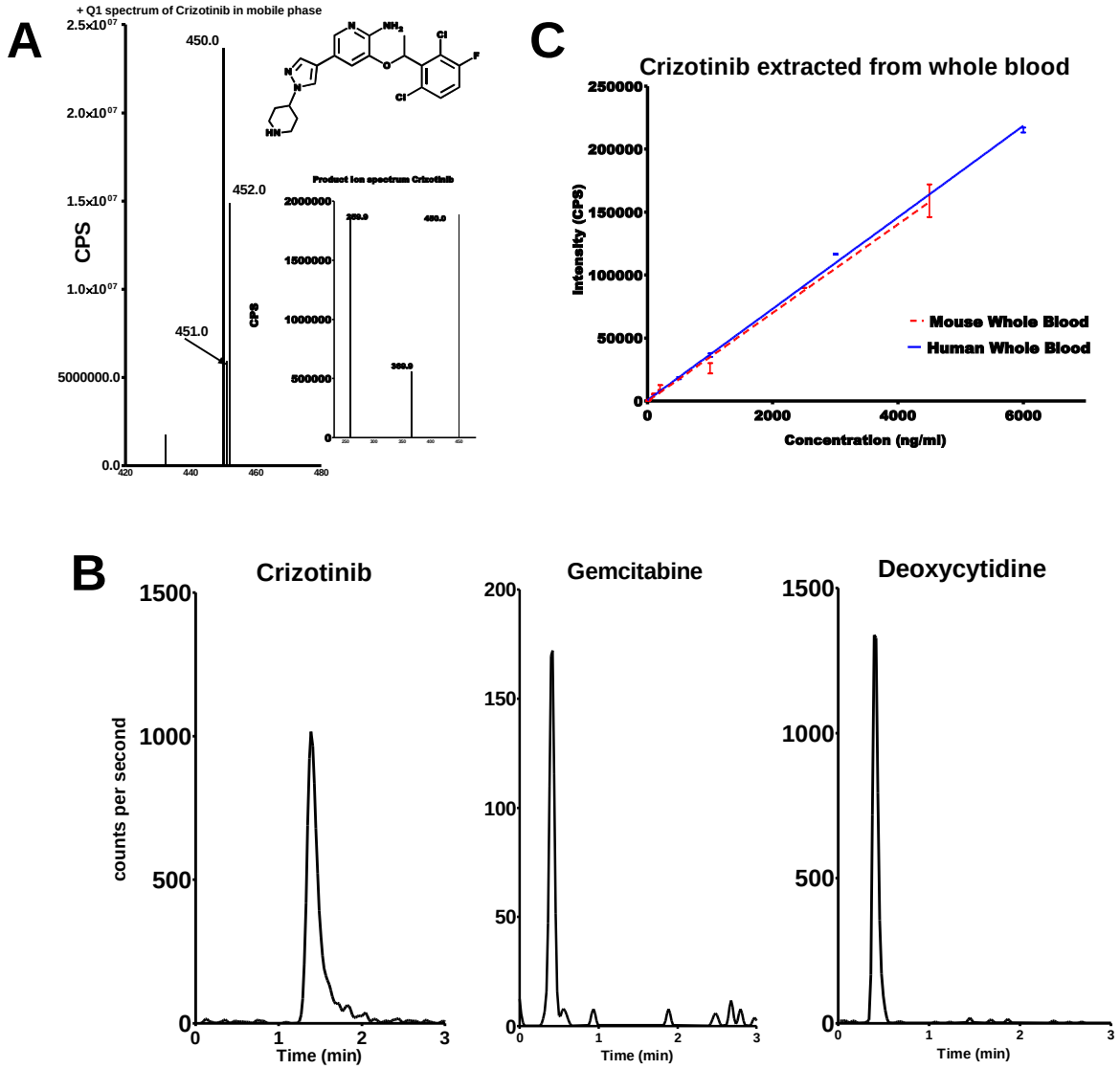

Figure 2. Ionisation spectrum and chromatography of crizotinib, gemcitabine and of the internal control deoxycytidine. (A) Ionisation spectrum of the parent compound (crizotinib) and the product ion spectrum of the $[M+H]^+$ ion for crizotinib in chromatographic mobile phase, consisting of acetonitrile, methanol, isopropyl alcohol and aqueous ammonium acetate at pH 7.80. (B) Chromatography of crizotinib, gemcitabine and deoxycytidine. (C) Linearity of crizotinib analysis in mouse and human whole blood.

Extraction and chromatography

Crizotinib chromatography was performed using the optimized chromatographic system for the multiple tyrosine kinase inhibitors (Figure 2B) [8]. Under these set conditions crizotinib exhibited less sensitivity

when compared to erlotinib, sunitinib and gefitinib. However, the crizotinib assay was linear up to 30 µg/ml in both plasma and serum (r^2 = 0.986, n= 10), and reproducible over a 3 day period. Crizotinib was more soluble in aqueous solutions than other reported TKIs. For whole blood the extraction was best performed in reverse, where the 20 µl sample of whole blood was pipetted directly into round bottomed 96 well already containing 80 µl of acetonitrile. This ensured each sample was homogeneously mixed prior to centrifugation and transfer of the supernatant for LC-MS/MS analysis.

Linearity

Linear regression for whole blood preparations was similar to plasma / serum / cell culture medium in the 1 – 5000 ng/ml and was acceptable (r^2>0.98). The standard error was within ± 15 % for each calibration point used. The limit of quantitation for crizotinib was 50 ng/ml, Due to lack of stock mouse whole blood preparations of human whole blood were compared to mouse whole blood standard curves. No difference was observed in the linear regression parameters determined (Figure 2C), hence human whole blood was used as an alternative stock matrix for the samples to be analyzed.

To limit the standard requirements per analytical run, standard curves for crizotinib and gemcitabine, and its metabolite difluorodeoxyuridine (dFdU) were prepared in either whole blood or cell culture medium. Separate extractions for gemcitabine/dFdU and crizotinib were just not possible because the sample volume available for the mouse blood samples ranged from 5-20 µl per time point. Therefore, extraction using acetonitrile precipitation was used for gemcitabine and dFdU. Gemcitabine/ dFdU sensitivity was lower (especially for dFdU in tissues) compared to the previously published procedure but was linear in response across multiple days. The limit of quantification for gemcitabine was 10 ng/ml. Linear regression for gemcitabine was not affected by the presence of crizotinib and chromatographically all compounds were separated (Figure 2C).

Tissue analysis

Preparation of a standard curve within a tissue matrix was not always feasible due to the lack of sufficient tissue from untreated mice. Therefore cell culture medium was compared with blank mouse tissue (heart), which was available in sufficient quantity for the validation procedure. Both were prepared as per protocol. Separate standard curves were prepared by spiking with crizotinib and gemcitabine dilutions in acetonitrile:water (5:1). No difference was observed. Therefore, for all tissue analysis cell culture medium was used for the standard matrix.

Recovery of crizotinib, gemcitabine and dFdU was verified using the extracted tissue of the combination treated animal, by testing whether subsequent extraction would yield any detectable drug. Therefore, tissues were extracted with acetonitrile:water (5:1) for a second and third time. After analysis no crizotinib, gemcitabine or dFdU could be observed within the second and third extracts, indicating that close to 100 % had been recovered on the first extraction (data not shown).

Results

Whole blood analysis

Limited sampling was performed at 1 and 2 hour, since the peak concentration of gemcitabine was expected around these time intervals [10,11]. Whole blood analysis of the treated animals demonstrated measurable concentrations for gemcitabine (2 h: 1.0 ± 0.3 µM), dFdU (2 h: 5.6 ± 1.4 µM) and crizotinib (2 h: 2.4 ± 0.8 µM) (Figure 3).

Tissue analysis

Gemcitabine was found in all tissues that could be analyzed, when given alone or in combination. The highest levels were found in skin and spleen. The concentration in the brain was relatively high compared to the other tissue types and even comparable to liver (Figure 4), showing a good brain penetration of gemcitabine. However, the primary metabolite of gemcitabine, dFdU, was not detectable for this group of samples. This was most likely due to the lack of sensitivity of uridine based compounds to mass spectrometric detection under the chromatographic conditions used, since we did not use the optimal LC-MS conditions as used earlier for tissues [14]. In the combination the highest levels of gemcitabine were found in the same tissues as at single drug treatment, with increased drug levels in blood, but lower levels in lung and brain (Figure 4).

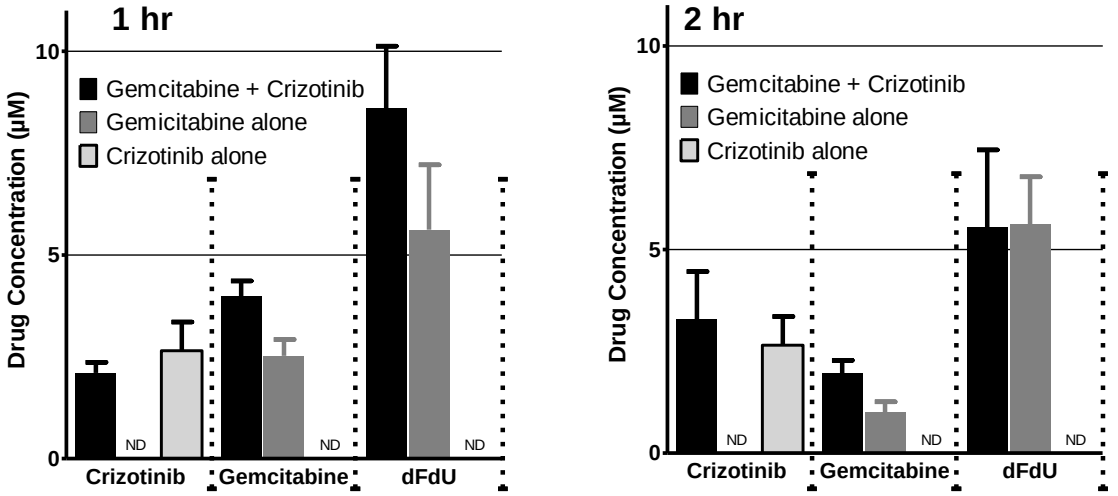

Figure 3. Concentrations of crizotinib, gemcitabine and dFdU in whole mouse blood. Blood was taken at 1 and 2 h after drug administration. Values are means ± SD of 3 animals.

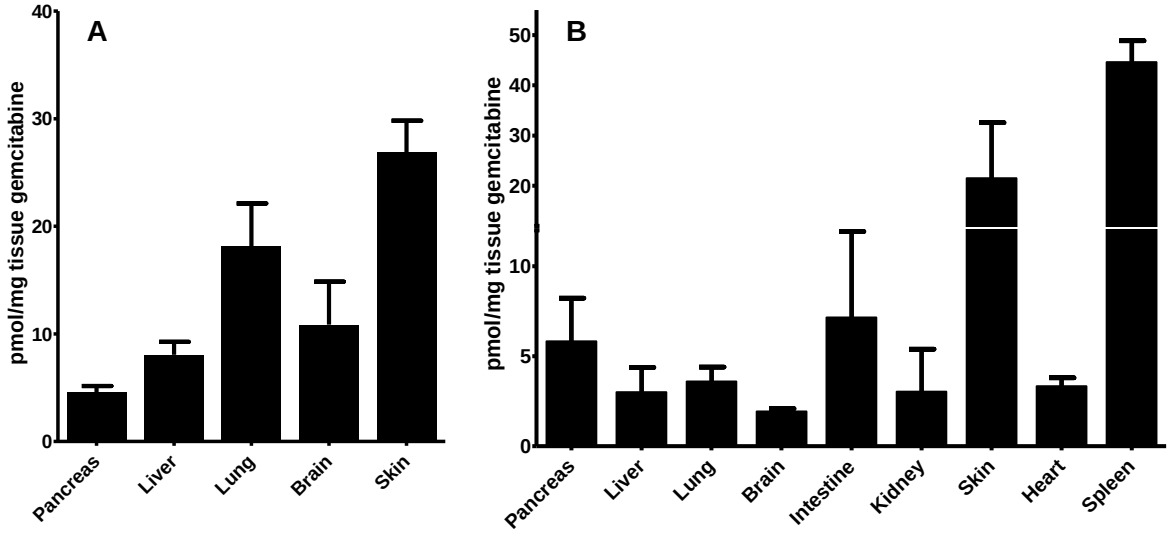

Figure 4. Tissue analysis (at 2 hour after drug administration) of gemcitabine at single drug administration (A) and in combination with crizotinib (B). Values are means ± SD of 3 animals.

At single treatment crizotinib accumulated at high levels in lung, liver and pancreatic tissues, and at very low levels in the brain (100-fold lower) (Figure 5A). In the combined treatment crizotinib (pmol/mg tissue) was also found in all major tissues at varying concentrations; intestine demonstrated the highest levels

(1084) followed by liver (215), lung (172) and kidney (167). In addition, crizotinib was found in the spleen (133), skin (28), heart (34), pancreas (48) and brain (4). Crizotinib demonstrated an increased accumulation compared to the single treatment in 4 of 5 tissues tested (Figure 5B and Table 1).

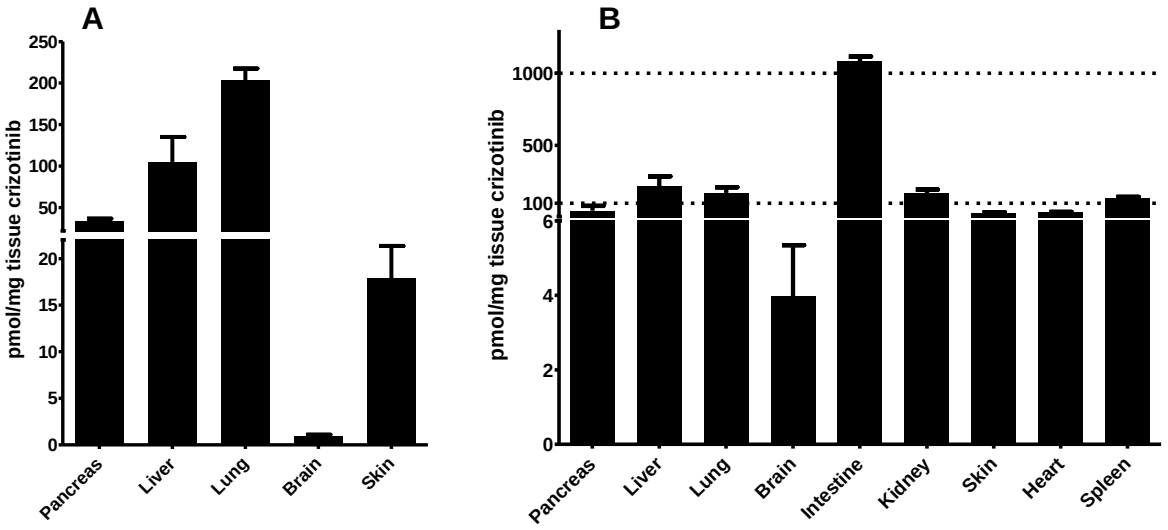

Figure 5. Tissue analysis (at 2 hour after drug administration) of crizotinib at single drug administration (A) and in combination with gemcitabine (B). Values are means ± SD of 3 animals.

Table 1. Effect of combination treatment on drug levels in normal tissues, the pancreatic tumor and blood.

	Blood	Pancreas	Liver	Lung	Brain	Skin	Tumor
Crizotinib	1.4	1.4	2.0	0.8	4.5	1.6	1.4
Gemcitabine	2.0	1.3	0.4	0.2	0.2	1.2	1.3

Values are ratios of the combination versus single drug. Tumor data are from [7]

Discussion

Tissue distribution of crizotinib alone and of the gemcitabine and crizotinib combination has not been reported previously. Only limited data are available for gemcitabine distribution in the range of tissues we have tested. Early data on gemcitabine tissue accumulation were obtained using radioactively labeled drug [12,13]; however, these data does not differentiate between gemcitabine and dFdU, which could overestimate the tissues exposure to gemcitabine. As expected, gemcitabine is accumulated into all tested tissues, in agreement with previous results in humans and animals [14-17], as also shown with 19F-NMR15. The use of the sensitive LC-MS/MS technique enabled acquisition of reproducible blood pharmacokinetics for all three compounds (crizotinib, gemcitabine and dFdU) simultaneously without interference [8,9], but tissue analysis of dFdU was not possible since we had to adapt the protocol to have one extraction procedure for all drugs. Using the optimal LC-MS/MS procedure for dFdU we could demonstrate accumulation of dFdU in human tissue [14]. Whole blood analysis proved consistent with expected pharmacokinetics of gemcitabine 1 and 2 hours after dosing [10,11]. Gemcitabine levels demonstrated a rapid decrease in concentration over the 2 hour time period corresponding to an increase in observable dFdU in agreement with an earlier pharmacokinetic study in mice [11]. For crizotinib, literature reported

data are limited, but since the methodology was adequate for blood and tissue pharmacokinetics of gemcitabine as well as blood crizotinib, it can be assumed that the crizotinib results also reflect the true situation. Crizotinib demonstrated an elevated concentration after 1 hour which was maintained after 2 hours indicating a steady state condition had probably been reached.

Crizotinib accumulated into all tested tissues at levels consistent with therapeutic concentrations, except for brain. Crizotinib accumulation in the brain tissue has not been previously reported, but low levels were expected since it is a good substrate for efflux pumps [18]. Hence, crizotinib does not show efficacy in patients with either brain tumors of liver metastases [19].

The combination of crizotinib with gemcitabine appears to enhance crizotinib exposure in the tissues of the pancreas, liver, brain and skin. The accumulation of crizotinib in the orthotopic tumor was about 37.4±30.3 pmol/mg tissue, while that of gemcitabine was about 5.84±2.41 pmol/mg tissue, demonstrating excellent tumor uptake. c-Met overexpression is observed in tumors from many different tissues such as brain, kidney, ovarian, breast and gastrointestinal cancers [4,20]. The use of crizotinib might be considered as an additional resource in these areas, especially in combination with gemcitabine. Crizotinib also appears to enhance the exposure of pancreas and skin towards gemcitabine, possibly by inhibiting the gemcitabine degradation enzyme cytidine deaminase [3]. The high accumulation of crizotinib in tissues might not reflect the actually accumulation in cells, particularly in the cytoplasm. Earlier we demonstrated in cancer cell lines that crizotinib might concentrate in lysosomes [18], similar to sunitinib [21]. Hence, in tissue with a high content of lysosomes the accumulation might be a lysosomal accumulation.

It is not clear what these data mean in view of toxicology, however, in general crizotinib is tolerated well, although the drug may induce gastrointestinal toxicity, edema and visual impairment [5,22,23]. The toxicity of gemcitabine in patients is predominantly hematological [24] which is in line with the high concentration observed in the spleen.

These results clearly show that distribution of crizotinib and gemcitabine occurs over a broad range of tissue types achieving therapeutic concentrations for both drugs. The accumulation in specific tissues (spleen, intestine) seems to be in line with the observed toxicity in patients. Moreover, in certain tissue types crizotinib appears to enhance the exposure to gemcitabine suggesting the need for further development of this combination, especially with regards to PDAC.

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