Chapter 39
PET Imaging of the Impact of Extracellular pH and MAP Kinases on the *p*-Glycoprotein (Pgp) Activity

Oliver Thews, Wolfgang Dillenburg, Frank Rösch, and Marco Fellner

Abstract The functional activity of *p*-glycoprotein (Pgp) can be increased in vitro by an extracellular acidosis via activation of MAP kinases (p38, ERK1/2). In order to study these effects in vivo a new ^{68}Ga-labeled PET tracer was developed which serves as a substrate of the Pgp and therefore indirectly mirrors the Pgp activity. For in vivo studies, experimental tumors were imaged under acidic conditions (inspiratory hypoxia, injection of lactic acid) and during inhibition of MAP kinases in a μ-PET system. In vitro, [^{68}Ga]MFL6.MZ showed an accumulation within the cells of about 20% which was increased to 30% by Pgp inhibition. In solid tumors a marked tracer uptake was observed showing spatial heterogeneity. When the tumors were acidified, the PET tracer accumulation was reduced by 20–30%. Changing the inspiratory O_2-fraction to 8% led dynamically to a decrease in pH and in parallel to a reduced tracer concentration. Inhibition of the p38 pathway reduced the Pgp transport rate. The new ^{68}Ga-labeled tracer is suitable for PET imaging of the tissue Pgp activity. In vivo imaging reveals that an acidosis activates the Pgp markedly, a mechanism in which the p38-MAPK pathway seems to play an important role.

Keywords *p*-Glycoprotein • Acidosis • MAP kinases • PET • ^{68}Ga

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O. Thews (✉)
Julius-Bernstein Institute of Physiology, University of Halle-Wittenberg,
Magdeburger Str. 6, 06112 Halle (Saale), Germany
e-mail: oliver.thews@medizin.uni-halle.de

W. Dillenburg
Institute of Physiology and Pathophysiology, University Medicine Mainz, Mainz, Germany

F. Rösch • M. Fellner
Institute of Nuclear Chemistry, University of Mainz, Mainz, Germany

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1 Introduction

Solid tumors show several pronounced differences as compared to normal tissues with respect to physiological characteristics at the cellular and tissue level. As a consequence of insufficient tumor perfusion [1] there is inadequate oxygen delivery with forced anaerobic metabolism resulting in an increased lactic acid formation [2] and extracellular acidosis [1]. Numerous studies have demonstrated that the abnormal physiological microenvironment reduces the cytotoxicity of chemotherapeutic drugs [3].

On the other hand, reduced chemosensitivity may result from drug transporters which actively pump amphiphilic xenobiotics out of the cell [4]. Modulation of these drug transporters (e.g., by inhibitors) can modify the cytotoxic efficacy of chemotherapy [5]. The best studied member of the ABC-transporter family is the *p*-glycoprotein (Pgp) responsible for a multidrug-resistant phenotype of many human tumors. Besides constitutional differences in the Pgp expression of tumor entities or cell lines, the expression as well as the functional activity of the transporter has been shown to be regulated. In vitro studies [6, 7] demonstrated that lowering the extracellular pH to 6.5 functionally increases the Pgp activity and by this reduces the cytotoxicity of chemotherapeutics which are a substrate of the *p*-glycoprotein. In vivo it was shown that lowering the tumor pH by forcing anaerobic glycolysis decreases the cytotoxicity of daunorubicin [6] which could be attributed to an acidosis-induced activation of the Pgp. However, in these experiments the Pgp transport rate itself could not be measured in vivo. The study also revealed that MAP kinases (p38, ERK1/2) are a signaling pathways responding to extracellular pH and that activate Pgp [6]. Inhibition of p38 or ERK1/2 reduced the Pgp activity in vitro.

From these results, there is strong evidence that extracellular acidosis functionally increases the Pgp activity via a p38-mediated pathway in vivo [6]. With functional imaging such as PET it might be possible to measure the functional Pgp activity noninvasively and to confirm the impact of extracellular acidosis on drug transport as well as to identify the role of MAP kinases in the signaling pathway in vivo.

2 Material and Methods

2.1 PET Tracer and Tumor Model

For functional Pgp imaging, six tracers (MFL1.MZ-MFL6.MZ) were synthesized which consist of hexadentate Schiff-bases labeled with the positron emitter ^{68}Ga ($T_{1/2}$ = 67.7 min, β^+ branching = 89 %). ^{68}Ga was eluted from a ^{68}Ge/^{68}Ga generator [8] with 10 mL HCl and on line–immobilized on an acidic cation exchanger. [AU1] Labeling was performed with 20 µL of each ligand solution (1 mg/mL in EtOH, 30 nmol). Radiochemical labeling yield and complex formation were determined by

thin layer chromatography (TLC). For injection the tracer pH was adjusted to 7.4 with NaOH and diluted with isotonic saline. The tracers themselves are substrates of the Pgp and are actively pumped out of the cell. The intratumoral concentration reflects therefore inversely the Pgp activity.

In all experiments the subline AT1 of the R3327 Dunning prostate carcinoma was used which functionally expresses the *p*-glycoprotein [7]. Cells were grown in RPMI 1640 medium (+10 % fetal calf serum) and passaged once per week. Solid carcinomas of this cell line were heterotopically induced by injection of AT1 cells (0.4 mL approximately 10^4 cells/μL) subcutaneously into the dorsum of the hind foot. Tumors were used when they reached a volume of between 1.0 and 2.0 mL approximately 10–14 days after tumor cell inoculation.

2.2 Acidosis Model and Tumor Treatment

In order to dynamically change the extracellular pH of the tumors, the inspiratory gas mixture was changed from pure oxygen (100 % O_2) starting 10 min prior to the PET tracer injection to a hypoxic gas containing 92 % N_2 + 8 % O_2 at 15 min after tracer injection (i.e., during the μ-PET measurement). It was continued until the end of the μ-PET measurement at 60 min. In parallel, the extracellular pH was measured with steel-shafted pH glass electrodes (type MI-418B, Microelectrodes Inc, Bedford NH, USA) with an outer diameter of 800 μm inserted into the center of the tumor. Before and after the PET imaging, the electrode was calibrated and pH measurements were corrected for signal shift.

In a second series the extracellular tumor space was acidified by small amounts of lactic acid injected directly intratumorally. Therefore, 50 μL of a 0.222 mM solution of lactic acid were injected in the tumor tissue at a depth of 2–3 mm about 5–10 min prior to the tracer application. The same amount of a 0.222 mM sodium lactate solution was applied in the contralateral tumor and this tumor served as intra-individual control.

In order to inhibit different MAP kinase pathways specific inhibitors (SB203580 and U0126 for inhibition of p38 and ERK1/2, respectively) were injected intratumorally. Therefore, inhibitors were dissolved in DMSO at a concentration of 1 mmol/L and tumors were treated with a single injection of a small amount (20 μL) of these inhibitors 5–10 min prior to the PET measurements. The tumor on the contralateral hind foot was treated with 20 μL DMSO alone and served as intra-individual control.

2.3 μ-PET Imaging

The μ-PET imaging was performed on a microPET Focus 120 small animal PET (Siemens/Concorde, Knoxville, USA). During measurements the anaesthetized

(pentobarbital 40 mg/kg, i.p.) rats were placed in supine position and breathed room air spontaneously through a tracheal tube. After a 15 min transmission scan with an external ^{57}Co source, dynamic PET studies were acquired in 2D mode. The radiotracer was administered as a bolus injection (0.4–0.7 mL, mean activity 44.0 ± 1.5 MBq) via a catheter placed in the left jugular vein. Time activity curves were obtained with varying time frames (1–5 min) for a total measuring interval of 60 min. Volumes-of-interests (VOIs) were defined for tumor and reference tissue in the field of view (testis). Ratios of tumor to reference tissue were calculated from integral image between 10 and 60 min after tracer injection.

3 Results

In order to measure the cellular uptake of the ^{68}Ga-Schiff base complexes, AT1 cells were incubated with the tracer in the presence or absence of the Pgp inhibitor verapamil (VPL) for 30 min after which the suspension was centrifuged and the activity in the cells and in the supernatant was determined. Figure 39.1a shows the fraction of ^{68}Ga-complexes detected inside the cells for all tracers. Tracers MFL2.MZ, MFL5.MZ, and MFL6.MZ were found to be substrates of Pgp since the active efflux could be inhibited by VPL resulting in a statistically significant higher intracellular concentration (Fig. 39.1a; e.g., 178 ± 5 % for [^{68}Ga]MFL2.MZ and 144 ± 3 % for [^{68}Ga]MFL6.MZ). From these in vitro experiments it became evident that [^{68}Ga]MFL2.MZ and [^{68}Ga]MFL6.MZ showed the best compromise of good passive uptake into the cells and pronounced Pgp-mediated efflux inhibitable by VPL. Subsequently, both these tracers were used in PET experiments with tumor-bearing rats. Figure 39.1b shows the averaged activity in tumor and testes indicating that [^{68}Ga]MFL6.MZ is strongly accumulated in the tumors resulting in a three-times

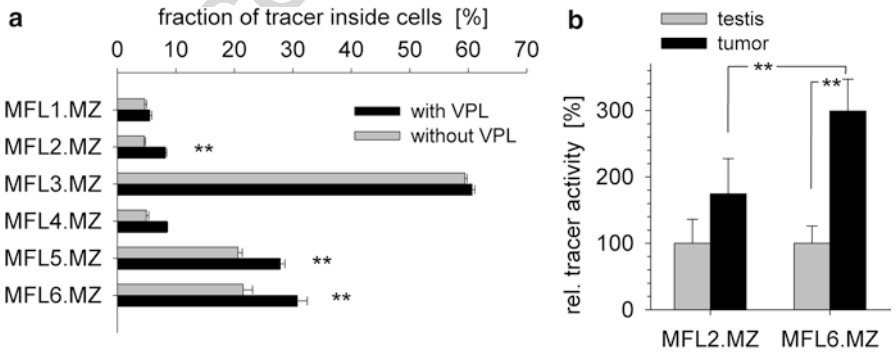

Fig. 39.1 (**a**) In vitro accumulation of various ^{68}Ga-labeled tracers in AT1 cells in the presence and absence of verapamil (VPL). (**b**) In vivo uptake of two tracers in AT1 tumors and a reference tissue (testis). Values are expressed as means ± SEM; (*double asterisk*) $p < 0.01$; $n = 3–12$

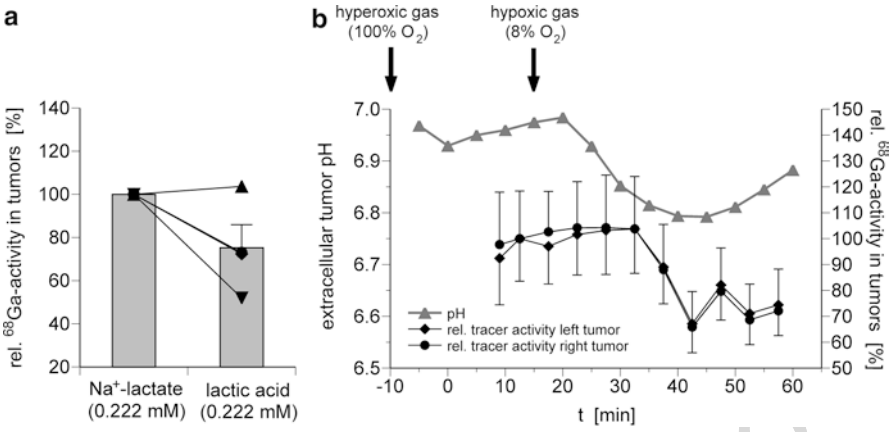

Fig. 39.2 (**a**) Intratumoral [^{68}Ga]MFL6.MZ concentration in tumors treated with lactic acid (50 µL, 0.222 mM) or with an equivalent volume of Na^+-lactate. *Each line* represents one individual animal with two tumors on each hind foot. *Columns* represent means ± SEM of all experiments. (**b**) Extracellular pH and [^{68}Ga]MFL6.MZ concentration during change of the inspiratory O_2-fraction

higher concentration as compared to the reference tissue whereas [^{68}Ga]MFL2.MZ is enriched by only 74 %. Therefore, [^{68}Ga]MFL6.MZ was chosen for further experiments.

As a weak indication of a pH dependency of Pgp-mediated transport the extracellular pH and the tracer concentration were analyzed in tumors of different volume. A clear inverse correlation was found between the pH and tumor volume ($r = -0.941$; data not shown) as well as between tracer concentration (inversely indicating the Pgp activity) and tumor volume ($r = -0.550$; data not shown). Direct injection of lactic acid into one tumor (and Na^+-lactate in the contralateral tumor) showed a markedly reduced tracer concentration in most of experiments (Fig. 39.2a) indicating a higher Pgp transport rate. Figure 39.2b illustrates an experiment where the pH was dynamically reduced during the imaging period by inspiratory hypoxia. Changing the inspiratory gas mixture from 100 to 8 % O_2 15 min after tracer injection reduced the pH from 6.95 to values around 6.8 (Fig. 39.2b, triangles). With a slight delay of 10 min the intratumoral tracer concentration decreased by 25–30 % in both tumors.

Figure 39.3a illustrates an example where in the right tumor the p38 inhibitor SB203580 was injected whereas the left tumor received only DMSO. The right tumor shows a much higher tracer concentration indicating a reduced Pgp activity. This effect was seen in all animals (Fig. 39.3b). On average, the SB203580-treated tumors showed a more than 70 % higher tracer concentration than the contralateral control tumors. Inhibition of the ERK1/2 pathway by U0126 had only a minor effect on the Pgp activity (increase of the tracer concentration by only 29 %; data not shown).

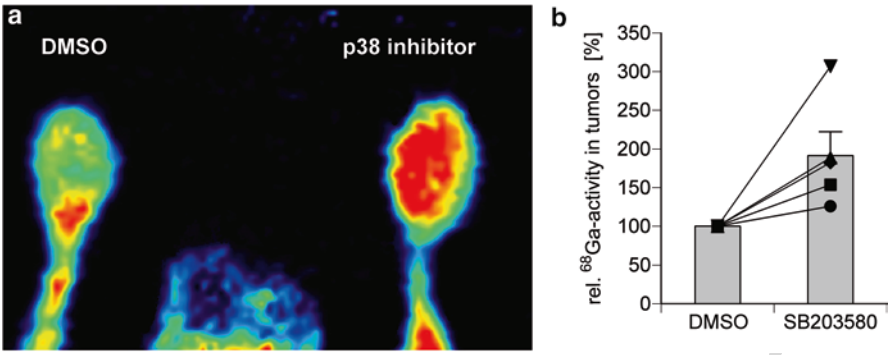

Fig. 39.3 (**a**) Example of [^{68}Ga]MFL6.MZ accumulation in AT1 tumors. In the right tumor 20 µL of a p38 MAPK inhibitor (SB203580) was injected whereas the contralateral tumor received the same volume of the vehicle (DMSO). (**b**) Relative [^{68}Ga]MFL6.MZ concentration in all tumors treated with the p38 inhibitor or with DMSO. *Each line* represents one individual animal with tumors on each hind foot. *Columns* represent means ± SEM of all experiments

4 Discussion

A suitable PET tracer for measuring the Pgp transport rate should fulfill two features: (1) it should enter the cell easily (by passive diffusion) and (2) the tracer should be a substrate of the Pgp and this transport should be inhibitable by Pgp inhibitors. All tracers show high cellular uptake but with marked concentration differences. Taking into account the volume fraction of the cells (~0.3 %) the concentration ratio between cells and medium was approximately 16:1 for most of the tracers but ~90:1 for [^{68}Ga]MFL3.MZ which is the most lipophilic compound. Since the intracellular concentration depends on the Pgp activity, the ratio of cellular tracer concentration in the presence and absence of verapamil could be used as an indicator of the Pgp-mediated efflux [9]. This ratio was ~1.2–1.8 for most tracers (Fig. 39.1a) but for the lipophylic tracer [^{68}Ga]MFL3.MZ Pgp inhibition had practically no impact. The uptake measurements in vivo correspond well with the in vitro data as indicated by a higher tumor concentration of the tracer [^{68}Ga]MFL6.MZ compared to [^{68}Ga]MFL3.MZ.

With this tracer µ-PET images were analyzed after acidifying the tumor tissue by direct injection of lactic acid. Since the interstitial fluid pressure is elevated in many human malignancies, the injection of even a small fluid volume of lactic acid into the tissue may somehow disturb tumor microcirculation. For this reason it cannot be excluded that the direct injection may reduce tumor perfusion locally and by this limit the tracer transport. For this reason, the contralateral tumor of each animal served as an intra-individual control by injecting the same volume of an equimolar Na^+-lactate solution leading to a comparable increase of the interstitial hydrostatic pressure. The comparison of the tracer concentration in both tumors therefore reflects solely the impact of the lower pH on Pgp activity.

The second method for acidifying the extracellular space was to force anaerobic glycolysis leading to an increased endogenous formation of lactic acid. Breathing a hypoxic gas mixture with 8 % oxygen is suitable to markedly lower the tumor-pO_2 [10] and to reduce the tumor pH [6]. In the present study hypoxia led to decrease in pH followed by an increase in Pgp activity resulting in a lower tracer accumulation (Fig. 39.2a). The increase of pH 30 min after the onset of the hypoxic breathing remains unclear at the moment. Perhaps changes in the blood pressure or a hypoxia-induced vasodilation led to an increase in tumor perfusion and by this to a better oxygen supply.

Previous in vitro data revealed that MAP kinases (p38, ERK1/2) play a relevant role for signal transduction in the acidosis-induced Pgp activation [6]. The present study clearly shows that MAP kinases are also important for the Pgp transport activity in vivo. Inhibition of p38 by SB203580 increased the intratumoral tracer concentration indicating a reduced Pgp transport rate (Fig. 39.3). A problem of the experimental design might be the direct intratumoral injection of drugs which could impair tumor perfusion. However, since in the contralateral tumor the same volume of DMSO was injected, the specific effect of SB203580 can be derived. In all tumors the activity in the SB203580 treated tumor was markedly higher (on average by the factor of 2). In the present study inhibition of ERK1/2 on the Pgp activity had only a minor impact on the Pgp activity which is in good accordance with previous indirect results on chemosensitivity of tumors [6].

The new ^{68}Ga-labeled tracer is suitable for PET imaging of the tissue Pgp activity. With this tracer it becomes possible to identify patients with multidrug-resistant tumors pre-therapeutically. In vivo imaging reveals that tumor acidosis activates the Pgp markedly, a mechanism in which the p38-MAPK pathway seems to play an important role. From these results new strategies for overcoming multidrug resistance (e.g., increasing tumor pH, inhibition of p38) may be developed.

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References

1. Vaupel P, Kallinowski F, Okunieff P (1989) Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. Cancer Res 49:6449–6465
2. Mueller-Klieser W, Vaupel P, Streffer C (1998) Energy status of malignant tumors in patients and experimental animals. In: Molls M, Vaupel P (eds) Blood perfusion and microenvironment of human tumors. Springer, Berlin, pp 193–207
3. Sanna K, Rofstad EK (1994) Hypoxia-induced resistance to doxorubicin and methotrexate in human melanoma cell lines in vitro. Int J Cancer 58:258–262
4. Schinkel AH, Jonker JW (2003) Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. Adv Drug Deliv Rev 55:3–29
5. Fojo T, Bates S (2003) Strategies for reversing drug resistance. Oncogene 22:7512–7523
6. Sauvant C, Nowak M, Wirth C et al (2008) Acidosis induces multi-drug resistance in rat prostate cancer cells (AT1) in vitro and in vivo by increasing the activity of the p-glycoprotein via activation of p38. Int J Cancer 123:2532–2542

7. Thews O, Gassner B, Kelleher DK et al (2006) Impact of extracellular acidity on the activity of p-glycoprotein and the cytotoxicity of chemotherapeutic drugs. Neoplasia 8:143–152
8. Zhernosekov KP, Filosofov DV, Baum RP et al (2007) Processing of generator-produced ^{68}Ga for medical application. J Nucl Med 48:1741–1748
9. Kunikane H, Zalupski MM, Ramachandran C et al (1997) Flow cytometric analysis of p-glycoprotein expression and drug efflux in human soft tissue and bone sarcomas. Cytometry 30:197–203
10. Thews O, Wolloscheck T, Dillenburg W et al (2004) Microenvironmental adaptation of experimental tumours to chronic vs acute hypoxia. Br J Cancer 91:1181–1189