DETERMINATION OF THE PLASMA INHIBITORY ACTIVITY OF DRUGS BASED ON THE CELLULAR PHOSPHORYLATION ASSAY

Kira Böhmer, Holger Weber, Daniel Feger, Marianne Birkle, Oliver Siedentopf, Melanie Müller, Sarah Umber, Jan E. Ehlert. Reaction Biology, Freiburg, Germany

Introduction

Anticipation of the in vivo activity of new investigational drugs is challenging. In vitro cell assays are performed in artificial cell culture media which lack components that influence drug activity in the human body. This is why we established a method to analyze the activity of kinase inhibitors in human or rodent plasma (plasma inhibitory activity, PIA) in high throughput compatible ELISA-based cellular phosphorlyation assays. By testing reference compounds such as Sunitinib or Sorafenib, we confirm expected correlations between the plasma binding of a compound and the loss of its activity. Further, samples from Sunitinib treated mice were tested to analyze the time kinetics of this inhibitor in the blood after oral application.

We were able to establish a reliable in vitro PIA assay to predict the in vivo effect of kinase inhibitors and to determine the amount of bioavailable test substance in the blood of treated animals.

Methods:

Standard cellular phosphorylation assay

MEF-FLT3-wt (FLT3 assay) or human chronic myelogenous leukemia K562 cells (BCR-Abl assay)

were plated in multiwell cell culture plates. After serum-starvation overnight, cells were incubated

with compounds for 90 min in serum-free medium. In the FLT3 assay cells were stimulated with human

FLT3-ligand to induce receptor tyrosine autophosphorylation before cells were lysed. BCR-Abl is

not stimulated. Phosphorylated substrates from lysed cells were quantified by ELISA and the IC50

was determined using the sigmoidal slope regression in the software GraphPad Prism 5.

PIA cellular phosphorylation assay

In the PIA cellular phosphorylation assay the cells were incubated with the compounds in 100% (FLT3) or 50% (BCR-Abl) heat inactivated plasma. Since K562 is a suspension cell line the medium can not be removed from the cells in the assay plate, which is why plasma was added 1:1 to the cells in serum-free medium. All other procedures remained the same as in the standard protocol.

Plasma preparation and heat inactivation

Plasma was prepared by centrifugation of Li-Heparin-blood for 10 min at 4000rpm. After addition of

the compounds the plasma was heat inactivated at 56°C for 30 min in a water bath.

Cell viability testing

MEF-FLT3-wt cells were plated and the following day calcein-AM (3 µM) was added to visualize live cells. After 30 minutes images were taken (time point 0). Thereafter the medium was removed and 50 µl of

either untreated or heat inactivated plasma was added. 50µl of medium served as control.

The plasma was spiked with calcein-AM (3 µM). Further green fluorescent images were taken 90 minutes

after plasma application.

In vivo pharmacokinetics

Blood was taken from mice at four time points after oral application of Sunitinib. Plasma was

prepared from Li-Heparin-blood and tested in the PIA FLT3 phosphorylation assay after heat inactivation.

Plasmas from three different mice per time point were tested in duplicates.

Result 1:
Standard cellular phosphorylation assay

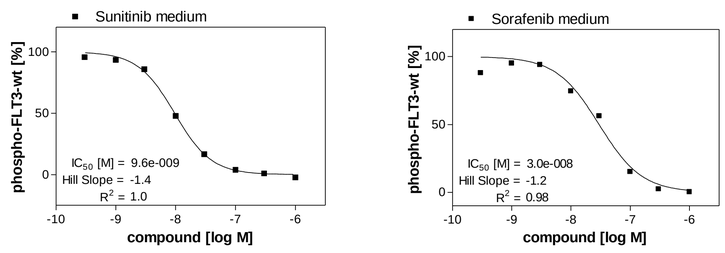


Figure 1: IC50 dose responses of Sunitinib and Sorafenib on FLT3-wt mediated autophosphorylation determined in the cellular phosphorylation assay using the standard assay set up.

Result 2:
Heat Inactivation of Plasma Prevents Plasma Induced Cell Death

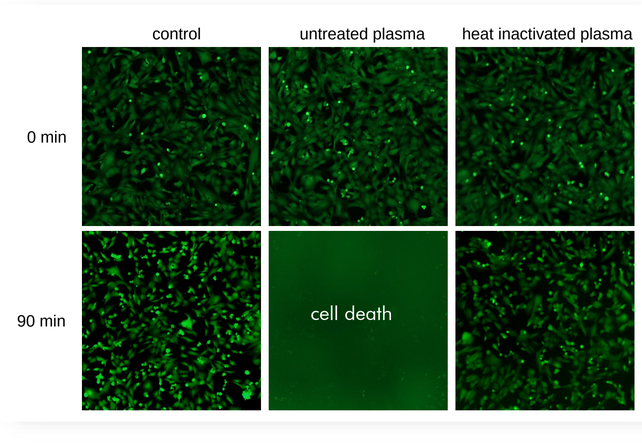


Figure 2: The cell viability testing showed that after a 90 min incubation of MEF-FLT3-wt cells with untreated human plasma the number of living cells decreases drastically in comparison to the control. This effect of the plasma can be prevented by heat inactivation at 56°C for 30 min.

Result 3:
PIA assay performed with FLT3-wt

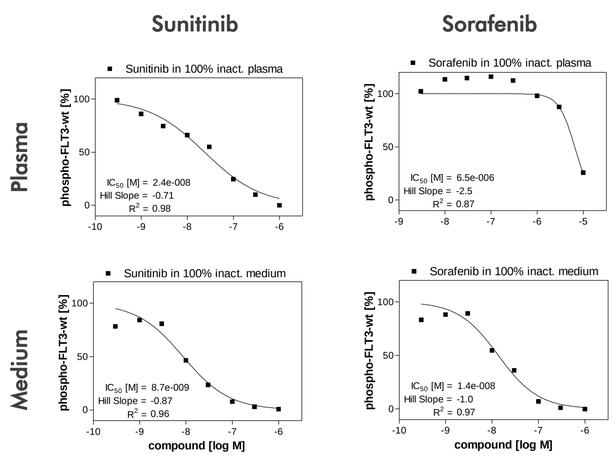


Figure 3: After heat inactivation of plasma and medium, dose response curves could be generated in the cellular phosphorylation assay. The activity of Sunitinib as a molecule with low plasma binding is only slightly affected by the presence of human plasma. The activity of Sorafenib which is highly plasma binding is reduced more than 460fold in the presence of human plasma.

Result 4:
PIA assay performed on BCR-Abl

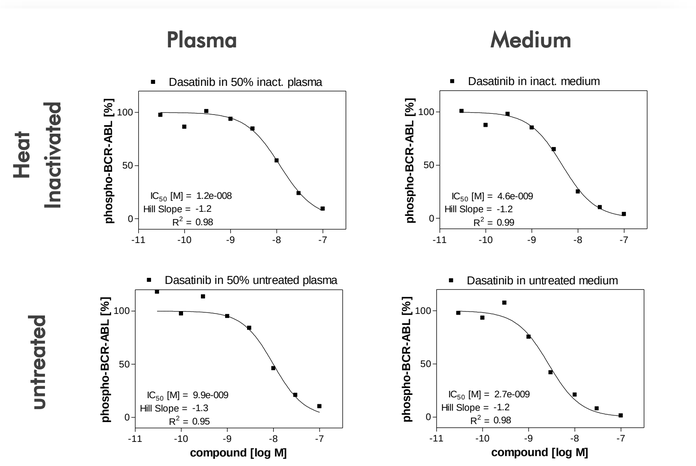
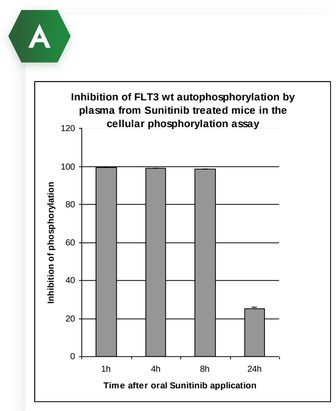
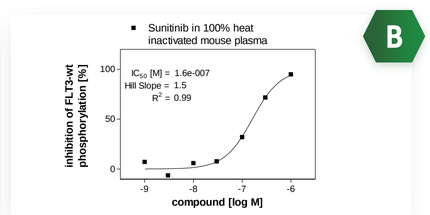


Figure 4: In the BCR-Abl cellular phosphorylation assay human K562 cells are used which are not sensitive to human plasma. Establishing the PIA method for this assay we could show that heat inactivation of plasma does not affect its interaction with the compounds.

Result 5:
PIA assay can be used to analyze time kinetics of compounds in vivo





C

Timepoint	Inhibition of phosphorylation	conc. Sunitinib (M)
1h	99,4	≥ 1,0E-06
4h	98,9	≥ 1,0E-06
8h	98,6	≥ 1,0E-06
24h	25,4	8,00E-08

Figure 5: A) The inhibitory effect on FLT3 autophosphorylation was assessed for plasmas from mice taken at four different time points after oral application of Sunitinib (80mg/kg). B) Dose response curve for Sunitinib spiked in mouse plasma is shown as control. C) Concentration of Sunitinib in mouse plasma after different time points.

Conclusion:

- PIA assay was successfully developed as tool to investigate the impact of plasma protein binding on the activity of compound in vitro and in vivo
- PIA assay can be used to determine the amount of active compound in the plasma of treated animals and potentially also humans
- The cellular phosphorylation assay measures the activity of kinase inhibitors in living cells on natural substrates and therefore provides translationable results
- Heat inactivation of plasma blocks plasma induced death of cell culture
- Besides FLT3-wt and BCR-Abl, the PIA assay can be performed to measure other kinase enzymes

Contact Information

Jan Ehlert, PhD, Head of Cellular Drug Discovery

Reaction Biology Europe GmbH
Engesserstr. 4
Freiburg, Germany

+49-761-769996-0
j.ehlert@reactionbiology.de
www.reactionbiology.com