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Implementing Reverse Phase Protein Array profiling as a sensitive method for the early pre-clinical detection of off-target toxicities associated with Sunitinib Malate

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List of abbreviations:

TKI	Tyrosine Kinase Inhibitor
RPPA	Reverse Phase Protein Array
VEGF	Vascular endothelial growth factor
PDGF	Platelet derived growth factor
AMPK	Adenosine monophosphate [AMP]-activated protein kinase
AEs	Adverse events
HTN	Hypertension
HFS	Hand-foot syndrome
EF	Ejection fraction
HIF	Hypoxia inducible factor
CLB	Cell lysis buffer

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BB	Blocking buffer
RFI	Relative fluorescence intensities
QC	Quality control
IPA®	Ingenuity Pathway Analysis
CO_2	Carbon dioxide
O_2	Oxygen
TEM	Transmission electron microscopy
Nm	Nanometer
mg/dL	milligrams per decilitre

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Abstract

The tyrosine kinase inhibitor (TKI) sunitinib is a multi-targeted agent approved across multiple cancer indications. Nevertheless, since approval, data has emerged to describe a worrisome side effect profile including hypertension, hand-foot syndrome, fatigue, diarrhoea, mucositis, proteinuria and (rarely) congestive heart failure. It has been hypothesised that the observed multi-parameter toxicity profile is related to 'on-target' kinase inhibition in 'off-target' tissues. Herein, to interrogate off-target effects in pre-clinical studies, a reverse phase protein array (RPPA) approach was employed. Mice were treated with sunitinib (40 mg/kg) for 4 weeks, following which critical organs were removed. The Zeptosens RPPA platform was employed for protein expression analysis. Differentially expressed proteins associated with damage and/or stress were found in the majority of organs from treated animals. Proteins differentially expressed in the heart were associated with myocardial hypertrophy, ischaemia/reperfusion and hypoxia. However, hypertrophy was not evidenced on histology. Mild proteinuria was observed, however no changes in renal glomerular structure were visible via electron microscopy. In skin, proteins associated with cutaneous inflammation, keratinocyte hyper-proliferation, and increased inflammatory response were differentially expressed. We posit that pre-clinical implementation of a combined histopathological/RPPA approach provides a sensitive method to mechanistically elucidate the early manifestation of TKI on-target / organ off-target toxicities.

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Statement of significance of the study

Despite clinical approval across a number of cancer indications, the side effect profile of sunitinib is more extensive than originally predicted, likely due to its impact on off target organs, kinases and proteins. This side effect profile was underestimated in preclinical studies, thus highlighting the need for improved models that can better predict toxicity. Herein, we report that when appropriate rodent models are combined with sensitive Reverse Phase Protein Arrays (RPPA) analyses, mechanistic insights into the side-effect profiles of TKIs may be elucidated. As RPPAs are high throughput and relatively cost-effective when compared to other proteomics methods the platform has potential for application within a toxicity "screen" providing clues towards future clinical adverse events (AE) profiles, whilst concurrently providing information on underlying toxicity mechanisms of action. We posit that appropriate animal models, used together with high-throughput RPPA methods will support better-informed clinical trials and more accurately predict clinical AEs. An improved understanding of toxicity mechanisms of action supports the optimal treatment of clinical adverse events, which neither delay nor compromise patient treatment protocols.

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Introduction

Sunitinib is an orally available tyrosine kinase inhibitor (TKI) approved for the treatment of advanced renal cell carcinoma, gastrointestinal stromal tumours and pancreatic neuroendocrine tumours [1] which is currently being studied in >500 trials across diverse cancer indications [2]. Although originally designed to target specific tyrosine kinases including vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), Fms-like tyrosine receptor kinase-3 and adenosine monophosphate [AMP]-activated protein kinase (AMPK) amongst others [3], it is now widely accepted that a more extensive range of kinases are targeted [4]. As a result, a significant toxicity profile has been reported [5] and although this may result from off-target effects, others potentially result from on-target actions in off-target organs i.e. “on-target toxicities”. The most commonly reported adverse effects (AEs) are fatigue diarrhoea, nausea/vomiting, mucositis, hypertension (HTN), planar-plantar erythrodysesthesia (hand-foot syndrome [HFS]), rashes and other skin toxicities, hypothyroidism, and decreased ejection fraction (EF) as well as a number of blood abnormalities [5].

Although several mechanisms underpinning sunitinib toxicity have been proposed, molecular pathways have yet to be fully elucidated. For example, we (and others) have previously shown that perturbations in cardiac energy metabolism as well as alterations in cardiac perfusion likely comprise two aspects of sunitinib induced cardiotoxicity [6, 7]. Other off-target interactions of sunitinib include hypoxia inducible factor (HIF)-1, glucose transporter 1 and AMPK, all of which perturb cellular energetics and metabolism [8]. Nevertheless, for the majority of AEs, underlying mechanisms are unknown and studies that investigate mechanisms of systemic sunitinib toxicity are sparse. Further elucidation would facilitate improved patient management, limit dose interruptions and thus improve outcomes. As it is not feasible to collect systemic tissue samples from patients on clinical trials, the use of appropriate pre-clinical models to investigate mechanisms of toxicity are warranted to provide additional information to drug development companies and the research community prior to conducting safety studies in patients.

Herein, using a clinically relevant mouse model we performed a pre-clinical “phase I” trial to recapitulate observed systemic effects following sunitinib treatment. To determine toxicity pathways a high throughput reverse phase protein array (RPPA) approach was employed. We demonstrate the utility of RPPA as a sensitive method to mechanistically interrogate the toxicity profile associated with TKIs.

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Materials and Methods

Animal studies

Female Balb/CJ mice (n = 39, 6 - 8 weeks, Charles River Laboratories, Sandwich, UK) were housed in groups of 3 - 5, maintained on a 12 hour light/dark cycle, with free access to standard rodent chow and water. Animal experiments conformed to guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Experiments were licensed and approved by the Department of Health and Children, Dublin, Ireland (License: B100/3654). Protocols were reviewed and approved by University College Dublin Animal Research Ethics Committee.

Animals were randomised and treated Monday-Friday for 4 weeks (oral gavage) with 40 mg/kg sunitinib (Sequoia Research Products, Pangbourne, UK) reconstituted in sterile water. Vehicle control cohorts received sterile water. We have previously shown that this model is physiologically relevant with animals having increases in blood pressure and decreases in EF.[7] *Reverse Phase Protein Arrays (RPPA)*

Following 4 weeks of sunitinib / vehicle treatment, protein was extracted from snap frozen tissue (heart, kidney, liver, brain, intestine, skin) (n = 5/group) and RPPAs were performed as previously described).[9] (Refer to Supporting Information Resource 1 for full details including methods, protein yield and normalisation).

For the arrays, 111 primary antibodies (refer to Supporting Information Resource 1, Table 1 for antibody information) were selected following an extensive literature search to ascertain key tyrosine kinase effector pathways in tissues. Previously generated antibody performance status was used to refine antibody selection. For the RPPA, following a blocking step, primary antibodies were incubated overnight and following wash steps, Alexa Fluor® 647 secondary antibodies were applied for a 45-minute incubation. Excess secondary antibody was removed and the fluorescence readout of the arrays was performed on the ZeptoReader. For full method descriptions please refer to Supplementary Information Resource 1.

Quality control and fold-change analysis

A number of quality control (QC) measures were applied to the generated data (please refer to Supplementary Information Resource 1 for specific details) and subsequently, to analyse the effect of

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sunitinib treatment on protein expression, the RFIs of sunitinib treated tissue samples were compared to vehicle treated samples to give an average fold change (FC) value. Differences were compared using Mann Whitney tests without multiple testing correction (GraphPad Prism 5).

Gene Ontology and Pathway Analysis

Initially all negative values were removed from the raw data and treatment ratios for sunitinib calculated alongside p-values (Student's t-test) while comparing vehicle versus sunitinib treated samples across all tissues. These ratios and their calculated p-values were uploaded to Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) using the provided Java client from QIAGEN Ingenuity website. Proteins and phospho-proteins were mapped to 'HUGO Gene Nomenclature Committee (HGNC) gene symbol'. IPA 'Core Analysis' with 0.1 p-value and 1.5 FC cut-off was performed with default settings and human as species. Analysed results from 'Canonical Pathways', and 'Gene Ontology Functions' sections were exported in raw text format.

Histopathologic and pathophysiologic analysis of sunitinib effects

In separate cohorts of mice, following completion of 4 weeks of sunitinib treatment as described, animals were euthanized using CO_2 overdose and necropsy performed to remove key tissues and fluids.

Cardiac puncture under terminal anaesthesia was performed on n = 8 mice to collect serum to analyse the effects of sunitinib treatment on circulating triiodothyronine (T3) and thyroxine (T4) levels. Please refer to Supporting Information Resource 2 for detailed descriptions. In N = 14 mice, the heart, kidney, liver, brain, skin, thyroid and intestine samples were formalin-fixed and paraffin-embedded. Sections of 5 µm were cut and routine H&E staining was performed to facilitate histological evaluation by a veterinary pathologist. Morphometric analysis of thyroid follicles was carried out in 6 of these mice, by determining the average height of a single follicle, as the average of 4 heights in each follicle. A total of 30 follicles (when possible) were randomly selected for each thyroid sample. The average height of the thyroid epithelium, of each thyroid sample, was determined as the average of the height of the 30 single follicles and it was used as a parameter correlated with the functional status of the thyroid gland. A software viewer was used for digital image analysis and analysis of the data was performed using Graph Pad Prism version 5 software (GraphPad Software, Inc., La Jolla, CA, USA). For proteinuria analysis 6

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mice from this cohort were housed in metabolic cages for 24 hours prior to euthanasia and urine was collected and analysed using urinalysis multi-sticks.

Finally for transmission electron microscopy (TEM), whole kidney samples were specially processed (N = 7). Please refer to Supporting Information Resource 2 for detailed methods).

Statistical Analyses

For all experiments a p-value of < 0.05 was deemed to be significant. Please refer to figure legends for statistical tests used. Error bars represent SEM unless otherwise indicated. As no correction for multiple testing was performed (for RPPA studies), p-values have been used for hypothesis generation. Literature searches were further performed to determine if and how proteins might play a role in toxicity.

Results

RPPA analysis reveals key proteins that may be involved in 'on-target toxicity' of sunitinib

Following 4 weeks of sunitinib (40 mg/kg, daily oral gavage) or vehicle (water, daily oral gavage) treatment, Balb/CJ mice (N = 5 per group) were euthanised and tissues snap-frozen for downstream analysis. RPPA analysis was performed on 6 tissues (heart, kidney, liver, brain, skin, intestine) to investigate whether pathways or proteins involved in sunitinib toxicity could be elucidated [7].

Following QC, 100 out of 111 proteins that were investigated by RPPA analysis were suitable for inclusion. RFI values from each antibody were grouped according to tissue/organ and for basic FC analysis; RFIs from sunitinib treated animals were divided by RFIs from vehicle treated animals. FCs above 1.5 (for up-regulation) or below 0.5 (for down-regulation) as well significance p-values of ≤ 0.05 when comparing vehicle and treatment groups were deemed to be of highest importance.

Ten proteins appeared to be differentially expressed in sunitinib versus vehicle treated groups across all tissue/organs (Table 1 and Figure 1). Please refer to Supporting Information Resource 3 for FCs of all proteins that did not reach statistical significance and/or FC criteria.

We next employed the Gene Ontology (GO) tool to investigate potential functions of differentially expressed proteins in the sunitinib treated group. Several categories of interest were identified that may be related to sunitinib toxicity. In the hearts of mice treated with sunitinib, categories identified relate to

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cell proliferation and differentiation, angiogenesis, vasculogenesis, fibroblast cell death, proliferation of epithelial and muscle cells and alterations in glycolysis. (Refer to Supporting Information Resource 4 Table 1 to see the implicated genes). In the kidneys of mice treated with sunitinib, categories identified relate to cell migration and movement, proliferation, cell cycle and angio-/vasculo-genesis (Supporting Information Resource 4, Table2). Similarly, in the brain (Supporting Information Resource 4, Table 3), categories identified relate to the cell cycle as well to neuronal cell death and cytotoxicity. In the intestinal tissue of mice treated with sunitinib, categories identified relate to cell cycle dynamics as well as cytotoxic cellular reactions, cell death, apoptosis and autophagy functions and to smooth muscle and epithelial cell proliferation (Supporting Information Resource 4, Table 4). Finally, in the skin of mice treated with sunitinib (Supporting Information resource 4, Table 5), categories identified relate to cellular proliferation and development of epithelial stromal cells, the proliferation of fibroblasts, the aetiology of fibrosis, hypoplasia and amyloidosis.

IPA® analysis highlighted a number of enriched pathways that may be implicated in the systemic toxicity of sunitinib treated patients (Table 2) including AMPK signalling and the NFAT and STAT3 pathways (heart), the STAT3 pathway and AMPK signalling (kidney), the neuregulin, ErbB, mTOR and p70S6K signalling pathways (brain) and the PI3K/AKT pathway (intestine).

Histologic and pathophysiologic effects of sunitinib treatment

We have previously shown that treatment with 40 mg/kg of sunitinib in the Balb/CJ mouse model leads to the development of HTN and also to decreased LVEF, mirroring the effects of sunitinib seen in the clinic.[7] Despite this, herein, following a 4 week sunitinib dosing period, gross histopathological analyses indicated no abnormalities in the heart, kidney, liver, brain, small and large intestine, or the skin that may be associated with sunitinib treatment (Figure 2 A - G).

Due to the presence of hypertension in this model, which is also evidenced clinically [5, 7], it was somewhat unexpected that no pathology was noted in the kidney. As such, additional studies were undertaken. Electron microscopy was performed on a cohort of samples (N = 7) to determine potential effects on the glomeruli capillary network: Basal lamina thickness, capillary lumen circumference and lumen area were analysed. Overall no differences were evident between the sunitinib treated and untreated groups (Figure 3A). Clinically, decreased kidney function can manifest as proteinuria; to assess this in mice, dipstick urinalysis was performed (N = 6). Sunitinib treatment resulted in human

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equivalent grade 2 proteinuria (30-100 mg/dL of protein in the urine; 1-2 g per day). The effect of sunitinib on thyroid morphology and function was also assessed to investigate preclinical evidence of clinically reported thyroid AEs. [10, 11] A significant decrease in thyroid epithelial cell height in the thyroids of mice (N = 3/group) treated with 40 mg/ kg sunitinib was observed (Figure 3C and 3D). Levels of the circulating thyroid hormones T3 and T4 were measured in another cohort (N = 3 vehicle, N = 5 sunitinib) of mice with no significant differences found between groups data not shown). These findings suggest presence of sub-clinical hypothyroidism, which has been previously been linked to poor glomerular filtration rates [10].

Discussion

The toxicity profile associated with sunitinib treatment in patients is extensive, notwithstanding its development as a "targeted" drug, having an assumed limited systemic toxicity profile as compared with standard chemotherapy drugs. This clinical toxicity profile, now commonly reported and acknowledged, was initially unexpected partly as these toxicities were not recorded in preclinical studies, which supported clinical trials [12]. Herein, we propose high throughput RPPA as a sensitive technique to reveal potential toxicities prior to overt patho-physiologic manifestation, and propose that this method may be appropriate in future preclinical testing of new TKIs to predict AEs in the clinic.

RPPA works on the premise of dot-blot probing for specific proteins in crude tissue lysates that are printed as small dots on a solid support.[13] This platform has evolved as a high throughput tool by incorporating robotic arraying techniques to achieve a higher accuracy and better signal to noise ratios [13], with the use of chemi-luminescence to improve signal detection and the integration of a bioinformatic analysis pipeline [14]. Currently, this highly adaptable and flexible platform may be employed to monitor changes in protein expression and activation over time, before and after treatment, between disease and non-disease states, and to compare proteomic changes between subject responders and non-responders [15]. Consequently RPPA technology is emerging as an important tool in the development of patient tailored therapies. Van Oostrum et al. have suggested that one of the powers of RPPA is in the early recognition of 'off target' side effects of drug candidates [16]. Nevertheless, to date, the use of RPPA as a tool to investigate off target toxicity effects remains under-utilized. Herein, we implement RPPA to detect early changes in protein expression that may be implicated in the development of 'on-target' toxicities.

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Sunitinib associated cardiotoxicity (HTN, decreased EF) and the mechanisms involved have been extensively studied. We have previously reported that glucose and oxidative metabolism are perturbed in the myocardium of sunitinib treated mice despite no overt pathology, implicating altered cardiac energy metabolism in the development of sunitinib associated cardiotoxicity [7]. In the current study, we reconfirmed that no overt pathophysiological differences manifest following sunitinib treatment (Figure 2A). Nevertheless, IPA analysis of cardiac RPPA data implicates pathways involved in cardiac necrosis, dysfunction, proliferation, hypertrophy and fibrosis, whilst the STAT3 pathway and AMPK signalling are also perturbed. AMPK signalling has been established as a mechanism underpinning perturbed cardiac function [6] due to its effect on energy homeostasis in the cardiomyocytes, whilst the STAT3 pathway has a role in cardiomyocyte protection and hypertrophy [17]. Individually, proteins that appeared to be modified by sunitinib treatment included increased PFKFB3, which is involved in the control of glucose metabolism and the cell cycle [18]. PFKFB3 has been shown to be up-regulated in hypoxia-stress conditions, which may represent a protective mechanism in an environment of depleted energy regulation [19]. This finding is in line with our previously reported observation of glucose metabolism alterations in the myocardium of sunitinib treated mice [7]. Increased eiF2α, Cyclin D1 and Src (with further evidence presented herein) have been implicated in endoplasmic reticulum stress, contributing to ischemia-induced cardiomyocyte apoptosis (eiF2α) [20], and in the induction of cardiomyocyte hypertrophy in rodent models (Cyclin D1/Src) [21]. FGFR3 has been shown to be most abundantly expressed in cardiomyocytes [22] however, the function of this receptor has not been described in detail.

RPPA analysis of kidney tissue indicated that pathways involved in renal necrosis, cell death, hyperplasia, hyper-proliferation, inflammation, nephritis, dysfunction and glomerular injury were affected by sunitinib compared to vehicle treatment. Elsewhere, the STAT3 and PI3K/AKT pathways have been implicated in drug induced nephrotoxicity [23], whilst HGF is involved in kidney repair mechanisms [24]. As VEGF and AMPK are known target pathways of sunitinib, their implication herein is not surprising and supports the “on-target” toxicity findings. In the current study elevated protein (equivalent to grade 2 proteinuria) was detected in the urine of mice treated with 40 mg/kg sunitinib for 4 weeks, appropriately replicating clinically observed TKI induced proteinuria [25]. Importantly, no significant pathophysiological findings were noted by microscopy at the cellular (Figure 2B and 2C) or subcellular level (Figure 3A), again demonstrating that RPPA analysis of preclinical models may detect sub-clinical manifestations of clinical toxicities.

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Hepatic failure is not commonly reported among sunitinib patients. We report neither notable pathway disruption (RPPA) nor histopathological findings (Figure 2D) in the livers of sunitinib treated mice. Similarly, low incidence of brain toxicities are reported in sunitinib treated patients and no histopathologic findings were seen herein (Figure 2E). Nevertheless, reversible posterior leukoencephalopathy syndrome (RPLS) has been sparsely reported with clinical symptoms manifesting as epileptic seizures, altered mental status, vomiting, visual disturbances and headache [26, 27]. The exact pathogenesis of RPLS remains unclear but it is most likely due to a disruption of cerebral vascular endothelial cells, impaired cerebrovascular autoregulation leading to oedema, and HTN [26, 28]. Known targets of sunitinib include those involved in endothelial cell proliferation whilst HTN as discussed, is common reported. Nevertheless, there were no pathophysiological changes observed in the brains of sunitinib treated mice in this study. ErbB3/HER3/EGFR appeared to be more highly expressed in sunitinib treated brain tissue compared to vehicle, whilst enriched pathways indicated by IPA analysis included neuregulin, ErbB and the p70S6K and mTOR signalling networks. Using the Gene Ontology (GO) tool to investigate potential functions of differentially expressed proteins in the sunitinib treated group, categories identified were those involved in necrosis, apoptosis of neuroglia, neuronal cell death and cytotoxicity (Supporting Information Resource 4, Table 3). Again, changes at the protein level (and detectable by RPPA) may precede overt pathophysiological changes. Further work (beyond the scope of the current study) are required to determine if perturbations in these pathways conclusively underpin sunitinib induced brain toxicity.

Intestinal toxicities during sunitinib treatment includes mucositis and diarrhoea. Bowel perforation is also a complication of sunitinib treatment in a small number of cases however the mechanism is unknown [29]. Herein, diarrhoea was not observed in animals undergoing treatment. Moreover, no evidence of toxicity was seen in the intestinal tissue of mice treated with sunitinib upon histopathological evaluation (Figure 2F). However, RPPA analysis of intestinal tissue showed that throughout the intestine, p21 appeared elevated in mice treated with sunitinib compared to vehicle treated mice (Table 1). Pathway analysis further implicates epithelial and smooth muscle cells pathways associated with cell death, apoptosis and autophagy.

HFS is a more widely reported and debilitating AE associated with sunitinib treatment. RPPA analysis of skin taken from sunitinib treated animals indicated that pathways involved in epithelial cell

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proliferation, connective tissue cell death, proliferation of fibroblasts, development of fibrosis and fibromatosis, differentiation of keratinocytes and the development of hypoplasia and amyloidosis were potentially involved (Supporting Information Resource 4, Table 5). When individual proteins were analysed, phosphorylated fibroblast growth factor receptor (pFGFR) appeared to be present at lower levels in sunitinib compared to vehicle treated skin tissue (Table 1). Our RPPA data thus support clinical observations where sunitinib induced HFS manifests as the skin being hyperkeratotic, and thickened in nature [30] with keratinocyte necrosis, inflammatory cell infiltration and dilated vessels being observed [31]. Moreover keratinocyte differentiation has been suggested as a potential mechanism of action in HFS as a result of sorafenib treatment [32]. In this context the RPPA data presented herein may indicate early changes in the pathways that ultimately result in HFS.

Concluding Remarks

Implementation of an RPPA platform provides a sensitive method to mechanistically elucidate the potential off-target and on-target toxicities associated with TKIs approved in the oncology setting. Furthermore, we propose that RPPA allows detection of drug-induced molecular changes prior to the onset of histological changes. Such molecular data emerging from appropriate pre-clinical studies could significantly enhance the clinical development of broad-spectrum TKIs. Using RPPA, we report on a panel of altered proteins that are likely involved in the systemic toxicity following sunitinib treatment. Orthogonal confirmation of findings (e.g. by IHC) could further strengthen these data, but were not possible herein due to lack of availability of appropriately processed tissue. Nevertheless, Bayer Technology Services (co-author) had previously considered the performance status of antibodies, based on signal intensities and quality across multiple studies. This information was considered when making selections for the current project.

To the best of our knowledge analysis of the systemic off-target effects of a TKI using a state of the art, highly sensitive, proteomic profiling approach in a clinically relevant preclinical model has not previously been undertaken. Although a number of pathways and proteins have been previously implicated, here we uncover potential novel pathways by which sunitinib exerts its off-target effects. Interestingly, a number of kinases are likely expressed at higher levels following sunitinib treatment, which may result from adaptation mechanisms.

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Conflict of Interest: M.G is employed at Bayer AG, Wuppertal. All other authors have declared no conflict of interest

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Figure Legends

Figure 1: **Differential expression of key proteins in organs of sunitinib and vehicle treated mice indicated by RPPA analysis, potentially implicates them in on-target toxicity.** Following 4 weeks of treatment with vehicle or 40 mg/kg sunitinib, tissues were removed, snap frozen and RPPA analysis performed as described. Representative graphs of the most significantly altered proteins are presented A) PFKFB3 RFI in the heart B) IκBa (ph. Ser 32/36) in the kidney C) ErbB-3/HER3/EGFR P Try 1289 in the Brain D) p21 CIP/WAF in the large and small intestine (grouped) and E) FGF-R p Tyr 653/654 in the skin.

Figure 2: **Sunitinib treatment does not cause gross pathohistologic changes in mouse organs.** Tissues were removed from animals treated with vehicle or 40mg/kg sunitinib after 4 weeks of therapy. All organs were fixed in 10% formalin and subsequently processed for histochemical analysis. Organs from N=3 animals were analysed in the vehicle group, whilst N = 5 in the sunitinib treated group. Representative images are shown for A) Heart, x40 mag, scale bars = 50uM B) Kidney, x5 mag, scale bars = 500uM, C) Kidney x40 mag, scale bars = 50uM D) Liver, x20 mag, scale bars = 100uM E) Brain, x20 mag, scale bars = 100uM F) Intestine, x40 mag, scale bars = 50uM G) Skin x 20 mag, scale bars = 100uM.

Figure 3: **Sunitinib treatment perturbs thyroid epithelial morphology but does not affect the glomerular capillary network in rodent models.** Following 4 weeks of sunitinib or vehicle treatment, tissues were removed and processed as described in materials and methods. A and B) Analysis of TEM images of the glomerular capillaries from sunitinib and vehicle treated mice indicated that as a group there were no overt changes to the morphology (mean luminal area, mean luminal circumference or mean basal laminal thickness) of the vessels (n = 4 sunitinib, 3 vehicle, error bars = SEM). B) Although no significant findings were found in the glomerulus of the majority of mice treated with sunitinib, one mouse out of 4 did display some basal laminal thickening and endothelial proliferation (black arrows) Scale bars = 1uM, Mag = x5000. C and D) In the thyroid, grouped analysis of thyroid epithelial height in mice treated with vehicle or 40 mg/kg sunitinib for 4 weeks indicated a thinner epithelial cell layer in mice treated with sunitinib ((C) *$p \leq 0.05$, n = 6, Error bars represent SEM.). D) Representative images of the thyroid epithelial cells (black arrows) from mice treated with vehicle or 40mg/kg sunitinib for 4 weeks. Scale bar = 50μm, magnification x56).

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Table 1 Proteins differentially expressed in tissue samples from sunitinib treated mice compared to vehicle treated (Mann Whitney *p ≤ 0.05,**p ≤ 0.01, without multiple testing correction).

Heart	Vehicle Mean Normalised RFI (SD)	Sunitinib Mean Normalised RFI (SD)	Fold change
eIF2α	0.13(0.05)	0.31(0.08)	2.46**
FGF-R3	1.70(0.41)	2.66(0.27)	1.57**
PFKFB3	0.71(0.15)	2.04(0.33)	2.88**
Cyclin D1	0.69(0.14)	1.23(0.26)	1.78*
Src	0.01(0.02)	0.09(0.04)	7.18*
Kidney			
IkBα phSer32/36	0.17(0.02)	0.26(0.03)	1.54**
Cyclin D1	0.74(0.37)	1.31(0.26)	1.78*
Brain			
ErbB3/HER3/EGFR phTyr 1289	0.05(0.02)	0.09(0.03)	1.97*
Intestine [grouped large / small intestine]			
p21 CIP/WAF1	0.37(0.15)	0.57(0.21)	1.54*
Skin			
FGF Receptor phTyr653/654	0.89(0.38)	0.41(0.15)	0.46*

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Table 2 IPA® identified enriched pathways that may be involved in the systemic toxicity of sunitinib

	-log(p-value)	Ratio	Genes
Heart			
STAT3 Pathway	7.52	0.0548	FGFR3,IGF1R,SRC,MAP2K1
NFAT in Cardiac Hypertrophy	5.95	0.0223	IGF1R,SRC,MAP2K1,CAMK2A
AMPK Signaling	4.13	0.0168	SRC,PFKFB3,CCND1
Kidney			
STAT3 Pathway	9.65	0.0685	FGFR3,MYC,SRC,CDKN1A,MAP2K1
HGF Signaling	6.68	0.0381	CCND1,CDKN1A,MAP2K1,PTK2
PI3K/AKT Signaling	6.41	0.0325	CCND1,CDKN1A,MAP2K1,NFKBIA
FGF Signaling	4.96	0.0353	FGFR3,MAP2K1,MAPKAPK2
VEGF Signaling	4.85	0.0326	SRC,MAP2K1,PTK2
AMPK Signaling	3.99	0.0168	SRC,CCND1,CDKN1A
Brain			
Neuregulin Signaling	8.8	0.0455	RPS6,PRKCD,ERBB3,RPS6KB1
ErbB Signaling	6.15	0.0349	PRKCD,ERBB3,RPS6KB1
p70S6K Signalling	5.72	0.0252	RPS6,PRKCD,RPS6KB1
mTOR Signalling	5.13	0.016	RPS6,PRKCD,RPS6KB1
Intestine			
PI3K/AKT Signalling	8.91	0.0325	AKT1,CDKN1A,PRKCZ,BAD

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