Article

Non-Targeted Metabolomics Analysis of the effects of Tyrosine Kinase Inhibitors Sunitinib and Erlotinib on Heart, Muscle, Liver, and Serum Metabolism In Vivo

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**Abstract: Background.** More than 90 tyrosine kinases have been implicated in the pathogenesis of malignant transformation and tumor angiogenesis. Tyrosine kinase inhibitors (TKIs) have emerged as effective therapies in treating cancer by exploiting this kinase dependency. The tyrosine kinase inhibitor erlotinib targets epidermal growth factor receptor (EGFR), whereas sunitinib targets primarily vascular endothelial growth factor receptor (VEGRF) and platelet-derived growth factor receptor (PDGFR). TKIs impact the function of non-malignant cells had have on- and off-target toxicities, including cardiotoxicities. Most of the reports of sunitinib have identified cardiotoxic effects, whereas erlotinib was less often found to have these effects. We hypothesized that the deleterious effects of TKIs were related to their impact on cardiac metabolism. **Methods.** C57BL/6 mice (10/group) were treated with therapeutic doses of sunitinib (40 mg/kg), erlotinib (50 mg/kg), or vehicle daily for 2 weeks. Echocardiographic assessment of the heart in vivo identified significant systolic dysfunction consistent with cardiotoxicity compared to vehicle treated controls. Heart, skeletal muscle, liver, and serum were flash frozen and prepped for non-targeted GC-MS metabolomics analysis. **Results.** Compared to vehicle treated controls, sunitinib treated mice had significant decreases in systolic function, whereas erlotinib treated mice did not. Non-Targeted metabolomics analysis of heart identified identified significant decreases in Docosahexaenoic acid (DHA), Arachidonic Acid/Eicosapentaenoic acid (EPA), O-Phosphocolamine, and 6-Hydroxynicotinic acid after sunitinib treatment. DHA was significantly decreased in skeletal muscle (quadriceps femoris), with elevations in cholesterol were identified in liver and elevated ethanol amine in serum. In contrast, erlotinib affected only one metabolite elevated (spermidine significantly increased). **Conclusions.** Mice treated with sunitinib had exhibited systolic dysfunction within two weeks, with significantly lower heart and skeletal muscle levels of long chain omega-3 fatty acids docosahexaenoic acid (DHA), arachidonic acid (AA)/eicosapentaenoic acid (EPA) and increased serum O-Phosphocholine phospholipid. This is the first link between sunitinib-induced cardiotoxicity and depletion in the polyunsaturated fatty acids (PUFAs) and inflammatory mediators DHA and AA/EPA in the heart, possibly by affecting mitochondria function where they have vital roles on calcium channels.

**Keywords:** erlotinib; sorafenib; kinase inhibitors; cardiotoxicity; metabolomics; serum; liver; muscle; heart

Non-standard abbreviations: DHA, Docosahexaenoic Acid; EPA, Eicosapentaenoic Acid, EGFR, epidermal growth factor receptor; IHD, ischemic heart disease; TKI, tyrosine kinase inhibitors; VEGRF, vascular endothelial growth factor receptor

1. Introduction

More than 90 tyrosine kinases have been implicated in the pathogenesis of malignant transformation and tumor angiogenesis [1,2]. Tyrosine kinase inhibitors (TKIs) have proven effective in cancer treatments by exploiting this kinase dependency [3]. The tyrosine kinase inhibitor sunitinib targets primarily vascular endothelial growth factor receptor (VEGRF) and platelet-derived growth factor receptor (PDGFR), whie erlotinib targets epidermal growth factor receptor (EGFR) [4]. Despite their specific targeting of tyrosine kinases, TKIs impact the function of non-malignant cells had have on- and off-target toxicities [4].

Recent population based observational cohort studies have assessed the rates of cardiovascular disease after treatment with sunitinib and erlotinib [5]. Examination of 141,601 individual case safety reports extracted from VigiBase® for TKIs (including sunitinib and erlotinib), 2594 cardiac failure cases were identified, with sunitinib among five KI (with dasatinib, imatinib, bosutinib, and nilotinib) disproportionally increased risk of cardiac failure (1.67 95% CL 1.51, 1.84) [6]. Subsequent studies tested sunitinib and erlotinib on force-generating engineered heart tissues from neonatal rat heart cells [7]. After 96 hours of incubation, a concentration and time-dependent decline in contractile force was identified with sunitinib, but not erlotinib, treatment [7]. This decline was associated with an impairment of autophagy and the appearance of autophagolycosomes. Studies directly testing cardiotoxicity of KIs in animals have found sunitinib cardiotoxic, but not erlotinib [8,9]. While sunitinib has consistently induced cardiotoxic effects, most studies of erlotinib have not found it cardiotoxic. However, one study has identified 11 erlotinib patients having ischemic heart disease [5]. In this population-based observational study with a 380-day median follow-up, 18 cases of ischemic heart disease (IHD) were identified, 11 which occurred in erlotonib patients (of 1046 total) and 5 in sunitinib treated patients (9 of 430 total) [5]. These cases occurred predominantly in the late follow-up period and do not provide a direct causal link between erlotinib and IHD [5]. Recent meta-analyses of clinical trials using sunitinib (1,077 people) identified an increased risk of heart failure (Relative Risk (RR) ratio of 4.3 with a number needed to harm (NNH) of 11) [10] . Neither sunitinib nor erlotinib was associated with an increased incidence of hypertension in these two studies [10].

Recent studies in human cardiomyocytes have identified sunitinib cytotoxicity due to sunitinib, but not erlotinib in vitro [11]. In these studies, sunitinib decreased cardiomyocyte viability, inhibited AMPK, increased lipid accumulation, disrupted beat pattern, and blocked hERG activity); in contrast, erlotinib demonstrated only minor changes (increased ACC phosphorylation, the rate limiting step in fatty acid biosynthesis) and did not impact ROS, caspase, or lipid levels and did not affect beat patterns) [11]. Similarly, sunitinb treatment in vivo enhances myocardial expression of pro-inflammatory cytokines and enhances the expression of pro-fibrotic factors, while decreasing factors that degrade collagen [12]. Interestingly, treatment with L-carnitine, which is known to shuttle free fatty acids from the cytosol into mitochondria for beta oxidation and energy production, is protective against these effects of sunitinib [12]. Collectively, these data suggest that sunitinib is cardiotoxic, whereas erlotinib is cardiosafe.

In the present study, mice were treated with sunitinib or erlotinib to determine their effects on metabolism at the level of the heart, liver, skeletal muscle, and serum using non-targeted metabolomics approach to find whether metabolic signatures distinguish cardiotoxic sunitinib from cardiosafe erlotinib and contribute to their distinct effects on the heart.

2. Results

Sunitinib is an orally delivered small molecule given in cycles at 50 mg per day for 4 weeks to maintain the therapeutic serum concentration (50-100 ng/ml) [13]. Dosing mice with 40 mg/kg yields comparable sunitinib concentrations and selectively inhibits VEGR2 and PDGF receptor phosphorylation [14,15]. Erlotinib is also orally delivered and given daily (150 mg) 1-2 hours after meals, resulting in Cmax of ~1500 ng/ml, with a half-life of ~16 hours [16]. In this study, we chose to treat mice with 50 mg/kg erlotinib daily, as this dosing achieves plasma concentrations within the therapeutic range for humans [17].

After two weeks of treatment, wild type female FVB-N mice treated with 40 mg/kg sunitinib were found to have impaired systolic function compared to vehicle-treated mice, whereas 50 mg/kg erlotinib did not affect contractile function (**Figure 1A**). Beyond significant increases in LV diameter after sunitinib (**Figure 1B**), conscious echocardiography analysis revealed no other alterations (**Table 1**). The apparent cardiotoxicity is consistent with previous studies demonstrating the much lower toxic threshold of sunitinib compared to erlotinib [7].

We next assayed heart, liver, skeletal muscle (quadriceps femoris), and serum collected after 2 weeks of TKI treatment using non-targeted metabolomics analysis to explore whether metabolic alterations may have contributed to the observed effects on cardiac function. In the hearts of mice treated with sunitinib, 92 metabolites were identified (**Figure A1, Table A1**), revealing primarily overlap between the sunitinib and vehicle control-treated mice (**Figure 2A**), consistent with only 5 metabolites identified as significant by t-test (**Figure 2B**). In erlotinib-treated mice, 87 metabolites were identified (**Figure A2, Table S2**), with little resolution between the erlotinib and vehicle-treated mice by principal components analysis (**Figure 2C**), and only one metabolite (spermidine) significantly increased by t-test (**Figure 2D**).

Given reports of both sunitinib-related hepatic failure [18] and erlotinib-related hepatotoxicity [19,20], we investigated the metabolic effects of sunitinib and erlotinib on liver. We identified 115 metabolites in sunitinib-treated livers (**Figure A3**) and 100 metabolites in erlotinib-treated livers (**Figure A4**). With considerable overlap in the metabolic features of sunitinib-treated and vehicle-control treated livers (**Figure 3A**), only cholesterol and sucrose (and similar disaccharides) were elevated with sunitinib treatment (**Figure 3B**). PCA analysis revealed considerable overlap between the liver metabolomes of erlotinib and vehicle-treated mice (**Figure 3C**), with homoserine and ornithine significantly decreased with erlotinib treatment (**Figure 3D**).

The effects of sunitinib treatment on skeletal muscle (quadriceps femoris) were investigated, where we identified 92 metabolites (**Figure A5, Table A1**), distinguished into two overlapping groups by PCA analysis (**Figure 4A**), and four significantly altered metabolites identified (**Figure 4B**), including significant decreases in dehydoralanine, adenosine, and docosahexaenoic acid. Eighty-three metabolites were identified from ertlotinib-treated quadriceps femoris (**Figure A6**), again largely overlapping with vehicle treatment (**Figure 4C**) two significantly altered metabolites by t-test, including dehydroalanine and C11 hydrocarbon (**Figure 4D**).

In serum from sunitinib and erlotinib treated mice, we identified 125 metabolites (**Figure A7, Figure A8**, respectively). Sunitinib treated serum had few changes from vehicle control treated mice (**Figure 5A**), with one ethanolamine being the only significantly increased metabolite (**Figure 5B**). Similarly, the metabolites identified in the ertlonib treated serum largely overlapped those of vehicle controls (**Figure 5C**), with only two significantly altered metabolites, including increased threonic acid and C14 hydrocarbon (**Figure 5D**).

3. Discussion

Sunitinib inhibits multiple tyrosine receptor tyrosine kinases including PDGFR, VEGFR, and CD117 (aka c-KIT) to reduce tumor burden through decreased vascularization and enhanced cancer cell apoptosis. Sunitinib has been approved by the FDA for the treatment of renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumor (GIST). Sunitinib cardiotoxicity has been reported in multiple clinical trials, but the specific mechanisms are not clearly understood. Erlotinib is a tyrosine kinase inhibitor that targets the EGFR in locally advanced or metastatic non-small cell lung cancer (NSCLC) that has failed a prior chemotherapy regimen. It has also been FDA approved for combination use with gemcitabine for the treatment of locally advanced, unresectable, or metastatic pancreatic cancer. Erlotinib is not clearly associated with cardiotoxicity.

Recent studies of the effects of sunitinib on isolated rodent hearts, revealed increases in TNFalpha and TnT in the perfusion buffer at the same time as impaired cardiac function, indicating direct cardiotoxicity [8]. In contrast, treating isolated hearts with erlotinib did not elicit increases in any of the biomarkers investigated (BNP, IL6, TNFalpha, cTnT, cTnI) [8]. Other studies have also reported that sunitinib affects cardiac function, which erlotinib does not, linked to effect on autophagic flux [7]. In the present study, we also find evidence that sunitinib is cardiotoxic, whereas erlotinib is cardiosafe in mice treated with doses comparable to those used in humans (**Figure 1**). We then identified significant changes in 9 heart and skeletal muscle metabolites when treated with sunitinib (**Table 2**), compared to 3 significantly altered metabolites in erlotinib treated heart and skeletal muscle (**Table 3**).

Mice treated with sunitinib had significantly lower heart and skeletal muscle levels of long chain omega-3 fatty acids docosahexaenoic acid (DHA) and arachidonic acid (AA) / eicosapentaenoic acid (EPA)(summarized in **Table 2**). Recent studies have confirmed the importance of omega-3 PUFAs in reducing cardiovascular disease and the associated inflammation [21,22]. Significant decreases in cardiac O-phosphocolamine were identified with sunitinib treatment (**Table 2**). O-Phosphocholine is the major concentrated phospholipid in human plasma (76% phosphocholine, 17% phosphoethanolamine) [23] and is the most abundant phospholipid in human erythrocytes [24]. As such, phosphocholine and phosphoethanolamine are the biggest reservoirs of dietary n-6 and n-3 PUFAS involved in inflammatory response. DHA and AA are critical regulators of cardiomyocyte membranes, responsible for the maintenance of cholesterol homeostasis [25]. The long-chain omega-3 fatty acid DHA (22:6n-3) is best known for its cardioprotective properties, due in part to its incorporation into cell membranes, resulting in a direct effect on calcium channels and their role in eicosanoid metabolism [26-28]. The mechanism by which AA / EPA and DHA are immunomodulatory seems to be due to derivatives of these PUFAs [29], including bioactive lipid mediators called resolvins, protectins, marescins, which have potent anti-inflammatory and immunoregulatory action in vitro and in vivo [30,31]. While the sunitinib-induced decreases in potent anti-inflammatory mediators AA/EPA, DHA, and O-phosphocholine cannot be directly linked to the observed cardiotoxicity, the findings do suggest one potentially novel mechanism. These findings suggest for the first time a link between sunitinib-induced cardiotoxicity and alterations in inflammation through its effects on DHA, arachidonic acid, and O-phosphocolamine.

In the present study, there is a significant elevation in liver cholesterol levels after sunitinib treatment (**Table 2**). Previous studies have made the clinical observation that sunitinib treatment causes hyperlipidemia in patients with metastatic renal cell carcinoma [34]. While the new-onset hyperlipidemia was found to be higher than in matched controls [34], no clear mechanism has been described. The results in the present study identify for the first time that sunitinib increases liver cholesterol in vivo, suggesting that that sunitinib’s direct effects on hepatic cholesterol biosynthesis may contribute to the hyperlipidemia recently reported in patients.

Sunitinib-treated rodents demonstrated elevated ethanolamine in the serum (**Table 2**). In serum, ethanolamine is converted to phosphoethanolamine by ethanolamine kinase, which represents the highest reservoir of phospholipids in human plasma [23]. However, the link between sunitinib treatment and serum ethanolamine is not clear, but may reflect a disrupted phospholipid metabolism in tissues (decreased AA/EPA, DHA, O-phosphocholine) resulting in elevated ethanolamine in the serum, and/or may reflect defects in the converstion of ethanolamine to phosphoethanolamine in the serum [35].

4. Materials and Methods

***Animals, experimental design, drug delivery, and harvest.*** Mice were 10-week old FVB/N females. They were fed the usual animal care diet per the UNC Animal Care facility and were gavaged daily by UNC Lineberger Animal Models Core staff with erlotinib 50 mg/kg/day or sunitinib 40 mg/kd/day (LC Laboratories E-4007 and S-8803) solubilized in dimethyl sulfoxide (DMSO). DMSO concentration in vehicle control was the same as in the group with the highest TKI/DMSO concentration. At baseline and on Day 14, 5 mice from each group underwent conscious echocardiography as previously described [36,37]. Mice were sacrificed under deep isoflurane anesthesia, blood was collected by cardiac puncture, clotted, and 100-200μL serum was snap frozen in liquid nitrogen. The heart was dissected, the atria and great vessels were excised, and blood was cleared from the ventricles in cold PBS. Liver and quadriceps femoris muscle were excised. Excess liquid was blotted from all tissues and they were snap-frozen in liquid nitrogen.

***Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS).*** All methods utilized a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. Samples were processed as previously described [40,41], sample extract reconstituted in solvents containing a series of standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic positive ion conditions, chromatographically optimized for more hydrophilic compounds. The extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 µm) using water and methanol, containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). Another aliquot was also analyzed using acidic positive ion conditions, however it was chromatographically optimized for more hydrophobic compounds. In this method, the extract was gradient eluted from the same C18 column using methanol, acetonitrile, water, 0.05% PFPA and 0.01% FA and was operated at an overall higher organic content. Another aliquot was analyzed using basic negative ion optimized conditions using a separate dedicated C18 column. The basic extracts were gradient eluted from the column using methanol and water, however with 6.5 mM Ammonium Bicarbonate at pH 8. The fourth aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH amide 2.1x150 mm, 1.7 µm) using a gradient consisting of water and acetonitrile with 10mM ammonium formate, pH 10.8. The MS analysis alternated between MS and data-dependent MSn scans using dynamic exclusion. The scan range varied slightly between methods but covered 70-1000 m/z.

Raw data were extracted, peak-identified by comparison to library entries of purified standards or recurrent unknown entities based on authenticated standards that contain the retention time/index (RI), mass to charge ratio (*m/z)*, and chromatographic data (including MS/MS spectral data). Biochemical identifications are based on retention index within a narrow RI window of the proposed identification, accurate mass match to the library +/- 10 ppm, and the MS/MS forward and reverse scores between the experimental data and authentic standards. The MS/MS scores are based on a comparison of the ions present in the experimental spectrum to the ions present in the library spectrum. The QC and curation processes were designed to ensure accurate and consistent identification of true chemical entities, and to remove those representing system artifacts, misassignments, and background noise. Peaks were quantified using area-under-the-curve. For studies spanning multiple days, a data normalization step was performed to correct variation resulting from instrument inter-day tuning differences. The data obtained in this study will be accessible at the NIH Common Fund’s Data Repository and Coordinating Center (supported by NIH grant, U01-DK097430) website, [http://www.metabolomicsworkbench.org](http://www.metabolomicsworkbench.org/).

***Metabolomic Statistical Analyses.*** Metaboanalyst (v3.0) run on the statistical package R (v2.14.0) used metabolite peak areas (as representative of concentration) [42,43]. These data were scaled using Pareto scaling feature. To detect systemic metabolic signature of the sorafenib treatment treatment, a t-test was performed using Metaboanalyst v3.0. T-test significant metabolites (p<0.05) were matched to metabolomics pathways using the Pathway Analysis and enrichment analysis features in Metaboanalyst 3.0. Only metabolites identified and detected in all groups were included in the statistical analysis. For heart and skeletal muscle samples, up to two missing values (of ten total) were imputed; for liver and serum up to three missing values (of ten total) were imputed with the group’s minimum value. If more values were missing, the analyte was not included in the statistical analysis. All data from this study are available in **Table A1**. All data are shown as mean +/- SEM, unless otherwise indicated.

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**Author Contributions:** B.J., M.W., C.P., and G.J. conceived and designed the experiments; T.L., W.H., A.I., J.B., C.N., and M.M. performed the experiments and wrote the materials and methods, B.J., M.W., J.B., M.M. analyzed the data; B.J., M.W., C.P., G.J., were involved with the interpretation of the data; M.W. and B.J. wrote and edited the work.

**Conflicts of Interest:** The authors declare no conflict of interest.

Appendix A

None

**Appendix B:** The following are available online at [www.mdpi.com/link](http://www.mdpi.com/link),

**Figure A1:** Heat map of all cardiac metabolites identified by GC-MS in sunitinib-treated animals.

**Figure A2:** Heat map of all cardiac metabolites identified by GC-MS in erlotinib-treated animals.

**Figure A3:** Heat map of all liver metabolites identified by GC-MS in sunitinib-treated animals.

**Figure A4:** Heat map of all liver metabolites identified by GC-MS in erlotinib-treated animals.

**Figure A5:** Heat map of all quadriceps femoris metabolites identified by GC-MS in sunitinib-treated animals.

**Figure A6:** Heat map of all quadriceps femoris metabolites identified by GC-MS in erlotinib-treated animals.

**Figure A7:** Heat map of all serum metabolites identified by GC-MS in sunitinib-treated animals.

**Figure A8:** Heat map of all serum metabolites identified by GC-MS in erlotinib-treated animals.

**Table A1.1-A1.8:** Non-targeted metabolomics performed on serum from mice treated with Vehicle, Sunitinib (40 mg/kg), or Erlotinib (50 mg/kg).

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