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Multitargeted kinase inhibitors imatinib, sorafenib and sunitinib perturb energy metabolism and cause cytotoxicity to cultured C2C12 skeletal muscle derived myotubes

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ABSTRACT

Tyrosine kinase inhibitors (TKIs) have advanced cancer treatment and prognosis but have also resulted in adverse effects such as fatigue, diarrhea, hypothyroidism, and other toxicities. We investigated TKI effects on skeletal muscle as a possible explanation of TKI induced fatigue. Changes in mitochondrial function due to inhibition of oxidative phosphorylation complexes, generation of superoxides, and inhibition of key transporters involved in uptake of glucose and/or nucleosides may result in alteration of energy metabolism and/or mitochondrial function. We investigated effects of imatinib, sorafenib and sunitinib on these processes in cultured C2C12 murine skeletal muscle cells. Imatinib, sorafenib and sunitinib were cytotoxic to C2C12 cells with IC_{50} values of 20, 8 and 8 μM, respectively. Imatinib stimulated glucose uptake and inhibited complex V activity by 35% at 50 μM. Sorafenib inhibited complex II/III and V with IC_{50} values of 32 and 28 μM, respectively. Sorafenib caused activation of caspase 3/7 and depolarization of mitochondrial membranes occurred very rapidly with complete loss at 5–10 μM. Sunitinib inhibited Complex I with an IC_{50} value of 38 μM and caused ATP depletion, caspase 3/7 activation, an increase in reactive oxygen species (ROS), and decreased nucleoside and glucose uptake. In conclusion, imatinib, sunitinib and sorafenib caused changes in mitochondrial complex activities, glucose and nucleoside uptake leading to decreased energy production and mitochondrial function in a skeletal muscle cell model, suggesting that these changes may play a role in fatigue, one of the most common adverse effects of TKIs.

1. Introduction

Tyrosine kinase inhibitors (TKIs) are designed to interfere with cellular signaling mechanisms in treatment of cancer. For example the TKI sunitinib, which targets vascular endothelial growth factor receptor (**VEGFR**) and c-kit, provided for the first time a survival advantage over interferon in treating metastatic renal cell cancer (**RCC**) [1]. Sorafenib was designed to target b-Raf but was subsequently shown to target both VEGFR and platelet derived growth factor receptor (PDGFR), and was the first therapy proven to prolong survival in patients with metastatic hepatocellular carcinoma (**HCC**) [2]. Imatinib has revolutionized treatment of chronic myelogenous leukemia (**CML**) by reducing requirements of a life threatening allogenic bone marrow transplant to a more manageable therapy [3]. A recent study has even suggested that some patients treated with imatinib are cured of their CML [4]. Although TKIs were designed to target tumor tissues, unexpected cardiac toxicity was shown with many targeted agents [5–9]. In addition, recent results also showed metabolic alterations such as changes in glucose levels in patients treated with imatinib, sorafenib and sunitinib [10–14]. It was shown that muscle wasting in cancer patients associated with sorafenib was likely due to inhibition of raf kinase in growth factor pathways regulating muscle size [15].

Mitochondrial dysfunction caused by many clinically used drugs are due to inhibition of electron transport/ATP synthesis, uncoupling of electron transfer from ATP synthesis, irreversible opening of the mitochondrial permeability transition pore (mPTP), oxidative stress,

Abbreviations: BCR, breakpoint cluster region; ABL, Abelson murine leukemia; PDGFR, platelet derived growth factor receptor; VEGFR, vascular endothelial growth factor receptor; NBMPR, nitrobenzylmercaptopurine riboside; TKIs, tyrosine kinase inhibitors; NTs, nucleoside transporters; IC_{50}, concentration of test compound that inhibited growth of treated cells by 50% relative to that of untreated cells

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inhibition of mitochondrial membrane transporters, and/or inhibition of fatty acid oxidation [16]. Complex I (45 subunits) appears to be a major site of interaction with these drugs because it offers multiple drug interaction sites. In addition, some drugs can also inhibit adenine nucleotide translocator (ANT) a protein involved in exchange of ATP for ADP in mitochondrial membranes. Many TKIs have structures that share similarities with ATP and have been shown to interfere with binding of natural ligands in several off-target proteins and enzymes, resulting in adverse effects. These include fatigue, cardiotoxicity, alterations in blood glucose levels, hypothyroidism, hypertension, hematologic, dermatologic and gastrointestinal toxicities. The occurrence of such adverse effects varies based on treatment schedule, amount of drug administered, route of administration, predisposing patient characteristics, renal or hepatic impairment and co-administered drugs and are due to TKIs acting on normal cells in tissues.

The major function of mitochondria, which are the power plants of cells [17], is to perform the final steps of oxidation of glucose, fats and amino acids to produce ATP from ADP. Some of the lipophilic TKIs that are weak acids or weak cations may cause uncoupling of electron transfer from ATP synthesis leading to loss of membrane potential. Passage of small molecules with molecular weights less than 5000 Da is accomplished by mitochondrial porins, or voltage-dependent anion-selective channels (VDACs) [18]. A highly conserved sequence in VDACs, the glycine-leucine-lysine (GLK) motif, was initially suggested to be an ATP binding site [19]. Because many TKIs have site(s) of action in several signal transduction pathways governing cell proliferation and metabolism, TKIs have the potential to generate toxicities in an unusually broad range of tissues, including liver, heart, kidney, muscle, and eye. Although mitochondria are key sites of cellular regulation, they are not included in conventional thinking about cancer treatment toxicities. We believe that various TKIs can interact with several nucleotide binding proteins in mitochondrial complexes and inhibit their activity leading to decreased ATP production and impaired muscle function, which patients experience as fatigue. Such TKIs could also lead to irreversible opening of the mPTP. TKIs may bind to complexes I to IV, inhibiting mitochondrial electron transport. Complex V catalyzes conversion of ADP to ATP and we believe that TKIs may bind to and inhibit complex V. If so, inhibiting mitochondrial electron transport will lead to decreased ATP production, which will decrease the function of various organs that are dependent on high levels of energy production, especially muscle and heart. In addition, some TKIs have effects on glucose and nucleoside uptake into cells [12,20–22]. The most important glucose transporters (GLUTs) in skeletal muscle are GLUT-1 and GLUT-4. GLUT-1 (SLC2A1) is constitutively active and is on plasma membranes whereas GLUT-4 is intracellular and can be recruited to plasma membranes in response to various stimuli [23]. Among human nucleoside transporters, hENT1 (SLC29A1) is widely distributed and appears to be the most important transporter in cellular uptake of nucleosides [24].

To understand adverse clinical effects of TKIs on skeletal muscle which manifest themselves primarily as TKI induced fatigue, we studied a murine C2C12 myoblast cell line, which is an experimental system widely used to produce a skeletal muscle cell culture model of C2C12 derived myotubes [25]. Mitochondrial dysfunction and alteration of energy metabolism can result from inhibition of oxidative phosphorylation complexes, generation of superoxide, and inhibition of key transporters involved in uptake of glucose and/or nucleosides. In this work, we investigated these and other related cellular processes in cultured C2C12 derived myotubes.

2. Materials and methods

2.1. Chemicals and reagents

NBMPR, dipyridamole, unlabeled nucleosides and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). Tissue culture (12-well) plates and flasks were from VWR International (Mississauga, ON) and cell culture media, horse serum, fetal bovine serum (FBS) were from Gibco BRL (Burlington, ON). Ecolite was from ICN Pharmaceuticals (Montreal, PQ). [^{3}H]2-Deoxy-D-glucose (2-DG) and [^{3}H]uridine were purchased from Moravek Biochemical's Inc. (Brea, CA). Imatinib, sorafenib and sunitinib were from LC Laboratories (Woburn, MA) and 10 mM stock solutions were made in dimethyl sulfoxide (DMSO) and diluted for experiments. Carrier solvent was used in controls for experiments. Cell Counting Kit-8 (CCK-8) was from Dojindo Molecular Technologies, Inc. (Rockville, MD). CellTiter-Glo Luminescent Cell Viability Assay kit and Caspase Glo 3/7® assay kit were purchased from Promega Corp., Madison, WI. MitoSOX™ Red and JC-1 were purchased from Molecular Probes, Eugene, OR. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. C2C12 cell culture

The mouse skeletal muscle myoblast cell line (C2C12) was obtained from American Type Culture Collection and was routinely passaged in growth media containing Dulbecco's Modified Eagle Medium (DMEM) with glucose (4.5 g/L), 1 mM sodium pyruvate, 2 mM L-glutamine, 10% fetal calf serum, and penicillin-streptomycin. Cultures were maintained at 37 °C in a 95% air and 5% CO_2 in a humidified atmosphere. Plating densities were such that cells typically reached 80–90% confluence within 48 h after plating. Differentiation to myotubes was induced by exposure of confluent cultures to differentiation media containing DMEM with 4.5 g/L glucose, 1 mM sodium pyruvate, 2 mM L-glutamine, with 2% horse serum and cells were further grown for 6–7 days before experiments. At this time the cell morphology changed to that of multinucleated myotubes. For experiments in 96-well plates, myoblasts were seeded at 7000 cells/well and grown for two days in growth media after which media was replaced with differentiation media and grown for 6–7 days before the experiment.

2.3. Cytotoxicity and ATP levels

Cytotoxicity assays were performed using Dojindo CCK-8 reagent as follows. C2C12 cells were plated in 96-well plates and, after differentiation to myotubes, were incubated for 72 h at 37 °C with or without graded concentrations (0–40 μM) of imatinib, sorafenib or sunitinib, and then incubated with CCK-8 reagent for 2–4 h. Absorbance at 450 nm was measured using a Spectramax M3 plate reader from Molecular Devices (Sunnyvale, CA). Three independent experiments, each with six replicates, were performed. IC_{50} values were determined by regression analyses of concentration-response curves. Graphs were generated using GraphPad Prism® 4.0 (GraphPad Software Inc.; San Diego, CA). ATP content in C2C12 cells was measured before and after treatment with imatinib, sorafenib or sunitinib by using CellTiter-Glo Luminescent Cell Viability Assay kit. Individual TKIs were dissolved in DMSO and a small portion was added to treated cells; the same volume of DMSO without TKIs was added to untreated (control) cells. After treatment for 24–72 h at 37 °C, ATP levels were measured. Data represent means of three experiments, each conducted with four replicates.

2.4. Caspase assay

For caspase assays, C2C12 cells were seeded in 96-well solid white plates. Varied concentrations of imatinib (10–40 μM), sorafenib (5–20 μM) or sunitinib (5–20 μM) were added to day 6 myotubes and incubated at 37 °C for 24 h, after which caspase 3/7 activity was measured using Caspase Glo 3/7® assay following manufacturer's directions. After the reagent was added to cells, they were mixed on a mechanical shaker and incubated at room temperature (RT) for at least 30 min. Caspase Glo 3/7® assay is a homogeneous, luminescent assay that measures caspase-3 and -7 activities and luminescence was