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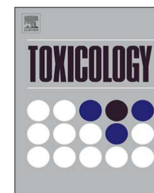

Involvement of mitogen activated kinase kinase 7 intracellular signalling pathway in Sunitinib-induced cardiotoxicity

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ABSTRACT

The tyrosine kinase inhibitor Sunitinib is used to treat cancer and is linked to severe adverse cardiovascular events. Mitogen activated kinase kinase 7 (MKK7) is involved in the development of cardiac injury and is a component of the c-Jun N-terminal kinase (JNK) signal transduction pathway. Apoptosis signal-regulating kinase 1 (ASK1) is the upstream activator of MKK7 and is specifically inhibited by 2,7-dihydro-2,7-dioxo-3*H*-naphtho[1,2,3-*de*]quinoline-1-carboxylic acid ethyl ester (NQDI-1). This study investigates the role of ASK1, MKK7 and JNK during Sunitinib-induced cardiotoxicity.

Infarct size were measured in isolated male Sprague-Dawley rat Langendorff perfused hearts treated for 125 min with Sunitinib in the presence and absence of NQDI-1. Left ventricular cardiac tissue samples were analysed by qRT-PCR for MKK7 mRNA expression and cardiotoxicity associated microRNAs (miR-1, miR-27a, miR-133a and miR-133b) or Western blot analysis to measure ASK1/MKK7/JNK phosphorylation.

Administration of Sunitinib (1 μM) during Langendorff perfusion resulted in increased infarct size, increased miR-133a expression, and decreased phosphorylation of the ASK1/MKK7/JNK pathway compared to control. Co-administration of NQDI-1 (2.5 μM) attenuated the increased Sunitinib-induced infarct size, reversed miR-133a expression and restored phosphorylated levels of ASK1/MKK7/JNK. These findings suggest that the ASK1/MKK7/JNK intracellular signalling pathway is important in Sunitinib-induced cardiotoxicity. The anti-cancer properties of Sunitinib were also assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay. Sunitinib significantly decreased the cell viability of human acute myeloid leukemia 60 cell line (HL60). The combination of Sunitinib (1 nM–10 μM) with NQDI-1 (2.5 μM) enhanced the cancer-fighting properties of Sunitinib. Investigations into the ASK1/MKK7/JNK transduction pathway could lead to development of cardioprotective adjunct therapy, which could prevent Sunitinib-induced cardiac injury.

1. Introduction

The tyrosine kinase inhibitor Sunitinib is used in the treatment of renal cell carcinoma and in gastrointestinal stromal tumours (Faivre et al., 2007). Sunitinib prevents tumour cell survival and angiogenesis by inhibiting a variety of growth factor and cytokine receptors, including platelet derived growth factor receptors, vascular endothelial growth factor receptors and proto-oncogenes c-Kit and RET. However, Sunitinib unfortunately is also associated with a lack of kinase selectivity resulting in the cardiotoxic adverse effects (Force et al., 2007). In the clinic, Sunitinib causes QT prolongation (Bello et al., 2009),left ventricular dysfunction (Shah and Morganroth, 2015) and heart failure (Ewer et al., 2014). These findings are consistent with many other successful chemotherapy agents linked with severe drug-induced cardiotoxicity (Hahn et al., 2014), including electrophysiological changes and left ventricular dysfunction which can cause heart failure in some patients (Aggarwal et al., 2013). Intracellular studies using animals have revealed that Sunitinib causes mitochondrial injury and cardiomyocyte apoptosis through an increase in caspase-9 and cytochrome C release in both mice and in cultured rat cardiomyocytes (Chu et al., 2007). Other indicators of apoptosis, such as an increase in caspase-3/7, have also been detected after Sunitinib treatment in rat myocytes

Abbreviations: ASK1, apoptosis signal-regulating kinase 1; ASK2, apoptosis signal-regulating kinase 2; DMSO, dimethyl sulphoxide; hERG, human ether-a-go-go-related gene; JNK, c-Jun N-terminal kinase; MKK7, mitogen activated kinase kinase 7; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NQDI-1, 2,7-dihydro-2,7-dioxo-3*H*-naphtho[1,2,3-*de*]quinoline-1-carboxylic acid ethyl ester; TTC, 2,3,5-Triphenyl-2*H*-tetrazolium chloride

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(Hasinoff et al., 2008).

MKK7 is a member of the mitogen-activated protein kinase kinase super family, which allows the cell to respond to exogenous and endogenous stimuli (Foltz et al., 1998), and furthermore MKK7 has shown to demonstrate a key role in protecting the heart from hypertrophic remodelling, which occurs via cardiomyocyte apoptosis and heart failure (Liu et al., 2011). The MKK7 activation of JNK results in many cellular processes including: proliferation, differentiation and apoptosis (Chang and Karin 2001; Schramek et al., 2011; Sundarrajan et al., 2003), and JNK signalling is vital for the maintenance and organisation of the cytoskeleton and sarcomere structure in cardiomyocytes (Windak et al., 2013). Interestingly, Sunitinib is an ATP analogue and competitively inhibits the ATP binding domain of its target proteins (Roskoski 2007; Shukla et al., 2009). MKK7 also contains a highly conserved ATP binding domain (Song et al., 2013). It is possible that Sunitinib binds as a ligand in the MKK7 ATP binding pocket, and thereby Sunitinib inhibits the MKK7/JNK transduction pathway, and as a result this could potentially cause myocardial injury. It is important to determine the relationship between MKK7 expression and Sunitinib induced cardiotoxicity by measuring the alteration of MKK7 mRNA and phosphorylated MKK7 levels in the presence of Sunitinib. Unravelling the relationship between Sunitinib and MKK7 could lead to a greater understanding of its off-target mechanism of action and lead to the improvement in the development of future drug discovery programmes or novel cardioprotective adjunct therapies.

Short non-coding RNA microRNAs carry out the negative regulation of mRNA transcripts by repressing translation (Bagga et al., 2005). Specific microRNAs expression patterns have been linked to cardiomyocyte differentiation and in response to stress (Babiarz et al., 2012) and have also been shown to be differentially expressed during the development of heart failure (Thum et al., 2007). The microRNAs miR-1, miR-27a, miR-133a and miR-133b produce differential expression patterns during the progression of heart failure (Akat et al., 2014; Tijsen et al., 2012). It is important to identify microRNA expression profiles in response to drug-induced cardiotoxicity as similar patterns in microRNA expression to those identified during heart failure may indicate the early onset of cardiotoxicity at a molecular level.

As MKK7 has no direct inhibitor, we have chosen to look at the upstream kinase ASK1 linked to MKK7 activation (Ichijo et al., 1997). ASK1 is activated in response to oxidative stress-induced cardiac vascular endothelial growth factor suppression in the heart (Nako et al., 2012). Izumiya et al. (2003) used ASK1 deficient transgenic mice to assess the role of ASK1 in angiotensin II induced hypertension and cardiac hypertrophy. Both the wild type and ASK1 deficient mice developed hypertension when stimulated with angiotensin II, however, the ASK1 deficient mice lacked cardiac hypertrophy and remodelling and activation of ASK1, p38 and JNK was severely attenuated, thus emphasising the importance of ASK1 in cardiac hypertrophy and remodelling signalling (Izumiya et al., 2003). ASK1 is selectively inhibited by NQDI-1 with high specificity with a K_i of 500 nM and IC_{50} of 3 μM (Volynets et al., 2011), however, as this is a relatively new drug, a complete pharmacological profile has not yet been fully characterised. ASK1 inhibition has previously been shown to offer protection against ischemia reperfusion injury (Toldo et al., 2012) and has also been shown to suppress the progression of ventricular remodelling and fibrosis in hamsters expressing severe cardiomyopathy phenotypes (Hikoso et al., 2007). These findings highlight the potential of NQDI-1 as a valuable asset to inhibit cardiac injury via the ASK1/MKK7/JNK pathway.

This novel study investigated the involvement of the ASK1/MKK7/JNK pathway in the Sunitinib-induced cardiotoxicity via the assessment of cardiac function and infarct in conjunction with relevant intracellular signalling mediators. Furthermore, we assessed the anti-cancer properties of Sunitinib and determined whether co-administration of Sunitinib with NQDI-1 affected the anti-cancer/apoptotic effect of Sunitinib in HL60 cells.

2. Materials and methods

2.1. Main reagents and kits

Sunitinib malate and NQDI-1 were purchased from Sigma Aldrich (UK). Both drugs were dissolved in dimethyl sulphoxide (DMSO) and stored at −20 °C. Krebs perfusate salts were from either VWR International (UK) or Fisher Scientific (UK). Total ASK1 (Catalogue no ab131506) was purchased from Abcam (UK). Phospho-ASK1 (Thr 845) (Catalogue no 3765S), Phospho-MKK7 (Ser271/Thr275) (Catalogue no 4171S), Total MKK7 (Catalogue no 4172S), Phospho-SAPK/JNK (Thr183/Tyr185) (Catalogue no 9251), Total SAPK/JNK rabbit mAb antibody (Catalogue no 9252), anti-rabbit IgG, HRP-linked antibody and anti-biotin, HRP-linked antibody were purchased from Cell signalling technologies (UK). All the primary antibodies were from a rabbit host, and MKK7 and JNK were monoclonal antibodies, whereas ASK1 was polyclonal (all antibodies were validated by the manufacturers). The Ambion MicroPoly(A)Puris kit, Ambion mirVana miRNA Isolation Kit and Reverse Transcription Kit were from Life Technologies (USA). The mRNA primers and the Applied Biosystems primers assays (U6, rno-miR-1, hsa-miR-27a, hsa-miR-133a, and hsa-miR-133b) were purchased from Invitrogen (UK). The iTaq Universal SYBR Green Supermix was purchased from BioRad (UK). The HL60 cell line were obtained from European Collection of Cell Culture (UK) (catalogue no. 98070106).

2.2. Animals

Adult male Sprague-Dawley rats (300–350 g in body weight); were purchased from Charles River UK Ltd (UK) and housed suitably. They received humane care and had free access to standard diet according to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). Animals were selected at random for all treatment groups and the collected tissue was blinded for infarct size assessment. The experiments were performed following approval of the protocol by the Coventry University Ethics Committee. All efforts were made to minimise animal suffering and to reduce the number of animals used in the experiments. Rats were sacrificed by cervical dislocation (Schedule 1 Home Office procedure). A total of 80 animals were used for this study and the data from 68 rats were included, while data from 12 rats were excluded from analysis due to the established haemodynamic exclusion criteria. A total of 16 animals were included for Langendorff perfusion experiments per main groups (Control, Sunitinib, Sunitinib + NQDI-1, and NQDI-1, where 6 of the animals were used for measurement of the area of infarct and the area of risk and the left ventricular tissue from another 10 animals was used for real time PCR and Western blot analysis). Furthermore, an additional 4 animals were used for Langendorff perfusion experiments with Sorbitol as a positive control for p-MKK7 Western blot analysis. No animals were culled due to ill health.

2.3. Langendorff perfusion model

The hearts were rapidly excised after the rats were culled and placed into ice-cold Krebs Henseleit buffer (118.5 mM NaCl, 25 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.7 mM $CaCl_2$, and 12 mM glucose, pH7.4). The hearts were mounted onto the Langendorff system and retrogradely perfused with Krebs Henseleit buffer. The pH of the Krebs Henseleit buffer was maintained at 7.4 by gassing continuously with 95% O_2 and 5% CO_2 and maintained at 37 ± 0.5 °C using a water-jacketed organ chamber. Each Langendorff experiment was carried out for 145 min: a 20 min stabilisation period and 125 min of drug or vehicle perfusion in normoxic conditions. Hearts were included in the study with a CF between 3.5–12.0 ml/g (weight of the rat heart) during the stabilisation period. Sunitinib malate (1 μM) was administered throughout the perfusion period in the presence or

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