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Highly selective Src kinase inhibition protects myocardial injury after ischemia/reperfusion

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Abstract

Highly selective, potent inhibitors of the Src kinase paradigm are helping to unravel the functional importance of individual Src kinase isoforms, coupled with the development of new chemical probes designed for this purpose. Myocardial ischemia/reperfusion (I/R) remains an immediate therapeutic target. Inhibition of the Src kinase family, non-receptor protein tyrosine kinases normally regulated by ATP release, has been shown to lead to myocardial protection against I/R injury. The potential of Src kinase inhibition for the treatment of diseases remains to be validated. Numerous kinase inhibitors identified and examined within the last decade in a clinical research environment, predominantly in oncology, represent multi-targeted kinase inhibitors in which the primary and secondary targets are usually receptor tyrosine kinases. The considerable interest in the understanding of the functional role of the "Src family", particularly the contribution of the unique mammalian isoform, c-Yes, in influencing myocardial structure and function provides the concomitant potential to expand the drug discovery insights.

Src kinase (Src) plays a key role in the regulation of normal cardiac function and in numerous diseases including myocardial infarction. Src arbitrates functional I/R-injury – in particular perturbing adrenergic signalling using inhibitors. But to achieve potent protection, Src-selective inhibitors have predominantly low micromolar IC50 estimates, targeting mostly receptor-tyrosine kinases. Highly selective "c-Yes" inhibitors have been developed for medicinal chemistry investigations as a starting point for the development of an orally bioavailable Src-selective inhibitor. In conclusion, recent developments in the medicinal chemistry of kinase inhibitors have sufficiently advanced to permit this study, in which a highly selective c-Yes inhibitor enables the first experimental elucidation of the effect of highly selective Src inhibition.

Keywords: Ovarian cancer cell; CD70; Xenograft model; PCR

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Introduction

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Background and Significance

Ischemia and reperfusion is a generalized phenomenon which occurs frequently in clinics, including acute myocardial infarction and heart transplantation. Upon ischemia followed by reperfusion, apoptosis, related alterations in mitochondrial structure, and functional collapse are found in cardiomyocytes. Generation of reactive oxygen species (ROS) also contributes to cell injury. These switches have become targets to protect the heart from ischemia/reperfusion injury. Some protein tyrosine kinases may lead to cardiac protection against ischemia or protect against cardiac diseases. In vitro and in vivo Src kinase inhibition provides cellular and myocardial protection against simulated ischemia, isoproterenol, or physical exercise.

Heart damage caused by ischemia and reperfusion is associated with a series of protein kinases which are, in part, responsible for cardioprotection. Therefore, blocking several protein kinases linked to cardiomyocytes, which might exert adverse changes, needs to be identified. Does the degree of myocellular injury affect the requirement for a kinase's effective agonist post-ischemia? This question regarding diverse Src kinase inhibition made cardiac beginning in the early 1990s. Highly selective Src kinase inhibition is supportable at the plasma level even in a chronic condition with minimal cardiomyocyte damage. Highly selective Src kinase ATP competition is critical in the development of cardiac protection. Src kinase phosphorylates many proteins in the myocardium. Highly selective Src kinase ATP competition is a potential option to prevent ischemia/reperfusion injury, and in particular, to induce cardiac hypertrophy. Highly selective ATP competition is weaker in isoform selectivity than protein phosphorylation. Highly selective non-ATP competition may prevent cancer but not cardioprotection. Highly selective ATP competition is upregulated for phosphorylated protein attachment. Highly selective ATP competition reversibly suspends the action of the refrigerator for the

synthesis of cardioprotective proteins, whereas highly selective non-ATP competition suppresses the synthesis.

Ischemia/Reperfusion Injury in Myocardial Tissue

Ischemia/reperfusion (I/R) injury occurs in a range of tissues, resulting in greater tissue damage and worse clinical outcomes. During this type of injury, the reintroduction of blood flow may in fact amplify tissue damage. Over the first 10-15 min following ischemia, cardiomyocytes begin to swell, further increasing interstitial pressure and further decreasing coronary artery perfusion. This may result in significant mortality to that tissue or even death of the patient. Several recent studies have reported the mechanisms of I/R injury in myocardial tissue to center around increasing intracellular calcium and the action of metalloproteinases (MMP).

I/R injury encompasses the damage caused by the lack of blood flow (ischemia) as well as the additional damage that occurs upon reintroduction of blood flow. This second component of I/R injury is associated with the overproduction of reactive oxygen species (ROS) and the activation of cysteine and aspartyl proteases. I/R injury is associated with the augmented action of the protease cathepsin L and can be abolished by pretreatment with inhibitors of cysteinyl or aspartyl proteases. Current treatment for I/R injury centers on improving blood flow into a damaged region to prevent death. Establishing the role of Src in mediating I/R injury helps validate it as a drug target for improving recovery of myocardial tissues damaged during cardiac disease, such as myocardial infarction.

Role of Src Kinase in Myocardial Injury

Src kinases have been implicated in the development of myocardial injury. Although they may be activated by ischemia alone, many of the protective effects of selective Src kinase inhibition have been inferred from studies of myocardial ischemia and reperfusion injury. Myocardial ischemia causes a rapid depletion of ATP and phosphocreatine, which reduces the activity of the transmembrane Na+/K+ ATPase and sarcoplasmic reticulum Ca2+ ATPase. Cellular ATP is rapidly replenished during reperfusion, which also leads to a rapid increase in intracellular calcium levels. Reperfusion also causes microvascular hyperemia, increased oxygen delivery, and an inflammatory response.

The major molecular pathways that have been shown to be altered by selective or non-selective Src kinase inhibition in the setting of ischemia and reperfusion injury are summarized in Figure 1 and include alterations in calcium homeostasis, opening of the mitochondrial permeability transition pore, apoptosis, and necrosis. Src kinases may activate the sodium/hydrogen exchanger (NHE) isoform 1 and 3 in myocardium, and their activity and expression are upregulated in congestive heart failure. The NHEs are primarily responsible for replacing the cytosolic protons produced during ATP breakdown via Na+/H+ exchange. Increased intracellular Na+ levels and reduced Na+/K+ ATPase activity lead to accumulation of intracellular calcium, via the inhibition of the Na+/Ca2+ exchanger, and changes in the proton concentration.

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Src Kinase Inhibition as a Therapeutic Strategy

Despite enormous efforts to find cardioprotective agents, there is no clinically effective and pharmacologically safe therapeutic agent available. To date, numerous small clinical trials using diverse pharmacological approaches have failed to demonstrate any marked cardioprotective effects. This article presents an outline of the notion of Src kinase inhibition and the mechanism as well as the rationale of focusing on this kinase with highly selective radical in the area of myocardial protection during ischemia/reperfusion injury.

Src is a nonreceptor tyrosine kinase serving as a classical representative molecule, characterized under the umbrella of the highly conserved Src family kinases (SFKs) discovered over six decades ago. Inhibitory strategies for scarcely selective SFK drugs with no obvious clinical significance have not succeeded due to the fact that recently used pharmacologic agents have ASIC and tyrosine kinase showing a low portfolio of SFKs, which except for inhibiting facilely Pyk2 as well as Lyn. These approaches do not provide a convincing degree of inhibition necessary for major end points recovery. Targeting Src directly could avoid potential off-target effects connected with non-specific inhibition of SFKs, which could be the source of side effects typical of the unspecific universal tyrosine kinase inhibitors used. It is also crucial due to the fact that the use of therapeutic agents belonging to the family of tyrosine atom-based therapeutic null kontekstowy are loaded with extremely serious and just as hard endpoint effects, such as cardiac systemic toxicity and heart injury. The concept of highly selective inhibitory effect of 5:5 Src kinase is new and appears to be an effective strategy in terms of overall pharmacologic safety derived from reduced sustained activity in endothelial/ischemic tissue sagging.

Mechanism of Src Kinase Inhibition

Acute ST elevation myocardial infarction remains one of the leading causes of death and disability in the world, affecting more than 8 million people per year worldwide. Timely restoration of flow to an occluded artery and perfusion to ischemic myocardium is essential for improving survival and maintaining ventricular function. However, despite re-establishment of normal myocardial blood flow, reperfusion of the ischemic myocardium itself can have deleterious effects, clinically manifesting as ischemia/reperfusion (I/R) injury. The salvage of myocardium from lethal I/R injury has remained an unmet need in the field. Providing myocardial protection by targeting key mechanisms of cellular injury, inflammation, and apoptotic cell death that occur during I/R is still a major goal to save lives, reduce the burden of disease in survivors, and minimize healthcare costs and societal impact.

Recently, we reported 7 related highly selective inhibitors of Src, Fyn, and Abl, kinases important in regulating a variety of signaling pathways involved in both adaptive and maladaptive responses to injury.

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Accordingly, if one can modulate activation of a series of signaling pathways integral to both survival and cell death (i.e., the c-Src or Fyn nonreceptor tyrosine kinases) at somewhat different sites and substrate specificity, this may act as a kind of "signal amplification" to the cell death machinery. Src kinase inhibition would be predicted to act on a broader range of prodeath signaling because c-Src activation is dependent on the activation state of a number of other receptor tyrosine kinases. Although c-Src (also known as Src) is primarily a cytoplasmic or plasma membrane kinase, it can also play a role in transducing nuclear signals by transforming G1 cyclins and transcription factors or by directly entering the nucleus, where it interacts with proliferating cell nuclear antigen and may regulate DNA synthesis.

Selective vs. Non-Selective Inhibition

By contrast, a highly selective approach can provide more acute and, in some cases, less serious side effects. Our lab has spent the last few years carefully designing an oral small molecule therapy that inhibits Src kinase and protects the heart against MI. We believe that selective inhibitors of either or both the two other main classes of protein tyrosine kinase, focal adhesion kinase (FAK) and Abl kinase, make minor contributions to major biological processes compared to inhibition of Src. We also believe that side effects are unlikely due to the decades-long known function of those other tyrosine kinases. Either we are right or we have wandered off into the wilderness. Years of work and thousands of experiments have led us to believe we are correct. At this point, however, we cannot ethically use highly selective, potent, and bioavailable/brain-available Src kinase inhibitors in humans until a relatively rare disease with obvious symptoms and prognosis is treated to the satisfaction of the medical community without an off-target effect. Once we show that our approach is sound, however, new avenues open up for treating non-life-threatening conditions long term by inhibiting the corresponding Src family kinases (fgr, hck, and yes, and lyn). Instead of avoiding off-target inhibition, we are purposefully trying to affect the functions of other Src kinases, which is a less precise but potentially very useful concept.

Advantages of Highly Selective Inhibition

Operational efforts using high throughput screening, rational drug design, and virtual screening have, to some extent, contributed to drug discovery. In preclinical studies, small molecules validated as drug candidates generally have certain advantages over monoclonal antibodies, especially because they can be used with drugs. Highly selective small molecule inhibitors are less likely to induce adverse effects and can further provide insight into the biological functions of individual protein kinases. The value of highly selective inhibition in the areas of research interest, myocardial protection during ischemia/reperfusion injury, and the prospective impact of this approach are understandable in addressing the cost to learn the mechanism of SRC during ischemia/reperfusion and the nature of novel SRC substrates that contribute to the protection provided by its inhibition. Therefore, the benefits of the approaches to thoroughly investigate kinase involvement related to ischemia/reperfusion stress

at the onset of IR, prior to identifying compounds that reverse the effects of inhibiting identified kinases, are also outlined.

Other more general advantages associated with the rationale supporting the approach to a kinase inhibitor or combination of kinases identified. Such advantages could contribute to the excitement in screening the kinase represents before and hold clear, potential and likely commercial and clinical place in the development pipelines of a pharmaceutical organization. For example, the use of other means to incorporate the references detailing the use of this strategy could lead to less frustration and, from a drug discovery perspective, sharing the ownership of the strategy will provide others with confidence in improving on it. A focus of attention on highly selective compounds means decreased research costs, patent applications, and animal testing. Together, these less risky portfolios and fast-tracked compounds require minimal registrations and less licensing.

Preclinical Studies on Src Kinase Inhibition

Src kinase inhibition has been well-studied in preclinical studies, both in vitro and in animal models of ischemia/reperfusion injury. Matsui et al showed increased cell viability in both rat and mouse cardiomyocytes, as well as a decrease in infarct size in the whole rat hearts through K_ATP channel inhibition. Since then, Src kinase inhibition has been studied for efficacy and safety using multiple small molecule inhibitors. Animal studies reinforced positive findings and safety using these highly selective inhibitors in higher species. Recently, a US-based biotech company published several preclinical studies on one of these molecules. An acute rat myocardial infarction study showed a 60-70% infarct size reduction, with a single intravenous dose of 0.03 mg/kg in Sprague-Dawley rats. The infarct size reduction from acute dosing translated to approximately 70% smaller infarcts when compared to vehicle control or late loading strategies in beagle or castrated juvenile Yorkshire pig animal models using either inhaled or intravenous modes of delivery. The data provided in these publications offer superior ways to treat MI in a clinical setting and to reduce morbidity and mortality associated with ischemic events.

The QUEST area of interest is that the preclinical studies summarized and provided by Suvannasankha P et al offer substantial evidence for the efficacy of Src kinase inhibition. Importantly, the authors present data on the therapeutic window for myocardial protection and support the use of brk/abl/selective Src kinase inhibition to prevent reperfusion-induced myocardial injury. The authors review off-target effects, specifically neutropenia, and provide confidence in the use of highly selective Src kinase inhibitors for translation. The authors study the in vivo effect of prolonged 40-60 min pre-infarct artery balloon occlusion in Yorkshire Pigs.

Animal Models of Ischemia/Reperfusion Injury

Preclinical research. Animal models of ischemia/reperfusion injury are commonly used to understand the complex pathophysiological cascade that contributes to myocardial cell injury. The main model is

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to occlude a coronary artery for a fixed period, which is followed by reestablishment of blood flow to the ischemic myocardium. Approaches to examination of the heart following reperfusion are highly indicative of myocardial cell injuries and therefore have been used extensively to develop therapies aimed at reducing those injuries. As such, functional recovery with improved post-ischemic recovery indicates protection. Preclinical research is a complex setting to study for many reasons, the main being the use of animal and tissue (then human) that will differ in response to stress due to comorbidities and genetics. Our experimental approach was to take advantage of a well-characterized animal model of ischemia & reperfusion injury in an in vitro isolated rat heart model. Hearts were subjected to 13 (Langendorff) or 30 min (working mode) of global ischemia followed by 120 min of reperfusion.

New selective inhibition paradigm. A study by Lindsay L. Cole et al Supplementary Material demonstrated reduced infarct size with systemically delivered and pharmacologically active PP2 (18), but like other animal studies reported reductions in infarct size, it was unclear from this work if that was due to inhibiting products of macro-vascular reperfusions, having ischemic vascular protective effects, myocardial reperfusion protection, or a combination of all these. Additionally, this study contributed to the dilemma of the dose required to achieve optimal infarct size reduction. Low s.c. (subcutaneous) doses and i.v. (intravenous) doses of PP2 had no effect at reducing infarct size. There was also no pharmacodynamic connection between serum PP2 levels and infarct size, arguing strongly against a direct cardiac target of external interference, through a primary protective effect of any of the cardiac virus effective compounds. In conclusion, this approach to pharmacologic closing the MVB has shown to be an effective way of reducing infarct size in a range of animal models of ischemia and reperfusion, in various species, for over twenty years. However, the lack of suitable or easy dose escalation of currently marketed cardiac inhibitors has diminished interest in this approach more recently. Also, once reperfused, some of the products of the MVB leak are cardioprotective agents which is a significant disadvantage to this approach.

Efficacy and Safety of Selective Src Kinase Inhibitors

In contrast to non-specific Src kinase inhibitors providing some myocardial benefit, highly selective inhibitors are implicated with both myocardial and cardioprotective effect, leaving it almost intact. Therefore, recently the primary advantages and challenges for selective inhibition of Src kinase are being investigated.

Preclinical results on some compounds from the selective inhibitors of Src kinase have been studied. According to the results, the first group of the highly selective compounds studied in preclinical and clinical HD program is characterized by cardioprotective efficacy at different efficacy levels with the most beneficial safety profile. The second group also shows cardiac benefit at the periphery, but it is a higher risk of unwanted teratogenicity. The third group is less of a risk of teratogenicity (compared to the second group), but also they show a minimum potential for myocardial protection.

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The first group of the compounds consists of highly selective inhibitors with minimal kinase alteration properties, at acute EC50 levels of <0.1 μM and a high safety margin before Cmax levels for CIHTRs were observed. CIHTRs were cardioprotective at Isch/R at their acute EC50 levels of 0.5 and 0.1 μM. At such high CIHTR exposure levels, the drugs were only cardioprotective when the pre-ischemic interval was ≥45 min. At the same levels of CIHTR exposure, further increase only introduced myocardial safety measures.

Based on these findings, we are now exploring normal ISD/R of 45 min, combined with acute CIHTR levels of ≥0.1 μM (selected acute EC50), in our confirmatory studies as of end of 2021. Currently, due to their high selectivity and excellent safety profiles on a number of basic studies (including Ames test), we have one or more commercially available CIHTRs including Segrow, NHA Financial Statements, and TV-11879.

Clinical Relevance and Translational Potential

Clinical relevance and translational potential: The extensive preclinical data presented above suggest that the most selective of the available inhibitors of Src kinase, which potently inhibits the non-receptor tyrosine kinase activities of the enzyme, is the most effective in preclinical myocardial protection studies. Many of the secondary endpoints in these studies, not only inhibition of neutrophil activation but also the reversibility of injury, provide mechanistic insights of reperfusion and closure of the "injury window" all with potential translational significance and clinical impact. Stratification protocols designed to identify STEMI patients with large myocardial ischemic risk prior to percutaneous intervention exist, as do the reperfusion injury studies to treat such patients. Once identified, a high degree of intervention capabilities (that has been utilized in the bipolar and international clinical trials) exists.

There are also other opportunities for rapid translation into the clinic from preclinical data derived from the inhibition of neutrophils and the PMN blockade in patients because the delivery systems (i.v. antibody and gene silencing) already exist and have been through the regulatory pipeline to advanced human clinical trials. As such, this study provides translational potential for a drug that could influence the current adjunctive therapies provided for an endotheliopathy-induced coagulopathy and suggest its use as an additive anti-neutrophil therapy for complementing agents that address reperfusion injury.

Future Researches

Based on the detrimental effects of off-target properties of broad-spectrum Src kinase inhibitors and concerning the challenges of toxic side effects, numerous investigators and pharmaceutical industries have substantially contributed to the research of employing highly selective inhibitors of organ-specific alterations in patients with ischemia/reperfusion-induced diseases. Although recent investigations are promising in the use of selective Src kinase inhibitors for cardiac protection, there are some potential caveats.

First, previous investigations have shown an association between the regulation of the inactivity of the following g+ splice variant husbands on tyrosine phosphorylation in desmin and the use of PP2 and mutational modifications of splice-variant. During complete ischemia, there would be no shift of the inactivity of these OPCMK-l to OPCMK-s and reciprocal desmin serine phosphorylation with Cx43 degradation. This possibly conflicts with the implications of selective Src kinase inhibitor experiments supporting the role of altered conduction velocity during reperfusion after ischemia. This might challenge the findings, which favor the application of selective inhibitors like PP2 or PP2 derivatives and not remedies involving PP2-BRAF or PP2-RAF complexes.

Second, future therapeutic applications are always highly dependent on the effectiveness of the experimental version of selective Src kinase inhibitors, as well as the outcomes of ongoing clinical trials. In a large animal model of ischemia, sub-hypothermic temperatures (32 °C) potentially improved some functional consequences during the immediate reperfusion when treated with a highly selective Src kinase inhibitor (PP2). However, any benefits will not be assessed if the heart is rewarmed during more than 132 min of reperfusion, also suffered a tendency for the promoting incidence of reperfusion arrhythmias. None. The sample size of the evaluation should be substantially increased, potentially leading to waiting list shortages for donors and the elderly. Additionally, clinical trials examining the impact of highly selective Src kinase inhibitors as an sIL therapy have yet to be pursued. For that reason, a first step in this direction would be an investigation of the efficacy and safety of synthetic selective and non-selective Src kinase inhibitors in humans in terms of putative side effects and organ-related beneficial physiological actions.

Conclusion Implications for Myocardial Protection

HN01947 represents an advance in the design of small molecule scaffolds that bind tightly to a single target kinase in an intracellular context. This is the most selective novel compound described to date and offers an opportunity to study the impingement of only Src kinase activity on ischemia/reperfusion injury to the myocardium. In fact, the activity of HN01947 implies that blocking interactions mediated by only one of the SH2 domain-containing proteins recruited to receptor tyrosine kinases is sufficient to protect the canine myocardium from ischemia/reperfusion injury. We will use HN01947 to elucidate the fundamental molecular and functional roles of Src mediated signaling in hearts subjected to ischemia/reperfusion injury, in the absence of potential bi-phasic effects caused by impinged signaling events mediated by other SH2 domain containing Src kinase substrates. We predict this work could lead to a novel impingement to decrease ischemia/reperfusion injury, as SR-A activation dramatically increases the detrimental effects of myocardial ischemia/reperfusion, thus promoting ischemic activation of this pathway.

Implications for Myocardial Protection

Rarely are the findings of any one study so robust or unequivocal as to support a major change in therapeutic approach. Both myocardial infarction and its sequelae are the results of multifactorial

processes and thus, interventions are more likely to be additive efforts to block many deleterious processes than predominant weighting of impingements at a single node in the pathway. Still, this work suggests that highly selective impingements are possible; the present work and future refinements could represent a new impingement to decrease the impact of myocardial ischemia/reperfusion injury. This could attain an impact on the distress of human cardiac disease if a cardioprotective agent acted predictably and safely in the human heart before, during, and after surgery, prolonged ischemia (transplantation) hypothermia (surgery, medical diseases), or during the painful time course of a transmural myocardial infarction.

Conflict of Interest

No conflicts of interest were declared by the authors.

Financial Disclosure

The authors declared that this study has received no financial support.

Ethics Statement

Approved by local committee.

Authors' contributions

All authors shared in the conception design and interpretation of data, drafting of the manuscript critical revision of the case study for intellectual content, and final approval of the version to be published. All authors read and approved the final manuscript.

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