MLR-1023 IS A POTENT AND SELECTIVE ALLOSTERIC ACTIVATOR OF LYN KINASE IN VITRO THAT IMPROVES GLUCOSE TOLERANCE IN VIVO

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Abbreviations:

ANOVA: analysis of variance; Csk: COOH-terminal Src kinase; DPP-IV: dipeptidyl peptidase-4; ELISA: enzyme-linked immunosorbent assay; GLP-1: glucagon-like peptide; IRS-1: insulin receptor substrate-1; ip: intraperitoneal administration; OGTT: oral glucose tolerance test; PPAR: peroxisome proliferator-activated receptor; PBS: phosphate buffered saline, T2D: type 2 diabetes

Section: Endocrine and Diabetes
ABSTRACT

MLR-1023 (2(1H)-Pyrimidinone, 5-(3-methylphenoxy)) is a candidate for the treatment of type 2 diabetes. The current studies were aimed at determining the mechanism by which MLR-1023 mediates glycemic control. In these studies, we showed that MLR-1023 reduced blood glucose levels without increasing insulin secretion in vivo. We have further determined that MLR-1023 did not activate PPARα, δ, γ receptors or GLP-1 receptors or inhibit DPP-IV or α-glucosidase enzyme activity. However, in an in vitro broad kinase screen, MLR-1023 activated the non-receptor linked Src-related tyrosine kinase Lyn. MLR-1023 increased the V_{max} of Lyn with an EC_{50} of 63 nM. This Lyn kinase activation was ATP binding site independent, indicating that MLR-1023 regulated the kinase through an allosteric mechanism. We have established a linkage between Lyn activation and blood glucose lowering with studies showing that the glucose lowering effects of MLR-1023 were abolished in Lyn knockout mice, consistent with existing literature linking Lyn kinase and the insulin-signaling pathway. In summary, these studies describe MLR-1023 as unique blood glucose lowering agent and that MLR-1023-mediated blood glucose lowering is dependent on Lyn kinase activity. These results, coupled with the results of the accompanying paper, suggest that MLR-1023 and Lyn kinase activation may be a new treatment modality for type 2 diabetes.
INTRODUCTION

MLR-1023 (Tolimidone; 2(1H)-Pyrimidinone, 5-(3-methylphenoxy); see Figure 1) is a small orally bioavailable compound that was originally developed as an anti-ulcer therapeutic (Lipinski et al., 1980). MLR-1023 reached Phase II clinical trials for this indication, exhibited good pharmacokinetic properties and was well-tolerated in patients when administered for up to 6 weeks (Saporito et al., 2012). In a phenotypic screening platform designed to uncover new therapeutically beneficial activities of small molecules, MLR-1023 administration significantly lowered blood glucose in a mouse oral glucose tolerance test (OGTT) suggesting that it might be useful as a therapeutic for the treatment of type 2 diabetes (T2D) (Saporito et al., 2012).

T2D is a heterogeneous disease of disrupted glucose homeostasis driven by insulin resistance, increased hepatic glucose output and pancreatic β cell dysfunction and loss (DeFronzo, 2010). Oral drugs approved for the treatment of T2D are heterogeneous in their mechanisms, safety profiles and tolerability, with their utility limited by side-effect profile and/or limited efficacy (Krentz and Bailey, 2005; DeFronzo, 2010). Among the new drugs in development for T2D are those that target protein kinases and kinase signaling pathways (Tahrani et al., 2011), including selective kinase activators (Sanders et al., 2007; Viollet et al., 2007; Simpson et al., 2009; Eglen and Reisine, 2011). Nearly all small molecule kinase activators act by binding to non-ATP allosteric binding sites on the target protein (Simpson et al., 2009; Eglen and Reisine, 2011). These allosteric regulatory interactions confer a high degree of selectivity to kinase activators as
compared to kinase inhibitors that typically bind to highly conserved ATP binding sites on the protein (Eglen and Reisine, 2011).

Lyn kinase is a Src-related non-receptor linked tyrosine kinase that is found in an array of cell types and tissues including smooth muscle, liver, brain, myeloid cells and adipose tissue (Pertel et al., 2006; Scapini et al., 2009; Yamada et al., 2010; Gibb et al., 2011). Although Lyn kinase has been primarily studied in myeloid lineage cells, there are key data that implicate Lyn kinase as a regulator of insulin signaling and glucose regulation. In adipocytes, Lyn kinase activation leads to a direct tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) (Muller et al., 2000; Muller et al., 2001b). IRS-1 phosphorylation amplifies the insulin receptor-signaling pathway by increasing Glut-4 translocation and glucose uptake and utilization (Muller et al., 2000; Muller et al., 2001b; Muller et al., 2005). This linkage between Lyn kinase activation and therapeutic efficacy in T2D has been noted for the oral diabetes therapeutic glimepiride (Muller, 2000; Muller et al., 2001a). Glimepiride-mediated Lyn kinase activation activity may be responsible for glycemic control activity independent of its insulin-secretagogue activity (Muller, 2000; Muller et al., 2005). However, glimepiride-mediated Lyn kinase activation occurs indirectly through regulation of lipid rafts and is likely to be non-specific (Muller et al., 2005). A compound that directly activates Lyn kinase would provide a new therapeutic modality that would increase insulin-signaling events without affecting insulin secretion and promote glucose lowering effects that would be therapeutically beneficial in T2D.
The present studies were designed to investigate the mechanisms involved in MLR-1023-mediated blood glucose lowering. These studies show that MLR-1023 selectively activated Lyn kinase through an allosteric mechanism \textit{in vitro}, and that Lyn kinase activity is critical for MLR-1023-mediated blood glucose lowering activity \textit{in vivo}.

**METHODS**

**Materials**

MLR-1023 was synthesized according to previously developed synthetic methods (Lipinski et al., 1980) at Advanced Synthesis Group (Newark, DE). Metformin was obtained from Sigma-Aldrich (St. Louis, MO). Insulin ELISA kits were obtained from Crystal Chem Inc. (Downers Grove, IL). Aviva glucose test strips and monitors were obtained from Roche (Nutley, NJ). All other compounds and reagents were obtained from Sigma-Aldrich Inc (St. Louis, MO).

**Animals**

All experiments were conducted in accordance with the National Institutes of Health regulations of animal care covered in "Principles of Laboratory Animal Care," National Institutes of Health publication 85-23 and were approved by the Institutional Animal Care and Use Committee.
CD1-ICR male mice 8-10 weeks of age (Ace Animals, Boyertown, PA) were used in studies of baseline glucose, glucose tolerance and insulin levels. Male and female Lyn kinase knockout mice (129S4-Lyn^{tm1Sor/J}) and wild-type controls (129S1/SvImJ) were acquired from Jackson Laboratories (Jackson, ME) (Chan et al., 1997). At the time of study, Lyn kinase knockout mice were phenotypically similar to wild-type mice. Mice were fed a standard diet and were 8 weeks of age at time of study. Mice were housed 2-4 per cage and given free access to food and water unless otherwise noted. Animals were fed with Harlan Chow (2016 Teklad Global 16% Protein Rodent Diet).

Drug Administration

MLR-1023 was formulated in carboxymethyl cellulose (0.5%), Tween 80 (0.025%) in PBS. MLR-1023 was administered intraperitoneally (ip) at dose volumes of 5 to 10 mL/kg.

Oral glucose tolerance test and glucose measurements

For OGGT studies, mice were fasted for 18 hrs, treated with drug or vehicle and then given a glucose solution (1.5 g/kg) via oral gavage. Blood glucose levels were measured from a drop of blood (5 µL) that was collected from the tail. Blood glucose levels were measured prior to drug administration, prior to glucose challenge and at various times after glucose challenge.

Blood glucose levels were measured using Accu-Chek Aviva Glucometers (Roche Diagnostics) and levels reported as mg/dL. Glucometers were calibrated prior to each study. Blood (5 µL) was acquired from a tail snip and directly applied to a glucose test.
strip. Blood levels of MLR-1023 were measured by LC/MS/MS and levels determined by comparing to a standard curve of MLR-1023 prepared in blood.

**Serum Hormone Measurements**

Serum was prepared from blood that was collected by retroorbital eyebled. Insulin levels were measured from mouse serum using an “Ultra sensitive Mouse/Rat Insulin Elisa Kit” (Crystal Chem Inc., Downers Grove, IL) according to the manufacturer’s directions.

**In Vitro and Cell-Based Measurements**

Studies of the effects of MLR-1023 on adipocyte differentiation, PPAR (α, δ, γ) activation, DPP-IV activity, adiponectin production, insulin production, α-glucosidase inhibition and GLP-1 receptor activation were conducted at ChemBiotek (Kolkata, India). All cell-based systems were validated with a reference compound (described for each assay below). The concentration of MLR-1023 was 10 μM for all assays. Reference compound concentrations are described in Table 2.

Briefly, adipocyte differentiation was assessed in mouse 3T3-L1 cells after 8 days of incubation with MLR-1023 or rosiglitazone (10 μM) (Tamori et al., 2002; Li et al., 2007). Adiponectin production was measured in mouse 3T3-L1 cells after 6 days of incubation with MLR-1023 or rosiglitazone (10 μM) (Xu et al., 2004). PPAR (α, δ, γ) transactivation studies were conducted in transiently transfected COS7 cells containing appropriate DNA constructs (pGAL4/ PPAR (α, δ or γ) co-transfected with a luciferase reporter vector). MLR-1023 or an appropriate reference compound (bezafibrate (100
μM), L-165,041 (10 μM) or rosiglitazone (10 μM)) was incubated with transfected cells for 24 hrs. Luciferase activity was monitored as a measure of PPARα, δ and γ activation (Staels et al., 1998; Gervois et al., 1999; Raspe et al., 1999; Carmona et al., 2007).

DPP-IV activity was assessed from protease derived from porcine kidney. MLR-1023 or P32/98 (10 μM) was incubated in the presence of substrate (Gly-Pro-7-amido-4-methylcoumarin) for 30 minutes. Formation of the fluorescent proteolytic cleavage product was monitored as a measurement of DPP-IV activity (Kieffer et al., 1995).

Insulin release was assessed in RINm5F cells. MLR-1023 or GLP-1 (5 μM) was incubated with these cells for 60 minutes and insulin levels measured by ELISA (Jehle et al., 1995). α-glucosidase activity was assessed from enzyme derived from Bacillus subtilis. This activity was measured by monitoring the cleavage of a p-nitrophenyl alpha-D-glucoside after 60-minute incubation with MLR-1023 or castanospermin (Ridruejo et al., 1989). GLP-1 receptor activation was monitored by measuring cAMP production in RINm5F cells after incubation with MLR-1023 or GLP-1 (5 μM) for 30 minutes (Watanabe et al., 1994).

**Kinase Assays**

MLR-1023 was evaluated for activity against 47 separate kinases in a Caliper Lifesciences kinase-profiling panel. The assay conditions for each kinase examined are described at the Caliper Lifesciences website (www.caliperls.com). Briefly, for each kinase assay, MLR-1023 (10 μM) was preincubated with kinase and fluorescein labeled protein substrate. The reaction was initiated with the addition of ATP (at a concentration
at or below the $K_m$ for each kinase) and the level of fluorescein phosphopeptide measured.

The assays were conducted in duplicate.

Subsequent Lyn kinase assays were conducted with Upstate Ltd (Dundee, Scotland).

Briefly, MLR-1023 at concentrations ranging from 1 nM to 100 µM was preincubated with Lyn kinase (Upstate, recombinant human Lyn A, catalog # 14-510, batch #: 25111U; final concentration: 2 µg/mL), reaction buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1 mM sodium orthovanadate, 0.1% 2-mercaptoethanol) and substrate Poly (Glu4-Tyr) at 4°C. After the preincubation step, the mixture was diluted 1:200 in reaction buffer that contained [$^{32}$P]ATP (final: 10 µM or as indicated) and incubated for an additional 30 min at 30°C or as otherwise indicated. The amount of $^{32}$P labeled substrate was used as a measure of Lyn kinase activity.

**Data analysis**

All data were analyzed by one or two-way ANOVA. A post-hoc Dunnett’s or Bonferroni test was used to determine statistical differences between treatment groups. Data are expressed as the mean ± SEM, unless otherwise indicated. Group sizes ranged from 6 to 8 mice per group.
RESULTS

Effects of MLR-1023 administration on blood glucose levels in euglycemic mice and in an oral glucose tolerance test

To investigate whether MLR-1023 modulates blood glucose lowering by increasing insulin levels the compound was administered to normal, freely fed mice. Freely fed mice (vehicle treated) exhibited blood glucose levels ranging from 138 mg/dL (baseline) to 177 mg/dL. Baseline insulin levels were 2.3 ng/mL. Administration of MLR-1023 (30 mg/kg; ip) significantly (p < 0.05) lowered blood glucose levels to 148 and 158 mg/dL, 30 and 90 minutes after administration, respectively (Figure 2A). Insulin levels were not significantly affected by MLR-1023 administration (Figure 2B).

In the OGTT study, glucose administration to fasted vehicle-treated mice evoked a 4-fold increase in blood glucose levels (56 to 227 mg/dL) with a peak increase observed 30 minutes after glucose challenge (Figure 2C). A single administration of MLR-1023 reduced blood glucose levels by 41% to 158 mg/dL. In this study, insulin levels did not increase but trended downward with MLR-1023 administration (Figure 2D).

Under these dosing conditions (ip; 30 mg/kg) MLR-1023 administration produced a $C_{\text{max}}$ level of 72.8 μM with a $T_{\text{max}}$ of 0.25 hrs (Figure 2E). Protein binding of MLR-1023 in mouse serum has been determined to be 99.0% (data not shown). Therefore the $C_{\text{max}}$ free plasma concentration of MLR-1023 can be estimated to be approximately 728 nM.
Effects of MLR-1023 on targets and mechanisms associated with known diabetes therapeutics

In an effort to identify mechanisms that may be associated with MLR-1023-mediated blood glucose lowering, MLR-1023 was evaluated in a battery of in vitro and cell-based assays that represented mechanisms associated with T2D drugs and metabolic disease (Table 1). In this broad evaluation, MLR-1023 did not directly affect PPARγ, α or δ activation. Moreover, MLR-1023 did not affect DPP-IV activity, GLP-1 receptor function or α-glucosidase activity. Consistent with in vivo findings (Figure 2), MLR-1023 administration did not increase insulin secretion in RINm5F cells. MLR-1023 significantly increased adipocyte differentiation and adiponectin production by 3.7 and 19-fold, respectively. These increases; however, were far less than those elicited by rosiglitazone which increased these parameters by 21 and 140-fold, respectively.

Evaluation of MLR-1023 in an in vitro kinase panel

MLR-1023, at a concentration of 10 µM, was evaluated for activity against 47 different kinases in a cell-free in vitro screen. Incubation of MLR-1023 with Lyn kinase elicited a repeatable 50% increase in enzyme activity. MLR-1023 did not significantly or meaningfully affect any of the other 47 kinases in the screen (Figure 3).

In studies designed to understand MLR-1023-mediated Lyn kinase activation it was found that the degree of activation was dependent on a preincubation step prior to initiation of the reaction with addition of ATP. MLR-1023-mediated activation of Lyn kinase increased in proportion to the length of preincubation period in the absence of ATP (Figure 4A). A 2.7-fold increase in Lyn kinase activation was achieved with a 30
min preincubation. Thus, in all subsequent studies, MLR-1023 was preincubated for 30 min with Lyn kinase in the absence of ATP prior to the start of the kinase reaction.

MLR-1023 elicited a concentration dependent increase in Lyn kinase activation with a 2.3 and 2.1-fold increase achieved at concentrations of 3 and 10 µM, respectively (Figure 4B). The data were analyzed by non-linear regression analysis using Graphpad Prism (version 5; San Diego, CA) was used to determine an EC$_{50}$ value. Using the log (agonist) vs response curve fitting analysis and constraining the lower value to 100 (as this represents the control value) an EC$_{50}$ of 63 nM was generated. A similar concentration response curve was generated using concentrations ranging from 1-100 µM and under identical conditions. In that study, the effect at 100 µM did not differ from that of 3 or 10 µM (data not shown).

**MLR-1023 mediated Lyn kinase activation: ATP kinetics**

MLR-1023 mediated Lyn kinase activation was studied with increasing concentrations of ATP (range 6.25 to 800 µM). In control conditions (in the absence of MLR-1023), the rate of Lyn kinase activity increased with increasing ATP concentrations from 132 U/mg to 881 U/mg. Inclusion of MLR-1023 (100 µM) increased Lyn kinase activity by 3-fold at each ATP concentration tested ($V_{\text{max}} = 2,601$ U/mg). MLR-1023 did not affect the $K_m$ for ATP (control= 22.4 µM; MLR-1023= 22.6 µM). These data are shown in Figure 5.

**Effects of MLR-1023 in Lyn kinase knockout mice**

To further test the hypothesis that activation of Lyn kinase was responsible for the glucose lowering activity of MLR-1023, Lyn knockout mice were evaluated for blood
glucose levels after fasting, after an oral glucose challenge, and after administration of MLR-1023 and metformin (Figure 6). In these studies, wild-type and Lyn knockout mice that were fasted for 18 hrs exhibited equivalent blood glucose levels (45.1 ± 4.2 compared to 47.4 ± 2.7 mg/dL, respectively). Likewise, oral glucose challenge produced an equivalent 4.5-fold increase in glucose levels (peak 224.2 ± 5.5 compared to 207.5 ± 8.6 mg/dL, respectively).

Acute administration of MLR-1023 (30 mg/kg) to wild-type mice treated with MLR-1023 showed a significant 35% reduction (MLR-1023=147.4 ± 11.8; Vehicle= 224.2 ± 5.5) in peak glucose on an OGTT compared to vehicle treated mice (p<0.05) (Figure 6A). In contrast, MLR-1023-treated Lyn knockout mice exhibited equivalent peak blood glucose levels (201.2 ± 13.7 mg/dL) to those of vehicle-treated Lyn knockout mice (207.5 ± 8.7 mg/dL) (Figure 6B). In a separate study, administration of the gluconeogenesis inhibitor, metformin, to Lyn knockout mice produced an equivalent 55% blood glucose lowering compared to vehicle in both Lyn knockout mice and in wild-type mice (Figure 6C and D). The pharmacokinetic profiles of MLR-1023 in knockouts and wild-type mice were equivalent (data not shown).
DISCUSSION

Defects in insulin receptor sensitivity and signaling are considered to be critical factors in the pathology of T2D. Restoration of insulin receptor sensitivity, mimicking insulin signaling or potentiating insulin receptor activity are considered to be relevant pharmacological approaches in the design of next generation therapies for T2D (Krentz and Bailey, 2005; DeFronzo, 2010). The current studies show that MLR-1023 does not interact with any known type II diabetic therapeutic target, elicits a blood glucose lowering response \textit{in vivo} in a Lyn-dependent fashion, and selectively activates Lyn kinase \textit{in vitro}. The accompanying manuscript shows that MLR-1023 functions as an insulin-sensitizing agent. These results taken together link MLR-1023-mediated Lyn kinase activation to increased insulin sensitivity and ultimately glycemic control.

In freely fed and in fasted mice subjected to an OGTT, MLR-1023 administration significantly reduced blood glucose levels. In both instances, serum insulin levels were unaffected. These data indicate that the effects of MLR-1023 were independent of insulin secretion. Other anti-diabetic mechanisms were excluded as possible contributors to MLR-1023 activity. In cell-based and \textit{in vitro} tests, MLR-1023 did not affect PPAR\(\alpha\), \(\delta\) or \(\gamma\) activation, insulin secretion, inhibit DPP-IV enzyme activity or bind to the GLP-1 receptor. Interestingly, MLR-1023 increased adiponectin production and adipocyte differentiation. Although these effects were significantly less than that elicited by rosiglitazone, they may ultimately link to the primary MLR-1023 mechanism.
In a broad *in vitro* kinase screening panel, MLR-1023 selectively activated Lyn kinase. MLR-1023 did not activate other kinases. Importantly, other Src-family kinases including Fyn, Lck and Src kinase were unaffected by MLR-1023. Direct effects of MLR-1023 on the substrate in the kinase reaction were excluded since the same fluorescein labeled peptide substrate was used in all of the kinase assays in the broad kinase-screening panel and MLR-1023 increased Lyn kinase activity in two different Lyn kinase assay systems that used two different substrates. Moreover, effects on phosphatase inhibition can be eliminated as a possible indirect mechanism of Lyn activation since these findings occurred in an *in vitro* system devoid of phosphatases.

*In vitro*, MLR-1023 increased Lyn activation with an EC\textsubscript{50} of 63 nM and with maximal activation at a concentration of approximately 1 \( \mu \)M. *In vivo*, administration of MLR-1023 at the active dose level (30 mg/kg) produced a free compound C\textsubscript{max} (i.e. unbound by protein) of 0.73 \( \mu \)M. This alignment between the concentration required to fully activate Lyn kinase *in vitro* and the concentration achieved *in vivo* for blood glucose lowering, are supportive of a relationship between MLR-1023-mediated Lyn kinase activation and blood glucose lowering.

MLR-1023 did not affect the K\textsubscript{m} of ATP for Lyn kinase in the activation assay indicating that MLR-1023 did not interact with the ATP binding site to either mimic or facilitate ATP binding with Lyn kinase. The lack of effect on the ATP K\textsubscript{m} suggests that MLR-1023 increased Lyn kinase activity through regulation at an allosteric binding site. Nearly all small molecule kinase activators act through binding to an allosteric site.
For example, the small molecule AMP kinase activator, PT-1, increases activity of the enzyme inhibiting the auto-inhibitory phosphorylation state of the enzyme (Pang et al., 2008). Another AMP kinase activator, A-769,662 acts by binding to an allosteric binding site, inducing a conformational change that prevents dephosphorylation and inactivation of the kinase (Cool et al., 2006; Goransson et al., 2007). There are also examples of small molecules directly activating a kinase through a conformational change independent of changing the phosphorylation state of the enzyme. For example, the insulin receptor tyrosine kinase activator L-783,281 and its structural analogs activate the enzyme by inducing a conformational change that removes auto-inhibition and allows ATP access to its binding site which in turn drives kinase activity (Zhang et al., 1999; Salituro et al., 2001).

Lyn kinase activity is tightly controlled by its phosphorylation state (Donella-Deana et al., 1998; Ingley, 2008). Phosphorylation of Lyn kinase at the Tyr397 residue within its activation loop results in increased activity (Donella-Deana et al., 1998). In contrast, phosphorylation at the C-terminal tail Tyr 508 residue inhibits activity by inducing its association with an internal SH2 domain (Donella-Deana et al., 1998). Lyn kinase is autophosphorylated at both sites (Ingley, 2008). In addition, Lyn kinase can exist in a non-phosphorylated, partially active state (Ingley, 2008). However, Lyn kinase is unlikely to exist in the non-phosphorylated form because of its propensity to autophosphorylate. Thus, it is unlikely that MLR-1023 induces a conformational change to the enzyme that shifts it to a fully activated non-phosphorylated form.
The existing data surrounding small molecule activators of kinases and data showing that Lyn is tightly regulated by phosphorylation state suggest several possible mechanisms by which MLR-1023 could modulate Lyn activation. MLR-1023 may affect one of the two known phosphorylation regulatory sites on Lyn. For example, MLR-1023 may modify the kinase to make it more amenable to phosphorylation at the activation site (Tyr 397) or inhibit phosphorylation at the inhibition site (Tyr 508). Alternatively, MLR-1023 could bind at the regulatory loop associated with Tyr 397 so as to stabilize the loop in its activating configuration independent of Tyr 397 phosphorylation state. Finally, MLR-1023 could affect kinase activity independent of modifying the phosphorylation state by, for example, inhibiting SH2 site interaction with the pTyr508 of Lyn and relieving the autoinhibitory effects of this interaction.

The most compelling data linking MLR-1023-mediated Lyn kinase activation to blood glucose lowering effects in vivo are provided by the results in Lyn kinase knockout mice. In these studies, MLR-1023 did not lower blood glucose levels in Lyn kinase knockout mice subjected to an OGTT. These data show that MLR-1023-mediated blood glucose lowering is dependent on the presence of Lyn kinase. Although these data do not necessarily indicate that MLR-1023 directly activates Lyn kinase in vivo, they do show a requirement of Lyn kinase for MLR-1023-mediated blood glucose lowering. Interestingly, fasted blood glucose levels and peak glucose levels were equivalent in both wild-type and Lyn knockout mice, suggesting the presence of Lyn is not a requirement for maintaining glucose homeostasis. Further, metformin lowered glucose following a glucose challenge in both wild type and knockout animals showing that MLR-1023 and
metformin administration affect glucose levels by different mechanisms and that these
mice are responsive to some forms of pharmacologically-mediated blood glucose
lowering.

The known involvement of Lyn kinase in insulin signaling and the data from the current
studies indicate that MLR-1023-mediates blood glucose lowering through a Lyn kinase
dependent mechanism. Src-related kinases, and especially Lyn kinase, are directly
involved in insulin signaling (Lebrun et al., 1998; Muller et al., 2001a; Muller et al.,
2001b; Muller et al., 2005). In adipocytes, activated Lyn kinase directly promotes the
phosphorylation of IRS-1 and in turn promotes insulin signaling including Glut-4
translocation (Muller et al., 2001b; Muller et al., 2005). However, the accompanying
paper clearly shows that MLR-1023 does not function as an insulin mimetic but as a
potentiator of insulin activity. It appears that MLR-1023-mediated Lyn kinase-activation
and IRS-1 phosphorylation, in the absence of insulin, are not sufficient to promote
glucose utilization in vivo. There are multiple phosphorylation sites on IRS-1 and it may
be that these phosphorylation site(s) acted upon by Lyn overlap or are distinct from those
sites phosphorylated by the insulin receptor. Thus, the coordinated phosphorylation of
IRS-1 by Lyn and the insulin receptor may induce a more active IRS-1 form that
manifests as a potentiated insulin-mediated blood glucose lowering response in vivo.

Glimepiride is a well-described insulin secretagogue that also promotes blood glucose
lowering activity independent of its action on insulin secretion (Muller, 2000).

Interestingly, this activity of glimepiride has also been related to activation of Lyn kinase
(Muller et al., 2001a; Muller et al., 2005). However, unlike MLR-1023, this Lyn kinase activation is non-selective and indirectly regulated through effects on membrane associated lipid rafts (Muller et al., 2005). Nevertheless, these data support the concept that activation of Lyn kinase is a viable mechanism for regulating blood glucose in T2D patients.

The data generated from the current studies and the results shown in the accompanying manuscript, coupled with the known involvement of Lyn kinase in insulin mediated signaling support a model where MLR-1023 promotes insulin signaling through activation of Lyn kinase. The data from these studies show that MLR-1023 activates Lyn kinase in vitro and show that its blood glucose lowering effects are dependent on the presence of Lyn kinase in vivo. The results from both manuscripts describe MLR-1023 as a mechanistically novel, putative therapeutic for the treatment of T2D.
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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Ochman, Lipinski, Handler, Reaume, Saporito

Conducted Experiments: Ochman, Saporito

Contributed new reagents or analytic tools: Lipinski

Performed data analysis: Ochman, Saporito

Wrote or contributed to the writing of the manuscript: Saporito, Reaume
REFERENCES


LEGENDS FOR FIGURES

Figure 1. Structure of MLR-1023 (Tolimidone; CP-26154; 2(1H)-Pyrimidinone, 5-(3-methylphenoxy).

Figure 2. Effects of a single administration of MLR-1023 on glucose levels in fed euglycemic mice and mice subjected to an OGTT. Figure A and B: euglycemic, freely fed mice were administered MLR-1023 and A) glucose-levels and B) insulin levels were measured 30 and 90 minutes after administration. The levels at -60 minutes represent baseline values. Figures C and D: fasted mice were administered vehicle or MLR-1023 at time 0 and then challenged with an oral glucose solution (1.5 g/kg) 15 minutes later. C) Glucose levels and D) serum insulin levels were measured 30 and 90 minutes after MLR-1023 administration. E) Blood levels of MLR-1023 (30 mg/kg) over time in euglycemic mice. The levels at -60 minutes represent baseline values. Data are expressed as the average ± SEM; n=6/group. *p<0.05, ***p<0.001 when compared to corresponding vehicle control at the same time point.

Figure 3. Effects of MLR-1023 on kinase activity in a broad panel screen. MLR-1023 at a concentration of 10 μM was evaluated for activity against 47 different kinases. The ATP was at or below the K_m of ATP for each individual kinase. The dotted lines represent the percent change criteria required to indicate a significant difference from control values.
Figure 4. Effects of MLR-1023 on Lyn kinase activation. A) MLR-1023 (100 µM) was preincubated with Lyn kinase and substrate without ATP for time periods between 30 seconds and 30 minutes at 4°C prior to the initiation of the reaction with ATP. B) MLR-1023 at concentrations ranging from 1 nM to 10 µM was preincubated with Lyn kinase and substrate without ATP for 30 minutes prior to the initiation of the reaction with ATP. The ATP concentration was 10 µM for both studies. To determine the EC<sub>50</sub>, data were analyzed by non-linear regression analysis with a log (agonist) vs response curve fitting and constraining the lower value to 100. The EC<sub>50</sub> for MLR-1023 was 63 nM.

Figure 5. ATP Kinetics of MLR-1023-mediated Lyn kinase activation. A) Effects of ATP concentration on MLR-1023-mediated activation of Lyn kinase. MLR-1023 (100 µM) and corresponding controls were preincubated with Lyn kinase and substrate without ATP for 30 minutes prior to the initiation of the reaction. ATP, at concentrations ranging from 10 to 800 µM, was added to initiate the kinase reaction. B) Lineweaver-Burk plot representation of ATP kinetics.

Figure 6. Effects of MLR-1023 and metformin on glucose levels in an OGTT in Lyn kinase knockout mice. Lyn kinase knockout and wild-type control mice were evaluated for response to MLR-1023 and metformin. A) MLR-1023 (30 mg/kg; ip) was administered to wild-type mice (Lyn +/+ ) and B) Lyn knockout mice (Lyn -/- ) at time 0 and then administered an oral glucose solution (1.5 g/kg) thirty minutes later (t=30 min).
C) Metformin (150 mg/kg; ip) was administered to wild-type mice (Lyn +/+ ) and D) Lyn knockout mice (Lyn -/- ) at time 0 and then administered an oral glucose solution (1.5 g/kg) thirty minutes later (t=30 min). Blood glucose levels were measured prior to glucose and after glucose administration at the indicated times. Data are expressed as the average ± SEM; n=6/group. *p<0.05 when compared to corresponding vehicle control at the same time point.
Table 1. Comparison of Activities of MLR-1023 and Reference Compounds in Cellular and Enzyme assays

<table>
<thead>
<tr>
<th>Assay (units)</th>
<th>Control</th>
<th>MLR-1023 (10 µM)</th>
<th>Reference Compound (concentration)</th>
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<td>Adipocyte Differentiation (%)</td>
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<td>22.0 ± 0.4</td>
</tr>
<tr>
<td>PPARα (fold activation)</td>
<td>1.0 ± 0.2</td>
<td>1.3 ± 0.1</td>
<td>Bezafibrate (100 µM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.2 ± 0.5</td>
</tr>
<tr>
<td>PPARδ (fold activation)</td>
<td>1.0 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>L165,041 (100 µM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>13.0 ± 0.8</td>
</tr>
<tr>
<td>Adiponectin Production (pg/mL)</td>
<td>10 ± 2.3</td>
<td>192 ± 11.6*</td>
<td>Rosiglitazone (10 µM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1398.2 ± 87.1</td>
</tr>
<tr>
<td>DPP-IV (% inhibition)</td>
<td>0.3 ± 0.4</td>
<td>0.7 ± 1.5</td>
<td>P32/98 (10 µM) 97.3 ± 0.8</td>
</tr>
<tr>
<td>Insulin Secretion (ng/mL)</td>
<td>3.6 ± 0.1</td>
<td>4.4 ± 0.08</td>
<td>GLP-1 (5 µM) 8.0 ± 0.01</td>
</tr>
<tr>
<td>α-glucosidase (% inhibition)</td>
<td>0.9 ± 0.2</td>
<td>14.6 ± 0.8*</td>
<td>Castanospermine (2.5 µM) 96.3 ± 0.19</td>
</tr>
<tr>
<td>GLP-1R (cAMP; pmol/mL)</td>
<td>17.8 ± 1.1</td>
<td>29.4 ± 1.2</td>
<td>GLP-1 (5 µM) 157 ± 11.1</td>
</tr>
</tbody>
</table>

MLR-1023 was tested for effects in *in vitro* and cell based assays. Experimental conditions are described in the methods section. *Indicates statistical difference (p<0.05) when compared to control value.
Figure 2.

Graph A: Comparison of glucose levels between Vehicle and MLR-1023 (30 mg/kg) over time (min).

Graph B: Comparison of insulin levels between Vehicle and MLR-1023 (30 mg/kg) over time (min).

Graph C: Comparison of glucose levels between Vehicle and MLR-1023 (30 mg/kg) over time (min).

Graph D: Comparison of insulin levels between Vehicle and MLR-1023 (30 mg/kg) over time (min).

Graph E: Time course of MLR-1023 levels in nanomoles (nM) over time (hrs).
Figure 3.
Figure 4.

A. Lyn Activity (% Control) vs. Preincubation Time (min)

- **MLR-1023**
- **Control**

B. Lyn Activity (% Control) vs. Concentration (log nM)

**EC$_{50}$ = 63 nM**
Figure 5.
Figure 6.