Inhibition of insulin receptor function by a human, allosteric monoclonal antibody

A potential new approach for the treatment of hyperinsulinemic hypoglycemia

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Introduction

Insulin initiates the regulation of cellular glucose metabolism by binding to the insulin receptor (INSR) on the cell surface, a process that activates the receptor’s intrinsic kinase activity.1 When activated, the INSR undergoes autophosphorylation, followed by the recruitment and phosphorylation of INSR signaling molecules, including the IRS proteins and members of the phosphotidylinositol 3-kinase (PI3K)-Akt pathway.2 In cells, activation of this pathway by insulin results in the translocation of glucose transporters to the cell surface with subsequent uptake of glucose.3,4

It has been suggested that monoclonal antibodies (mAbs) to the INSR may be useful in various disorders of glucose metabolism.3 Hypoglycemia due to insulin excess from both exogenous and endogenous sources is not an infrequent clinical condition.5-8 In some instances, the current treatments for insulin-induced hypoglycemia do not adequately restore normoglycemia, resulting in prolonged hospitalization or neurological damage.6 Although not currently available, therapies that attenuate insulin signaling via inhibition of the INSR, such as antagonist mAbs, could prove to be effective for the treatment of sustained and life threatening hyperinsulinemic hypoglycemia.

Studies of antibodies that inhibit insulin activation of the INSR, including both spontaneously occurring human autoantibodies and mouse mAbs, have been reported.9-11 In humans, autoantibodies to the INSR typically bind at the insulin binding site (the orthosteric site), and directly compete with insulin for binding. In most cases, these antibodies cause severe insulin resistance and diabetes despite compensatory hyperinsulinemia.12-15 Orthosteric INSR autoantibodies isolated from humans have been studied in rats, and have been shown to be weak agonists...
that cause hypoglycemia at low concentrations and hyperglycemia at high concentrations.\(^8\) Thus, these types of orthosteric antibodies are not likely clinical candidates for the treatment of hyperinsulinemic hypoglycemia.

Allosteric antibodies, antibodies that do not bind at the ligand binding site of receptors, can regulate cell signaling.\(^13\)\(^,\)\(^18\) In theory, these allosteric antibodies have the potential to bind and regulate receptors more selectively than orthosteric antibodies due to lower sequence and structural homology at allosteric sites relative to orthosteric sites.\(^19\) Allosteric regulation of the INSR by glucose and peptides has been previously described.\(^20\)\(^-\)\(^23\) We recently generated an allosteric, human mAb that activated the INSR both in vitro and in vivo, and normalized fasting glucose levels in diabetic mice.\(^24\)\(^,\)\(^25\) These types of mAbs have been classified as selective insulin receptor modulators.\(^26\) It is possible, therefore, that allosteric antibodies to the INSR that inhibit its activation could also be generated, and be useful for the treatment of hyperinsulinemic hypoglycemia. To date, such antibodies have not been reported.

In the study reported here, we identified allosteric antibodies that antagonized INSR signaling and selected one, XMetD, for further characterization. This allosteric antibody antagonized insulin action both in vitro and in vivo.

**Results**

**XMetD discovery**

Allosteric modulating antibodies targeting the human INSR (hINSR) were identified by panning naïve human antibody phage display libraries using the recombinant extracellular domain of the hINSR complexed to insulin. Antibodies binding the hINSR-insulin complex were identified by FACS screening of bacterial periplasmic extracts. Six that had high affinity to both the human and the mouse INSRs were reformatted to fully human IgG2 antibodies. The most efficacious of these antibodies, XMetD, was employed in all subsequent studies described herein.

**Binding of XMetD to the hINSR**

Binding of XMetD to Chinese hamster ovary (CHO) cells expressing either isoform of the hINSR (hINSR-A or hINSR-B) was demonstrated using FACS (Fig. 1). XMetD bound similarly to both isoforms of the hINSR. In contrast, XMetD bound minimally to parental CHO cells (Fig. 1) and to CHO cells expressing the insulin-like growth factor-1 receptor (IGF-1R) (data not shown). Most likely this minimal binding was due to the interaction with the low concentration of endogenous hamster INSR.

To quantify the binding affinity of XMetD to the hINSR and to measure the effect of insulin on the kinetics of XMetD binding to the hINSR, we employed surface plasmon resonance (SPR) analysis using solubilized hINSR (B isoform) that had been captured on the sensor surface (Fig. 2). In the absence of insulin, the on-rate for XMetD binding to the hINSR was \(3.8 \times 10^4 \text{ M}^{-1}\text{sec}^{-1}\) and the off-rate was \(3.1 \times 10^{-4} \text{ sec}^{-1}\) (\(K_D = 8 \text{ nM}\)). In the presence of insulin, the affinity of XMetD was -3-fold lower (\(K_D = 26 \text{ nM}\)). With insulin, the on-rate for XMetD binding to the hINSR was \(4.4 \times 10^4 \text{ M}^{-1}\text{sec}^{-1}\) and the off-rate was \(1.6 \times 10^{-3} \text{ sec}^{-1}\).

**The effect of XMetD on the binding of insulin to the hINSR**

We next studied whether XMetD inhibited the binding of insulin to the hINSR. Employing biotinylated human insulin in a FACS-based assay, we observed that 1.0 \(\mu\)M XMetD only partially inhibited insulin binding to the hINSR, with a \(-3\)-fold reduction in the observed affinity of biotinylated insulin (Fig. 3). No additional inhibitory effects on insulin binding to the hINSR were observed at higher XMetD concentrations. The EC\(_{50}\) for insulin binding in the absence of XMetD was 47 nM (95% CI: 31 to 71 nM) and increased to 125 nM (95% CI: 99 to 166 nM) in the presence of 1.0 \(\mu\)M XMetD. The observations that insulin only partially inhibits XMetD binding to the hINSR and, reciprocally, that XMetD only partially inhibits insulin binding to the hINSR, are characteristic of negative allosteric modulation rather than competitive orthosteric inhibition.\(^19\)\(^,\)\(^27\)

Monoclonal antibodies can induce desensitization of the INSR.\(^18\)\(^,\)\(^29\) We therefore studied the effect of XMetD on internalization (Fig. S1A) and downregulation (Fig. S1B). Insulin in the presence of a negative control IgG had little or no effect on these functions. XMetD as well did not induce either INSR internalization or downregulation in the presence or absence of insulin. In contrast a positive control IgG, in the presence of insulin, did induce both INSR internalization and downregulation by greater than 50%.

**The effects of XMetD on insulin-induced hINSR signaling**

We then assessed the effect of XMetD at 333 nM (the maximal inhibitory concentration for this function) on insulin activation of the hINSR. The EC\(_{50}\) for insulin-stimulated hINSR autophosphorylation in the absence of XMetD was 7.3 nM (95% CI: 6.0 to 8.9 nM). XMetD treatment resulted in a greater than 40-fold decrease in the sensitivity of insulin-stimulated hINSR autophosphorylation with an EC\(_{50}\) of 310 nM, (95% CI: 230 to...
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As a control, IGF-1R autophosphorylation by IGF-1 was studied. XMetD did not influence this autophosphorylation (data not shown). The much greater inhibition by XMetD of insulin-mediated hINSR autophosphorylation, relative to its inhibitory effect on insulin binding to the hINSR, indicated that the antagonistic effects of the antibody were mediated only in part by its negative modulation of insulin binding affinity. Thus, XMetD was also a negative modulator of hINSR activation.19,27 Accordingly, the negative modulating effects of XMetD on hINSR activation were further characterized by investigating how XMetD influenced insulin-dependent activation of intracellular signaling pathways. The serine phosphorylation of Akt is a critical downstream mediator of the metabolic effects of the activated hINSR.1 To characterize the effect of XMetD on insulin-dependent Akt phosphorylation, we employed CHO-hINSR cells exposed to XMetD concentrations ranging from 0 to 3333 nM (Fig. 5A). XMetD, at a maximal concentration for this function (3333 nM), induced a greater than 100-fold decrease in the sensitivity of insulin-dependent Akt phosphorylation. The EC50 for insulin-stimulated pAkt in the absence of XMetD was less than 1 nM (95% CI: 0.04 to 0.34 nM) and increased to 197 nM (95% CI: 174 to 223 nM) in the presence of 3333 nM XMetD. XMetD alone had no effect on Akt phosphorylation (Fig. 5A).

The receptor for IGF-1 has structural and sequence similarity to the INSR.30 We therefore assessed whether XMetD affected IGF-1-activatitvation of Akt in CHO cells expressing the human IGF-1R (CHO-hIGF-1R). IGF-1 treatment induced 416 nM (Fig. 4). As a control, IGF-1R autophosphorylation by IGF-1 was studied. XMetD did not influence this autophosphorylation (data not shown). The much greater inhibition by XMetD of insulin-mediated hINSR autophosphorylation, relative to its inhibitory effect on insulin binding to the hINSR, indicated that the antagonistic effects of the antibody were mediated only in part by its negative modulation of insulin binding affinity. Thus, XMetD was also a negative modulator of hINSR activation.19,27

Erk, when activated by tyrosine phosphorylation, is another critical downstream mediator INSR activity.1 We therefore evaluated the effect of XMetD on insulin-dependent Erk phosphorylation. As with Akt phosphorylation, XMetD induced a similar decrease in the sensitivity of insulin-dependent Erk phosphorylation in CHO-hINSR cells. In the presence of control IgG, the EC50 for insulin-induced Erk phosphorylation was 1.8 nM (95% CI: 1.4 to 2.3 nM); whereas, in the presence of XMetD, the EC50 for insulin-induced Erk phosphorylation was 107 nM (95% CI: 97 to 117 nM), (Fig. 5C).

The receptor for IGF-1 has structural and sequence similarity to the INSR.30 We therefore assessed whether XMetD affected IGF-1-activatitvation of Akt in CHO cells expressing the human IGF-1R (CHO-hIGF-1R). IGF-1 treatment induced
Akt phosphorylation in these cells, but unlike the prior studies with hINSR, XMetD exhibited little or no antagonism of IGF-1-mediated activation of Akt in CHO-hIGF-1R cells (Fig. 6). These data, in concert with the aforementioned lack of both XMetD binding to the IGF-1R, and XMetD inhibition of IGF-1R phosphorylation, strongly suggested that the inhibitory effect of XMetD was specific for the INSR.

To study whether XMetD inhibited glucose transport in muscle, a major target tissue for insulin, we employed cultured L6 muscle cells. These cells express both the hINSR and human GLUT-4, providing robust insulin sensitivity and responsiveness. In these cells, XMetD antagonized insulin stimulation of glucose uptake (Fig. 7) by 70-fold. The EC$_{50}$ for insulin-stimulated glucose uptake in the absence of XMetD was 0.25 nM (95% CI: 0.17 to 0.35 nM) and increased to 18 nM (95% CI: 12 to 26 nM) in the presence of 333 nM XMetD. Thus, the above studies indicated that inhibition of insulin-stimulated INSR activation by XMetD resulted in greatly diminished metabolic signal activation. In these studies, XMetD had no effect on INSR signaling in the absence of insulin (data not shown).

In addition to its effects on metabolic functions such as glucose transport, insulin can also stimulate the growth and proliferation of cancer cells. In COLO-205 human colon cancer cells, insulin stimulated growth with a half maximal effect at 0.5 nM. In contrast to the observed impact of XMetD on insulin-stimulated glucose transport, XMetD had little or no effect on insulin-stimulated proliferation (Fig. S2). Similar data were observed with other cancer cell lines including Saos-2 osteosarcoma cells (data not shown).

Binding of XMetD to mINSR and effects on mINSR-cell signaling

To determine if the mouse would be a suitable species for the characterization of XMetD in vivo, we evaluated the ability of XMetD to act as a negative modulator of the mouse INSR (mINSR) in vitro. XMetD bound to the mINSR in a manner similar to that of hINSR (data not shown). As with the hINSR, XMetD inhibited insulin-dependent Akt activation by the mINSR (Fig. 8). The EC$_{50}$ for insulin-stimulated Akt activation in the absence of XMetD was 0.97 nM (95% CI: 0.72 to 1.30 nM) and increased to 103 nM (95% CI: 88 to 119 nM) in the presence of 333 nM XMetD.

Effect of XMetD in normal mice

To assess whether the inhibitory effects of XMetD on the INSR observed in vitro were also observed in vivo, studies were performed in normal C57BL/6 mice. In fasted animals treated with control IgG, blood glucose levels were under 100 mg/dL (Fig. 9A). Treatment with XMetD resulted in slightly elevated glucose levels that were not significantly higher than controls. In mice treated with control IgG, plasma insulin levels were 0.73 ± 0.13 ng/ml (mean ± SEM) (Fig. 9B). In fasted mice treated with XMetD, plasma insulin levels were much higher at 12.6 ± 0.2 ng/ml.

We also studied whether XMetD inhibited the INSR activity in non-fasted mice. In non-fasted animals treated with control IgG for 24 h, blood glucose levels were under 200 mg/dL (Fig. 9C). In contrast, non-fasted animals treated with XMetD had significantly higher glucose levels. In mice treated with control IgG, plasma insulin levels were 1.1 ± 0.4 ng/ml (mean ± SEM) (Fig. 9D). In fasted mice treated with XMetD, plasma insulin levels were much higher at
66.0 ± 14.1 ng/ml. These studies indicated that both in fasted and non-fasted mice, XMetD induced insulin resistance with concomitant hyperinsulinemia.

**Effect of XMetD in mice with insulin implants**

To explore whether XMetD would antagonize hypoglycemia induced by insulin excess, we created a mouse model of hyperinsulinemic hypoglycemia via the insertion of slow-release insulin implants into normal mice. In this model, the implants continually released insulin for over 2 weeks, providing sustained exposure to high concentrations of the hormone. Three days post-implantation, and after a 6 h fast, mice became hypoglycemic with blood glucose levels in the range of 50 mg/dL (Fig. 10). Following a one week treatment with control IgG, blood glucose levels remained in the range of 50 mg/dL in fasted mice with implants. In contrast, following a one week treatment with XMetD, fasted mice with implants were no longer hypoglycemic, having blood glucose levels of approximately 100 mg/dL; these levels were not significantly different from control mice (Fig. 10).

**Discussion**

In this study, we identified and characterized a fully human mAb, XMetD, and found it to be a novel antagonist of insulin activation of the INSR. XMetD demonstrated the properties of an allosteric antibody. To identify this type of antibody, we employed the insulin-INSR complex rather than the INSR alone to screen phage libraries. This approach reduced the potential for obtaining antibodies that bound to the orthosteric site of the INSR, because the latter site was occupied by its ligand, insulin. XMetD bound directly to the INSR, and its binding was only partially reduced by a saturating concentration of insulin. Conversely, XMetD only partially inhibited insulin binding to
its receptor. These observations are characteristic of a reciprocal relationship between a negative allosteric modulator (XMetD) and an orthosteric ligand (insulin). 19,35

After insulin binds to the INSR, it stimulates receptor auto-phosphorylation and intrinsic kinase domain activation. The metabolic effect of insulin signaling through the INSR on both glucose transport and other aspects of metabolism occurs in large part through the PI3K-Akt pathway. 21,36 In cultured cells, XMetD markedly antagonized insulin-dependent INSR auto-phosphorylation and downstream metabolic effects, including Akt phosphorylation and glucose transport. Therefore, these studies indicated that XMetD was a highly effective inhibitor of insulin-mediated INSR signaling in vitro. It was notable that XMetD did not inhibit insulin-stimulated cancer cell growth in vitro. The reason for the lack of inhibition of this function has not been determined but may reflect an intrinsic aspect of malignant transformation. Further studies will be needed to understand this process.

The inhibitory effects of XMetD on insulin-mediated Akt activation with cells expressing the mINSR were similar to cells expressing the hINSR. Therefore, we undertook studies in mice to determine if this antibody would induce insulin resistance in vivo. In normal non-fasted mice, XMetD elevated blood glucose to levels within the diabetic range. Extreme insulin resistance was induced as reflected by the marked elevation of plasma insulin levels. In normal fasted mice treated with XMetD, hyperglycemia was not observed, but there was strong evidence of insulin resistance, as insulin levels in the fasted state were also markedly elevated.

Thus, the results of these studies indicated that XMetD is a highly effective INSR antagonist, causing insulin resistance both in vitro and in vivo. These findings suggested that this type of antibody would be effective at blocking hypoglycemia induced by insulin excess. Since XMetD was active on the mINSR, we tested XMetD in mice that were made hyperinsulinemic by the insertion of sustained-release insulin implants. As a result of this procedure, the animals became hypoglycemic, but glucose levels returned to the normal range when these hyperinsulinemic mice were treated with XMetD.

Not infrequently, diabetic patients treated with either insulin or insulin-releasing agents develop symptomatic hypoglycemia. This hypoglycemia can be severe and sustained, requiring visits to the emergency department and or prolonged hospitalization. 7,8 Our results indicate that allosteric antagonist antibodies to the INSR, such as XMetD, can inhibit INSR signaling in vivo. This observation raises the possibility that this class of antibodies may be useful for the short-term treatment of hypoglycemia caused by exogenous insulin.

In addition, rare conditions of sustained endogenous hyper-insulinemia where current therapies are not always adequate can occur. These conditions include insulinoma, 6,8,37 excess secretion of IGF-II 38 and congenital hyperinsulinemia (CHI). 39-41 In the latter condition, particularly in those patients with defects of the ATP-regulated potassium channel, control of hypoglycemia can be extremely difficult. 41,42 Preliminary studies, in the SUR-1 knockout mouse (an animal model of CHI), indicate that XMetD treatment blunts the effect of exogenous insulin. 43 Moreover, this antibody prevents fasting hypoglycemia in this animal model. 43 It is therefore possible that antagonist allosteric INSR antibodies could be a potential new treatment for patients
with hypoglycemia due to both exogenous and endogenous hyperinsulinism. Additional long-term studies with this class of antibodies will be needed to understand their use and clinical utility.

Materials and Methods

XMetD discovery

The extracellular domain of the human insulin receptor (hINSR; R&D Systems, #1544-1R) was biotinylated with Sulfo-NHS-LC-Biotin (Pierce, #21327) according to the manufacturer’s protocol. To obtain allosteric antibodies to the INSR, panning and subsequent screening were performed with the biotinylated extracellular domain of the hINSR maintained in the presence of a saturating insulin concentration (10 micromolar; Sigma-Aldrich, #19278) to block the orthosteric binding site, and prevent the selection of orthosteric antibodies. This biotinylated receptor-ligand complex was immobilized on streptavidin-coated magnetic Dynabeads® M-280 (Invitrogen Dynal AS, #11205D) and panned against antibody phage display libraries (XOMA Corporation).44,45 Following each round of panning, phage were deselected against streptavidin-coated magnetic Dynabeads® M-280 to remove non-specific phage antibodies. After three rounds of panning and deselection, bead-bound phage were eluted and used to infect TG1 bacterial cells according to standard methods.45 The lysate supernatants were assayed for INSR binding to suspension-adapted CHO cells (vide infra) by flow cytometry. The CHO cells employed were transfected with either the human or mouse INSR. Antibodies with ortholog cross-reactivity were reformatted into fully human IgG2 mAbs and tested for antagonist activity. The IgG2 with the greatest INSR inhibitory activity was XMetD.45

Engineered cells used to study the activity of XMetD

CHO cells were engineered to express either human INSR iso type A (CHO-hINSR-A), human INSR iso type B (CHO-hINSR-B), or mouse INSR iso type B (CHO-mINSR-B). INSR transfected cell lines had ~250,000 receptors per cell compared with the untransfected cells, which had less than 5,000 INSR per cell as determined by flow cytometry.46 For the majority of the subsequent functional studies shown herein, we employed CHO cells transfected with the B isoform of either the hINSR (CHO-hINSR) or mINSR (CHO-mINSR). The B isoform of the INSR was employed because it is the predominant isoform of the INSR in adult metabolic insulin responsive tissues.47-49 CHO cells were also engineered to express human IGF-1R (CHO-hIGF-1R) at 200,000 receptors per cell. Rat L6 myocytes were engineered to express both human GLUT-4 and the hINSR (100,000 receptors per cell).24

Binding of XMetD to the INSR by FACS

For flow cytometry (FACS), CHO-hINSR-A, CHO-hINSR-B and CHO-mINSR-B cells (2 × 10^6 per ml) were washed and resuspended in phosphate buffered saline with 0.5% fatty-acid-free bovine serum albumin (BSA) and 0.1% sodium azide (FACS buffer). XMetD was then added and cells were incubated for 120 min at 15 °C. Cells were washed and resuspended in Alexa Fluor® 647-conjugated goat anti-human IgG (1:200; Invitrogen, #A-21445) followed by a 30 min incubation at 4 °C. Subsequently, the cells were washed and analyzed on a FACScan™ flow cytometer (Becton Dickinson). All FACS data were analyzed using Prism software (GraphPad, Inc) and multiplied by a common factor so that the Bmax value for the curve fit with the greatest plateau (CHO-hINSR-B cells) was normalized to 100%.

Quantitative kinetic analysis of XMetD binding to the hINSR by SPR

XMetD kinetics and binding affinity were determined by SPR performed on the ProteOn XPR™ instrument (Bio-Rad) at 25 °C using the one-shot kinetics method.50,51 SPR measures biomolecular interactions in real-time in a label-free environment.52 CHO-hINSR-B cells were solubilized in Tris lysis buffer (TLB) consisting of 150 mM NaCl, 20 mM Tris pH 7.5, 1 mM EDTA, 1 mM EGTA, and 1% Triton® X-100 supplemented with a protease inhibitor cocktail (Roche, #058927901). Briefly, 1 ml of TBL was added to ~2 × 10^6 cells, and the cell suspensions were agitated on a rocker at 4 °C. After 2 h, this solution was centrifuged at 4 °C for 20 min at 14,000 rpm using a tabletop centrifuge. The supernatant containing solubilized INSR was filtered and kept frozen at -80 °C until it was thawed just prior to analysis. To prepare the SPR detection surface for INSR capture, CT-3 mAb (Fisher, #MS-636-PABX), which recognizes the carboxy-terminal moiety of INSR, was covalently immobilized on the sensor chip.
(Bio-Rad GLM, #176–5012) using standard amine-coupling chemistry. Briefly, the GLM sensor chip was preconditioned with successive injections of 10 mM SDS, 50 mM NaOH, 100 mM Tris pH 9.5, and running buffer consisting of 10 mM HEPES, 150 mM sodium chloride, 3 mM EDTA, 0.05% Polysorbate 20 (Teknova, #H8022) at a flow rate of 100 µL per min. The chip surface was then activated with a five min injection of a freshly prepared 1:1 solution of 0.1M N-hydroxysuccinimide (NHS) and 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide at a flow rate of 25 µL per min. Following activation, 10 µg/mL of CT-3 antibody in pH 4.5 acetate buffer was injected at a flow rate of 30 µL per min until ~10,000 RU of the capture antibody was immobilized on the sensor surface. To block the surface, 1 M ethanolamine hydrochloride-NaOH pH 8.5 was injected for five min.

Solubilized INSR diluted 1:1 in running buffer supplemented with 1 mg/ml BSA, with or without 1 µg/ml human insulin (Sigma, #A30590), was captured at a density of 1000-1200 RU on a vertical flow channel coated with the CT-3 antibody. After switching to a horizontal orientation and following 10 min of baseline stabilization, XMetD was injected over the captured solubilized INSR at concentrations of 133, 44, 14.7, 4.9, and 1.64 nM at a flow rate of 30 µL per min. Association was monitored for 5 min, and dissociation was monitored for 10 min. Surfaces were regenerated with 100 mM HCl following the XMetD injections. Double-referenced data were curve fit with a simple 1:1 binding model using ProteOn™ software to yield kinetic parameters for on-rate ($k_a$) and off-rate ($k_d$). Equilibrium binding constant ($K_D$) values were calculated from a ratio of the kinetic parameters ($k_d / k_a$).

The effect of XMetD on insulin binding affinity for the INSR

The effect of XMetD on insulin binding to the INSR was assessed using a FACS-based assay. CHO-hINSR-B cells were preincubated for 10 min at 4 °C with a saturating concentration of XMetD or an anti-keyhole limpet hemocyanin (KLH) IgG2 isotype control IgG (XOMA Corporation) followed by a 30 min incubation with increasing concentrations of biotinylated insulin (R&D Systems, custom reagent). Cells were then washed and resuspended in allophycocyanin-conjugated streptavidin (1:200 Invitrogen, #SA1005) followed by a 30 min incubation at 4 °C. Subsequently, the cells were washed and analyzed as above.

The effect of XMetD on INSR internalization and downregulation

To study the effect of XMetD on INSR internalization, we employed IM-9 human lymphoblasts. These cells were maintained in RPMI media (Gibco, #22400-089) containing 10%...
fetal bovine serum (FBS) (HyClone, #SH3007.03). To assess INSR internalization, the cells were incubated for 5 h in RPMI media without serum supplemented with 0.25% BSA. Next, IM-9 cells were treated for 2 h at 37 °C with antibodies plus a range of insulin concentrations. In addition to XMetD, we included both a negative control IgG (anti-KLH) and a downregulating positive control IgG (anti-INSR mAb, XOMA Corporation) all at 66 nM. After 2 h the cells were washed and then stained with the anti-INSR mAb MA-20 (Santa Cruz Biotechnology, #SC-57344) for 30 min at 4 °C. MA-20 does not compete with either XMetD or the positive control mAb for binding to the INSR. Following this incubation, cells were washed twice and resuspended in APC-conjugated goat anti-human IgG (1:200; Jackson ImmunoResearch, #115-136-146) and then incubated for 30 min at 4 °C. Subsequently, the cells were washed and analyzed on an AccuriTM C6 flow cytometer (Becton Dickinson). Fluorescence data reflecting surface INSR were analyzed using the instrument software (CFlow Plus).

To further study the effect of XMetD on INSR downregulation, we employed 3T3-L1 mouse preadipocyte cells (ATCC, #CL-173). These cells were grown on collagen coated plates, differentiated and cultured as described, except that cells were incubated without insulin following the removal of the differentiation media. Differentiated adipocytes were treated for 2 h at 37 °C in DMEM + 0.25% BSA media with antibodies at plus a range of insulin concentrations. In addition to XMetD, we employed the aforementioned negative and positive control IgGs, all at 66 nM. After 2 h the cells were lysed with Tris-lysis buffer (TLB) and analyzed for total INSR content using the Millipore, STAR ELISA Assay Kit (#17-483).

**INSR signaling in cultured cells**

To evaluate the effect of XMetD on hINSR autophosphorylation, CHO-hINSR-B cells were preincubated at 37 °C with either 333 nM XMetD or control IgG for 30 min, followed by incubation with increasing concentrations of insulin for 10 min. The phosphotyrosine content of the INSR was measured by ELISA (Millipore, #CBA038). To evaluate the effect of XMetD on insulin mediated Akt phosphorylation, CHO-hINSR-A, CHO-hINSR-B or CHO-mINSR-B cells were preincubated at 37 °C with either XMetD or control IgG for 30 min followed by an incubation with increasing concentrations of insulin for 10 min. Both total Akt and Akt phosphorylated at Ser473 were measured using an electrochemiluminescent assay (Meso Scale Discovery, #K150MND). The same basic protocol was employed for investigating the effect of XMetD on IGF-1 mediated IGF-1R signaling. Insulin-dependent Erk phosphorylation was performed as previously described using an electrochemiluminescent assay (Meso Scale Discovery, #K151DWD).

**Glucose transport assay**

To measure 2-deoxy-glucose uptake, rat L6 muscle cells expressing both hINSR and human GLUT-4 were preincubated at 37 °C with either 333 nM XMetD or control IgG for 60 min followed by incubation with increasing concentrations of insulin for 10 min. [3H]-2-deoxy-D-glucose was then added for 20 min and its uptake measured.

**Proliferation assay**

COLO-205 colorectal carcinoma cells (ATCC, #CCL-222), were cultured in RPMI 1640 media supplemented with 10% FBS. For proliferation assays, cells were seeded in microtiter plates (Costar, #3917) at a density of 20,000 cells per well. The media was then changed to RPMI (Gibco, #12676) supplemented with 0.5% FBS that had been both charcoal-dextran stripped and heat inactivated. After 24 h, increasing concentrations of insulin were added in the presence and absence of XMetD. Cells were then incubated for 48 h at 37 °C. Proliferation was measured using the CellTiter-Glo® luminescent cell viability assay (Promega, #G7571).

In all the aforementioned in vitro studies, the viability of the cells was approximately 90% as assessed by both microscopic observation and by trypan blue exclusion. Incubation with XMetD did not influence cell viability. All in vitro studies were performed at least 3 times and representative studies are shown. The in vitro signaling, downregulation and proliferation data were analyzed using Prism software (GraphPad, Inc) and normalized so that the curve fit maximal values equaled 100%.

**Effects of XMetD in normal fasted and non-fasted mice**

All animal experiments were approved by the XOMA Institutional Animal Care and Use Committee (IACUC) and performed in accordance with IACUC guidelines. All animals were maintained in a pathogen-free environment and allowed free access to food and water prior to study. In the first series of experiments, male C57BL/6 mice 6 to 8-weeks of age (The Jackson Laboratory, #000664) were employed and acclimated...
to a 12 h light-dark cycle. Twenty-four animals were randomly divided into two cohorts (n = 12); one cohort was treated with XMetD and the other with the aforementioned anti-KLH isotype control IgG (both antibodies were administered intraperitoneally at 10 mg/kg). All mice were then allowed free access to food for 10 h. Subsequently, each cohort was divided into two groups (n = 6). One group from each cohort was fasted for 14 h, while the other group was allowed free access to food during the dark cycle. At 24 h after antibody administration, blood from the tail vein was obtained and glucose was measured using a glucometer. Plasma was also obtained at this time by cardiac puncture, and insulin levels measured by ELISA (Alpco Diagnostics, 80-INSMSU-E01). XMetD did not cross-react with either capture or detection antibodies used in these systems (data not shown).

**Effects of XMetD in mice with insulin implants**

Male C57BL/6J mice, 6- to 8-weeks of age (The Jackson Laboratory, #000664) were randomly divided into two groups. The first group (n = 6) went untreated. In the second group (n = 12), two insulin implants (LinBits, Linshin Inc, #Re-1-T) were placed subcutaneously under the mid-dorsal skin as recommended by the manufacturer. According to the manufacturer, a single implant continually releases 0.1 units of insulin over 24 h in vivo for over 2 weeks. Insulin levels in control mice were less than 500 pg/mL compared with greater than 3000 pg/mL in mice with implants. After 3 d, both untreated and implanted mice were fasted 6 h and blood glucose was measured. On the next day, half of the implant group (n = 6) was treated intraperitoneally (10 mg/kg) with XMetD and the other half of the group (n = 6) was treated with the control IgG: this injection was repeated 5 d later. One week after initiation of antibody treatment, mice were fasted for 6 h and blood glucose was subsequently measured.

**Disclosure of Potential Conflicts of Interest**

Corbin JA, Bhaskar V, Goldfine ID, Issafras H, Bedinger DH, Lau A, Michelson K, Gross LM, Kuan HF, Tran C, Lao L, Handa M, Watson SR, Narasimha AJ, Zhu S, Levy R, Webster L, Wijesuriya SD, Liu N, Wu X, Chemla-Vogel D, Lee SR, Wong S, Wilcock D, Rubin P, and White ML have been or are employees of XOMA Corporation.

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Corbin JA led the research team, designed the in vitro experiments and wrote the manuscript. Bhaskar V designed the in vivo experiments, researched the data and edited the manuscript. Goldfine ID wrote and edited the manuscript and contributed to the discussion. Wilcock D, Rubin P, and White ML edited the manuscript and contributed to the discussion. Issafras H, Bedinger DH, Michelson K, Maddux BA, Kuan HF, Tran C, Lao L, Handa M, Watson S, Zhu S, Levy R, Webster L, Wijesuriya SD, Liu N, Wu X, and Chemla-Vogel D researched the in vitro data. Lau A, Gross LM, Narasimha AJ, Lee SR, and Wong S researched the in vivo data. Corbin JA takes responsibility for the content and originality of this manuscript. The authors wish to thank Dr Patrick J Scannell and Dr Marina K Roell, XOMA Corporation, for critical reading of the manuscript and helpful comments.

**Supplemental Materials**

Supplemental materials may be found here: www.landesbioscience.com/journals/mabs/article/26871

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