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Characterization of monoacylglycerol acyltransferase 2 inhibitors by a novel probe in binding assays

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ABSTRACT

Monoacylglycerol acyltransferase 2 (MGAT2) is a membrane-bound lipid acyltransferase that catalyzes the formation of diacylglycerol using monoacylglycerol and fatty acyl CoA as substrates. MGAT2 is important for intestinal lipid absorption and is an emerging target for the treatment of metabolic diseases. In the current study, we identified and characterized four classes of novel MGAT2 inhibitors. We established both steady state and kinetic binding assay protocols using a novel radioligand, [^{3}H]compound A. Diverse chemotypes of MGAT2 inhibitors were found to compete binding of [^{3}H]compound A to MGAT2, indicating the broad utility of [^{3}H]compound A for testing various classes of MGAT2 inhibitors. In the dynamic binding assays, the kinetic values of MGAT2 inhibitors such as K_{on}, K_{off}, and $T_{1/2}$ were systematically defined. Of particular value, the residence times of inhibitors on MGAT2 enzyme were derived. We believe that the identification of novel classes of MGAT2 inhibitors and the detailed kinetic characterization provide valuable information for the identification of superior candidates for in vivo animal and clinical studies. The current work using a chemical probe to define inhibitory kinetics can be broadly applied to other membrane-bound acyltransferases.

Monoacylglycerol acyltransferase 2 (MGAT2) is a membrane-bound lipid acyltransferase that is an emerging molecular target to treat obesity and type 2 diabetes [1]. MGAT2 is highly and selectively expressed in the small intestine, where it exerts an important role in the monoacylglycerol pathway of triacylglycerol (TG) synthesis for the absorption of dietary fat [2,3].

MGAT2 knockout (KO) mice exhibit a multitude of healthy metabolic phenotypes relative to wild-type (WT) controls, including resistance to high-fat diet-induced obesity, improvement in insulin sensitivity, and decreased fat accumulation in liver and adipose tissue [1]. To provide further insights into the therapeutic utility of MGAT2 inhibition, Yen's group recently generated two additional strains of *Mogat2* gene-modified mice. In one study, they generated an inducible *Mogat2*-inactivating mutation where they temporally controlled *Mogat2* inactivation only in adult mice [4]. Importantly, the adult onset MGAT2 deficiency also mediated the resistance to high-fat diet-induced obesity and insulin resistance [4]. In the second study, they generated intestinal-specific MGAT2 KO mice, which had a very similar phenotype compared with the whole body KO, which also exhibited a healthier metabolic phenotype with less weight gain, improved insulin sensitivity, and lipid profiles compared with their wild-type littermates under the high-fat diet challenge. In addition, the intestinal MGAT2 KO mice also showed a delay in fat absorption, a decrease in food intake, and a propensity to use fatty acids as fuel when first exposed to a high-fat diet. These findings illustrate that intestinal MGAT2 is the key target for pharmaceutical intervention [5].

Multiple pharmaceutical companies have identified MGAT2 inhibitors as therapeutic agents to combat obesity and type 2 diabetes, and many structurally diverse small molecules as inhibitors of MGAT2 have been published, where structure–activity relationships (SARs) were primarily evaluated based on results

Abbreviations used: MGAT2, monoacylglycerol acyltransferase 2; TG, triacylglycerol; KO, knockout; WT, wild-type; SAR, structure–activity relationship; DGAT1, diacylglycerol acyltransferase 1; AWAT2, acyl-CoA wax alcohol acyltransferase 2; DMSO, dimethyl sulfoxide; 2-MOG, 2-monooleoylglycerol; LC/MS, liquid chromatography/mass spectrometry; BMS, Bristol–Myers Squibb; HTS, high-throughput screening; ACAT, acyl CoA:cholesterol acyltransferase.

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obtained using in vitro MGAT2 enzymatic assays [6–10]. In the current study, we report the identification of additional novel MGAT2 inhibitors.

Because MGAT2 is a membrane protein and its substrates are hydrophobic, there is little information regarding the detailed mechanism of the acyltransferase enzyme reaction. Using a conventional enzyme assay, it is very difficult to generate detailed kinetic values for MGAT2 inhibitors.

To fill this gap, we synthesized a novel radioligand, [^{3}H]compound A. Exploiting the classic principle of mass action to model ligand binding to receptor proteins [11,12], we established binding assay protocols of [^{3}H]compound A for MGAT2 enzyme. In the steady state competition assay, multiple chemotypes of MGAT2 inhibitors all caused ligand displacement, indicating that this radioligand can be applied to studying diverse MGAT2 inhibitors. Furthermore, kinetic values such as K_{on}, K_{off}, and $T_{1/2}$ were systematically defined.

Materials and methods

Materials

All recombinant acyltransferases (human, rat, and mouse MGAT2, human MGAT3, human diacylglycerol acyltransferase 1 [DGAT1], and human acyl-CoA wax alcohol acyltransferase 2 [AWAT2]) used in the current study were expressed with an N-terminal FLAG epitope fused with the respective open reading frame sequences in the insect cell baculovirus system. The enzymes were prepared using microsome preparation as described by Cheng and coworkers [13]. Compound A, [^{3}H]compound A, and all other MGAT2 inhibitors studied in the current work are synthesized according to the online supplementary material or Turdi and coworkers [7]. All other chemicals and materials were obtained from Sigma–Aldrich.

Saturation binding of [^{3}H]compound A to recombinant human MGAT2

All binding assays described in the current article were conducted in 96-well, 2-ml deep well polypropylene plates (VWR). For 1-ml binding reactions, aliquots of 890 μl of binding buffer (100 mM potassium phosphate buffer [pH 7.5] and 5 mM ethylenediaminetetraacetic acid [EDTA]), 1 μl of dimethyl sulfoxide (DMSO, final concentration 0.1%), or 1 μl of 10 mM compound A (final concentration 10 μM in assays for determining nonspecific binding) and 100 μl of 0.005, 0.01, 0.02, or 0.05 mg/ml recombinant human MGAT2 microsomal membranes (for final amount of 0.5, 1, 2, or 5 μg in each assay, respectively) were added and incubated at room temperature for 10 min. The binding reactions were started by adding 10 μl of serial diluted [^{3}H]compound A (specific activity at 32 μCi/nmol) at 100× concentrations. The reaction mixtures were incubated at room temperature for 2 h and then were filtered through a GF/B filter plate (presoaked in 0.3% polyethylenimine) by using a Unifilter-96 Cell Harvester (PerkinElmer). The filter plate was washed three times with ice-cold washing buffer (phosphate-buffered saline [pH 7.5] and 0.15% CHAPS) and air-dried. The bottom of the filter plate was sealed, and 30 μl of MicroScint-20 (PerkinElmer) was added into each well. The plate was counted in a TopCount for 1 min/well. Specific binding was calculated by subtracting nonspecific binding from the total binding. Saturation binding curves were plotted for each human MGAT2 concentration. Observed K_d was calculated using the "one-site binding" model with the GraphPad Prism program. The final K_d for [^{3}H]compound A was the average of the K_d values obtained at each human MGAT2 concentration.

Steady state inhibition of MGAT2 binding by MGAT2 inhibitors

For 1-ml binding reactions, 885 μl of binding buffer, 5 μl of half-log serial diluted MGAT2 inhibitors at 200 × concentrations or DMSO (0.5% final concentration for vehicle control), and 100 μl of 0.05 mg/ml human MGAT2 microsomal membranes (5 μg) were added and incubated at room temperature for 10 min. The binding reactions were started by adding 10 μl of 150 nM [^{3}H]compound A (1.5 nM final concentration with specific activity at 32 μCi/nmol). Excess compound A at 1 μM final concentration was included for determining nonspecific binding. The reaction mixture was incubated at room temperature for 2 h. Bound radioligand was separated from free ligand by the filtration procedure as described above. The percentage of specific binding in the presence of MGAT2 inhibitors at various concentrations was calculated against that of DMSO control (100%). The binding IC_{50} was calculated using the "dose–response" model with GraphPad Prism. K_i values were calculated as $IC_{50}/(1 + L/K_d)$, where L is the concentration of [^{3}H]compound A used in the assay and K_d is the binding dissociation constant determined separately for [^{3}H]compound A.

Rate of dissociation of [^{3}H]compound A/MGAT2 complex

To assemble 1-ml binding reactions in a 96-well, 2-ml deep well plate, aliquots of 95 μl of binding buffer, 100 μl of 0.05 mg/ml human MGAT2 microsomal membranes, 5 μl of DMSO (final concentration at 0.5% at time 0) or 0.2 mM compound A (1 μM final concentration for determining nonspecific binding), and 800 μl of [^{3}H]compound A at 1.25 × concentrations (1 × in the assays with specific activity at 32 μCi/nmol) were added. The reaction mixture was incubated at room temperature for 2 h. To initiate the dissociation of [^{3}H]compound A, aliquots of 5 μl of 0.2 mM compound A (1 μM final concentration) were added at various time points. Bound radioligand was separated from free ligand by the filtration procedure as described above. Dissociation reactions under several concentrations of [^{3}H]compound A were generated, and the dissociation constant (K_{off}) at each [^{3}H]compound A concentration was calculated using the "one phase exponential decay" model with GraphPad Prism. The final dissociation constant for [^{3}H]compound A was the average of the K_{off} values obtained at each concentration.

Rate of association of [^{3}H]compound A/MGAT2 complex

To assemble 1-ml binding reactions in a 96-well, 2-ml deep well plate, aliquots of 95 μl of binding buffer, 100 μl of 0.05 mg/ml human MGAT2 microsomal membranes (5 μg each reaction), and 5 μl of DMSO (0.5% final concentration for assays to determine total binding) or 0.2 mM compound A (1 μM final concentration for determining nonspecific binding) were added. Aliquots of 800 μl of [^{3}H]compound A (specific activity at 32 μCi/nmol) at 1.25 × concentrations were added at various time points to start the binding reaction. The reaction mixtures were incubated at room temperature for various lengths of time. Bound radioligand was separated from free ligand by the filtration procedure as described above. Association reactions under several concentrations of [^{3}H]compound A were conducted, and the observed association constant (K_{obs}) at each [^{3}H]compound A concentration was calculated using the "one phase exponential association" model with GraphPad Prism. A plot with K_{obs} at the y-axis and [^{3}H]compound A concentration at the x-axis was generated and analyzed for linear regression, of which the slope was resolved as the final association constant (K_{on}) for [^{3}H]compound A.