Identification and Characterization of NDT 9513727 \([N,N\text{-}bis(1,3\text{-}benzodioxol-5-ylmethyl})\text{-}1\text{-}butyl\text{-}2,4\text{-}diphenyl\text{-}1H\text{-}imidazole\text{-}5\text{-}methanamine]\), a Novel, Orally Bioavailable C5a Receptor Inverse Agonist\(^S\)

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ABSTRACT

The complement system represents an innate immune mechanism of host defense that has three effector arms, the C3a receptor, the C5a receptor (C5aR), and the membrane attack complex. Because of its inflammatory and immune-enhancing properties, the biological activity of C5a and its classical receptor have been widely studied. Because specific antagonism of the C5aR could have therapeutic benefit without affecting the protective immune response, the C5aR continues to be a promising target for pharmaceutical research. The lack of specific, potent and orally bioavailable small-molecule antagonists has limited the clinical investigation of the C5aR. We report the discovery of NDT 9513727 \([N,N\text{-}bis(1,3\text{-}benzodioxol-5-ylmethyl})\text{-}1\text{-}butyl\text{-}2,4\text{-}diphenyl\text{-}1H\text{-}imidazole\text{-}5\text{-}methanamine]\), a small-molecule, orally bioavailable, selective, and potent inverse agonist of the human C5aR. NDT 9513727 was discovered based on the integrated use of in vitro affinity and functional assays in conjunction with medicinal chemistry. NDT 9513727 inhibited C5a-stimulated responses, including guanosine 5'\text{-}3\text{-}O\text{-}(thio)triphosphate binding, Ca\textsuperscript{2+} mobilization, oxidative burst, degranulation, cell surface CD11b expression and chemotaxis in various cell types with IC\textsubscript{50} from 1.1 to 9.2 nM, respectively. In C5a competition radioligand binding experiments, NDT 9513727 exhibited an IC\textsubscript{50} of 11.6 nM. NDT 9513727 effectively inhibited C5a-induced neutropenia in gerbil and cynomolgus macaque in vivo. The findings suggest that NDT 9513727 may be a promising new entity for the treatment of human inflammatory diseases.

The complement system is part of the innate immune system that plays an important role in host defense mechanisms and consists of more than 30 blood-borne and cellular proteins activated via three biochemical cascades of activation, named the classical, alternative, and mannose-binding lectin pathways. Effector mechanisms of the complement system include two anaphylotoxin G-protein-coupled receptors, the C3a receptor and the C5aR (also known as CD88), and the membrane attack complex, C5b-9. C5a, a 74-amino acid four-helix bundle glycoprotein, is generated after local complement activation brought about by divergent stimuli including immune complexes, infectious agents, various bio- chemicals including polysaccharides and the amyloidogenic β-peptide, and physical injury (Makrides, 1998). The C5aR is expressed on several cells of myeloid lineage including neutrophils and mast cells, on Kupfer andstellate cells of the liver, and on subsets of smooth muscle, endothelial, epithelial, lymphocytic, astrocytic, and neuronal cells (Gerard and Gerard, 1994; Zwirner et al., 1999; Schieferdecker et al., 2001). C5aR is a G-protein-coupled receptor that functionally couples ligand binding to intracellular responses, including decreases in intracellular AMP levels, increases in intracellular calcium

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**ABBREVIATIONS:** C5aR, C5a receptor; AMD, age-related macular degeneration; r, rat; h, human; NDT 9517011, 4-(2-[(cyclohexylphenyl)methyl]amino)-2-oxoethyl-N-(diphenylmethyl)-2-piperazinecarboxamide; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; BSA, bovine serum albumin; GTP\textsuperscript{S}, guanosine 5'\text{-}O\text{-}(3\text{-}[\text{35S}]\text{thiotriphosphate; GTP}\textsuperscript{S}[\text{[35S]}], guanosine 5'\text{-}O\text{-}(3\text{-}[\text{35S}]\text{thiotriphosphate; KRH, Krebs}\text{-}Ringer-HEPES; FLIPR, fluorometric imaging plate reader; m.o.i., multiplicity of infection; ROS, reactive oxygen species; Bay K8644, S-(--)1,4-dihydro-2,6-dimethyl-5-nitro-4-(2-[[trifluoromethyl]phenyl]-3-pyridine carboxylic acid methyl ester.
levels, and activation of phosphatidylinositol 3-kinase and mitogen-activated protein kinase activities (Gerard and Gerard, 1994). The cellular consequences of C5aR activation include classic neutrophil activation events, such as chemotaxis, cellular adhesion, and degranulation. In addition, C5aR activation has been shown to influence cytokine and chemokine gene expression and cellular apoptosis (Guo et al., 2000; Grant et al., 2002).

Because of the proinflammatory nature of C5a, C5aR antagonists have been sought as potential therapeutic agents for disorders, such as rheumatoid arthritis, asthma, respiratory distress syndrome, ischemia-reperfusion injury, sepsis, psoriasis, inflammatory bowel disease, and neuroinflammatory disorders (Guo and Ward, 2005). Several research advances, including C5aR knockout mice, recombinant C5a protein antagonists, and C5a and C5 convertase-neutralizing antibodies, have provided important evidence for the involvement of the C5aR in various inflammatory disorders (Wang et al., 1995; Pellas et al., 1998; Gerard and Gerard, 2002; Guo and Ward, 2005; Solomon et al., 2005; Köhl, 2006). In the case of rheumatoid arthritis, the role of the C5aR in disease pathogenesis and progression has been elucidated using the K/BxN T-cell receptor transgenic mouse model of inflammatory synovitis (Ji et al., 2002). In a series of elegant studies using several complement pathway deficiencies in mice, these investigators demonstrated that the inflammatory arthritis state was critically dependent on C5aR, whereas disruption of the C5b-9 complex had no effect on disease pathogenesis. Human genetic studies implicate increased complement activity in rheumatoid arthritis, asthma, and cellular apoptosis. In addition, C5aR antagonists have been shown to influence cytokine and chemokine gene expression and cellular apoptosis (Guo et al., 2000; Grant et al., 2002).

**Materials and Methods**

**Materials.** Recombinant expression of rat (r) and human (h) C5a and hC5a desArg was performed in an Escherichia coli system as described previously (Okinaga et al., 2003). Recombinant (r) human C5a was purchased from Sigma-Aldrich (St. Louis, MO). NDT 9513727 was synthesized at Neurogen according to procedures reported in Thurkauf et al. (2002) and Luke et al. (2003); concentrations of this compound are stated as the free-base equivalent. The cyclic peptide C5a antagonists AcF[OPdCHAWr] and F[OPdChaWr] (Finch et al., 1999) were synthesized as described previously (Waters et al., 2005). The small-molecule agonist NDT 9517011 was synthesized at Neurogen.

**Cell Lines and Cell Culture.** CHO, U937, HL-60, and AtT-20 cells were obtained from the American Type Cell Culture (Manassas, VA). For the functional studies described below, U937 cells were differentiated in RPMI 1640 containing 10% fetal bovine serum and 1 mM dibutyryl cAMP for 48 h at 37°C, and HL-60 cells were differentiated in RPMI 1640 containing 20% fetal bovine serum and 1 mM dibutyryl cAMP for 48 h at 37°C. AtT-20 cells were cultured in F10 medium containing 15% horse serum and 2.5% fetal bovine serum. hC5aR was isolated from a human fetal brain cDNA library by standard hybridization techniques, and its sequence was determined. The hC5aR cDNA was cloned into the pcDNA3.1 mammalian expression plasmid (Invitrogen, Carlsbad, CA) and transfected into CHO cells. A CHO cell line expressing high levels of hC5aR was subsequently selected by G418 (Sigma-Aldrich) resistance. The CHO cell line expressing C5L2 was described previously (Okinaga et al., 2003).

**Recombinant Expression of the Human C5a Receptor in Baculovirus-Infected S/9 Cells.** The hC5aR cDNA was cloned into the baculovirus expression vector, pBacPak9 (Clontech, Mountain View, CA). Specific C5a receptor- and G-protein subunit-containing baculoviral expression vectors were cotransfected along with BaculoGold DNA (BD Biosciences Pharmingen, San Diego, CA) into S/9 cells. The S/9 cell culture supernatant was harvested 3 days after transfection. The recombinant virus-containing supernatant was serially diluted in Hink’s TNM-FH insect medium (JRH Biosciences, Lenexa, KS)-supplemented Grace’s salts and with 4.1 mM 1-glutamine, 3.3 g/l lactalbumin hydrosolate, 3.3 g/l ultrarfiltered yeastolate, and 10% heat-inactivated fetal bovine serum (hereafter “insect medium”) and plaque assayed to titer the viral stock for recombinant plaques. Recombinant baculoviral clones were identified, passage 3 baculoviral stock was titered via plaque assay, and a multiplicity of infection (m.o.i.) and infection time course experiment was carried out to determine conditions for optimal receptor expression. Results from these studies showed that an m.o.i. of 0.1 and a 72-h incubation were parameters that achieved optimal hC5a receptor expression in up to 1 liter of S/9 cultures.

**Log-phase S/9 cells were infected with recombinant baculovirus followed by culture in insect medium at 27°C. Infections were carried out either with the hC5a receptor alone or with the hC5a receptor in combination with G-protein-encoding virus stocks obtained from BioSignal Packard, Inc. (Montreal, QC, Canada). The hetero-ericogenic G-protein subunits expressing viral stocks were rat Go αs, encoding virus stock (BioSignal Packard, Inc. no. V5J008), bovine Gβ1, encoding virus stock (BioSignal Packard, Inc. no. V5H012), and human Gγ2, encoding virus stock (BioSignal Packard, Inc. no. V6B003). The infections were carried out at an m.o.i. of 0.1:1.0:0.5:0.5 for hC5aR, Go αs, Gβ1, and Gγ2, respectively, and harvested 72 h after infection. A cell suspension aliquot was analyzed for viability by trypan blue dye exclusion, and the remaining S/9 cells were harvested via centrifugation (3000 rpm/10 min/4°C).

**Affinity Binding with [125I]IhC5a.** S/9 cell pellets expressing hC5aR, Go αs, γ1, and γ2 were resuspended in homogenization buffer (10 mM HEPES, 250 mM sucrose, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin, 200 μM phenylmethylsulfonyl fluoride, and 2.5 mM EDTA, pH 7.4) and homogenized using a Polytron homogenizer (setting 5 for 30 s). The homogenate was centrifuged (5360g/10 min/4°C) to pellet the nuclei, and the supernatant containing plasma membranes was decanted to a clean centrifuge tube, centrifuged (48,000g/30 min, 4°C), and resuspended in 30 ml of homogenization buffer. This centrifugation and resuspension step was repeated.
twice. The final pellet was resuspended in ice-cold Dulbecco's PBS containing 5 mM EDTA and stored in frozen aliquots at −80°C until needed. The protein concentration of the resulting membrane preparation was measured using the Bradford protein assay (Bio-Rad, Hercules, CA). P2 membranes were resuspended by Dounce homogenization in binding buffer (50 mM HEPES, pH 7.6, 120 mM NaCl, 1 mM CaCl2, 5 mM MgCl2, 0.1% BSA, pH 7.4, 0.1 mM bacitracin, 100 KIU/ml aprotinin). P2 membrane preparations were obtained for tissue binding studies. Canine, rat, rabbit, mouse, gerbil, and cynomolgus macaque monkey (Macaque fascicularis) lung tissues were obtained from Covance Research Products (Princeton, NJ) or Pel-Freeze, Inc. (Rogers, AR) and were stored at −80°C until preparation of membranes. Lung was minced into small pieces, thawed in ice-cold phosphate-buffered saline, rinsed with the same buffer, and homogenized at setting 5 in a Polytron homogenizer in a buffer containing 1 mM phenanthroline in 50 mM Tris, pH 7.0. The homogenate was centrifuged at 2300 rpm in a Sorvall SC 5C rotor (Sorvall, Newton, CT) for 30 min at 4°C, and the supernatant was harvested and filtered through gauze. The filtered supernatant was centrifuged at 20,000 rpm in the same rotor for 90 min. The supernatant was decanted, and the pellet was resuspended in the buffer described above. The suspension was centrifuged 10 min at 20,000 rpm, and the P2 membranes were resuspended at a protein concentration of 2 mg/ml in 25 mM HEPES, pH 7.2, containing 0.2 mg/ml each of aprotinin and leupeptin.

For saturation binding analysis, membranes (5 μg of protein) were added to polystyrene tubes containing 0.005 to 0.5 nM [35S]C5a (recombinant; PerkinElmer Life and Analytical Sciences, Waltham, MA) and the binding buffer with the final concentration of additives as stated above. Non-specific binding was determined in the presence of 300 nM hC5a (Sigma-Aldrich) and accounted for less than 10% of total binding.

For competition analysis, S/9 membranes (5 μg) and lung membranes (150 μg) were added to polystyrene tubes containing 0.030 nM [35S]C5a for S/9 membrane binding studies or 0.050 to 0.10 nM [35S]C5a for tissue binding analysis. NDT 9513727 was added to separate assay tubes at concentrations ranging from 10−10 to 10−5 M to yield a final volume of 0.250 ml. Non-specific binding was determined in the presence of 300 nM hC5a (Sigma-Aldrich) and accounted for less than 10% of total binding. After a 2-h incubation at room temperature, reactions were terminated by rapid vacuum filtration. Samples were filtered over preassembled (in 1.0% polyethyleneimine for 2 h before use) GF/C Whatman filters and rinsed twice with 5 ml of cold binding buffer without BSA, bacitracin, or aprotinin. Remaining bound radioactivity was quantified by gamma counting. IC50 and Hill coefficient (nH) were determined by plotting the data as a log-logit function, and the 50% inhibition point was determined by using the linear portion of data plot. The Hill coefficient was determined as the slope factor of the resultant linear transformation.

**GTPγS Binding Assays.** GTPγS binding activity was measured using a modification of a previously described method (Weiland and Jacobs, 1994). S/9 cells recombinantly expressing the human C5αR and the G-protein subunits αi, βi, and γi were used to prepare P2 membranes as described in the affinity binding protocol. On the assay day, thawed membrane preparations were resuspended in GTPγS binding assay buffer (50 mM Tris, pH 7.0, 120 mM NaCl, 2 mM MgCl2, 2 mM EGTA, 0.1% BSA, 0.1 mM bacitracin, 100 KIU/ml aprotinin, 5 μM GDP) and added to reaction tubes at a concentration of 30 μg/reaction tube. After adding NDT 9513727 at concentrations ranging from 0.1 nM to 10 μM, reactions were initiated by the simultaneous addition of 100 μM GTPγS and human recombinant hC5a (Sigma-Aldrich) ranging in concentration from 0.001 nM to 10.0 μM (final assay volume of 0.250 ml). After 60-min incubation at room temperature, the reaction was terminated by vacuum filtration over GF/C filters (preassembled in wash buffer containing 0.1% bovine serum albumin) with ice-cold wash buffer (50 mM Tris, pH 7.0, containing 120 mM NaCl). Bound GTPγS was determined by liquid scintillation spectrometry. Nonspecific binding was defined by 10 μM GTPγS and represented less than 5% of the total binding.

Inverse agonist activity of NDT 9513727 was assessed in a modified GTPγS binding assay, wherein the baseline activity was elevated by lowering the GDP concentration in the assay to 1.0 μM and performing an NDT 9513727 dose-response study in the absence of C5α. NDT 9513727 was assessed as to its mechanistic type of antagonism by evaluation in the GTPγS binding assay using the Schild analysis (Arunlakshana and Schild, 1959). The Schild analysis evaluated C5α-stimulated GTPγS binding dose-response curves at four different NDT 9513727 concentrations.

**Intracellular Calcium Mobilization.** Differentiated U937 cells were pelleted by centrifugation and resuspended in Krebs-Ringer-HEPES (KRH) solution (25 mM HEPES, 5 mM KCl, 0.96 mM NaH2PO4, 1 mM MgSO4, 2 mM CaCl2, 5 mM glucose, pH 7.4) containing Fluo-3 (Invitrogen, Eugene, OR) calcium-sensitive dye (10 μg/ml). The cell suspension was incubated at 37°C for 30 min. Cells were pelleted, washed, repelleted, and resuspended at a density of 1 × 106 cells/ml. Cells were seeded into 96-well plates suitable for use in the fluorometric imaging plate reader (FLIPR, model no. 1; Molecular Devices, Sunnyvale CA). Fluorescence responses, measured by excitation at 480 nM and emission at 530 nM, were monitored upon the addition of 0.01 to 30 nM human C5α to an FLIPR.

Differentiated U937 cells were incubated with varying concentrations of NDT 9513727, ranging from 0.1 nM to 4 μM, for 1 h before the addition of 0.3 μM hC5α (an EC50 concentration). The fluorescence response, upon the subsequent addition of the EC50 concentration of hC5α, was then monitored using an FLIPR. The IC50 and Hill coefficient of the C5α response were determined as with the radioligand binding data.

**C5α-Induced U937 and Neutrophil Chemotaxis Assays.** NDT 9513727 was evaluated for inhibition of C5α-induced chemotaxis of either freshly prepared human neutrophils or differentiated U937 cells. Fresh human neutrophils were isolated from whole blood using a Polymorphrep gradient (Nycomed, Oslo, Norway). Forty milliliters of human blood yielded approximately 30 million neutrophils with greater than 95% viability as assessed by trypan blue staining. Cells were sedimented by centrifugation and incubated with 8 μg/ml calcine-AM fluorescent dye (Molecular Probes) for 30 min at 37°C. Cells were sedimented by centrifugation, resuspended in phenol red-free RPMI medium (Invitrogen) containing 0.1% fetal bovine serum, sedimented again by centrifugation, and resuspended at a density of 3 × 106 cells/ml in phenol red-free RPMI 1640 medium containing 0.1% fetal bovine serum. One hundred fifty-microliter cell suspensions were mixed in assay tubes containing 1.5 μl of NDT 9513727 (10−11 to 10−6 M final concentrations) in dimethyl sulfoxide. Cells were incubated with compound for 30 min at room temperature. Neutroprobe ChemoTx 96-well plates (Neuro Probe, Gaithersburg, MD) were used to assess hC5α-activated chemotaxis. The wells in the plate contained either 0.4 or 0.5 nM C5α (for U937 and human neutrophils, respectively), concentrations of C5α previously determined to be EC50 to achieve a maximal chemotaxis response in these assays. An 8-μm pore diameter membrane was placed on top of the wells, and aliquots of cell suspension were transferred in quadruplicate onto spots on the membrane directly over each well. The plate was placed at 37°C for 1 h. The tops of the plate membranes were then rinsed with phosphate-buffered saline and gently wiped with cheesecloth to eliminate residual cells that did not migrate. Fluorescence measurements were made in the plate wells with the Fluoroskan Ascent 96-well plate fluorometer (excitation 485 nm, emission 530 nm; Thermo Electron Corp, Hartford, CT). The extent of fluorescence present provides an index of the extent of cellular chemotaxis. The data were plotted as a function of drug concentration to determine the IC50 using a logistic equation, with the aid of the KaleidaGraph Program 3.0 from Synergy Software.

**C5α-Induced Cell Degranulation Assay.** U937 cells were differentiated as described above for 72 h. Cells were centrifuged and...
resuspended in HEPES buffer containing 0.5 mM glucose at 107 cells/ml. Cells were then equilibrated for 30 min at 37°C. Approximately 3.5 million cells were added to 1.5 ml tubes containing 3.5 μl of NDT 9513727 (10-11 to 10-6 M final concentrations). Cells with or without the presence of drug were incubated at room temperature for 30 min. One hundred microliters per well of cells was added in triplicate to a 96-well Falcon clear bottom plate containing 5.0 μg of cytochalasin B and 5 nM C5a, which was previously determined as the EC50 dose for β-glucuronidase release. Plates were incubated for 30 min at 37°C. After incubation, 100 μl of supernatant was carefully removed and transferred to a fresh 96-well clear bottom plate (containing 50 μM fluorescein di-β-D-glucuronide) for the determination of β-glucuronidase activity. Plates were incubated for 16 h at 37°C. Released fluorochrome was measured using the Fluoroskan plate reader (excitation, 485 nm; emission, 530 nm). The data were plotted as a function of drug concentration to determine the IC50 using a logistic equation as described above.

Reactive Oxygen Species Assay. Differentiated HL-60 cells were washed several times and resuspended in KRH containing 10 μM dihydrorhodamine 123 (Molecular Probes). Aliquots of the cellular suspension were transferred to individual wells in a 96-well plate. Some wells contained varying concentrations (0.01-100 nM) of NDT 9513727, and others did not contain drug. Recombinant hC5a was diluted in KRH buffer containing 200 nM phorbol 12-myristate 13-acetate and added to each of the wells. The C5a concentration in all wells containing NDT 9513727 was 2.5 nM, the EC50 for C5a in this assay. Plates were incubated at 37°C for 30 min. Fluorescence was then measured using the FluorSkan (excitation, 488 nm; emission, 530 nm) and normalized to negative control wells by subtracting the background fluorescence observed (no C5a added). Normalized data were plotted as a function of drug concentration to determine the IC50 using a logistic equation, with the aid of the KaleidaGraph Program 3.0 from Synergy Software (Reading, PA). The assay typically resulted in a signal to background noise of between 60 to 90 relative fluorescence units using maximal C5α concentrations of 100 nM.

Granulocyte CD11b Flow Cytometry Assay. Human blood was collected in Vacutainers containing 143 USP units of sodium heparin anticoagulant (BD Biosciences, San Jose, CA) and processed within 4 h of collection. These experiments were performed at room temperature to minimize the conversion of C5a to C5a desArg by plasma carboxypeptidase activity. BioHit precision micropipets were used for accuracy and reproducibility. Using 12 × 75-mm polystyrene tubes, drug or dimethyl sulfoxide vehicle was added to 100 μl of whole blood (1% dimethyl sulfoxide final) for 15 min before the addition of recombinant human C5a (Sigma-Aldrich). C5a stock solution was prepared in PBS containing 0.25 mg/ml bovine serum albumin (Sigma-Aldrich). For C5a concentration-response analysis, 1 to 300 nM C5a was added to stimulate granulocytes for 2 to 5 min. For drug inhibition experiments, 10 nM C5a (approximate EC40) was added. Direct immunofluorescent granulocyte labeling was accomplished with conjugated monoclonal antibodies CD11b-PE-Cy5 and CD15-fluorescein isothiocyanate from BD Biosciences Pharmingen. Twenty microliters of a neat working solution of each antibody was added to each sample and incubated for 10 min. Subsequently, the samples were lysed for 10 min in 2 ml of NH4Cl, pH 7.3, buffer and centrifuged at 300g for 5 min, washed in 1 ml of 0.1% sodium azide/PBS buffer, recentrifuged, and fixed in 1% paraformaldehyde/0.1% sodium azide/PBS buffer before flow cytometry analysis. Samples were run on a Beckman Coulter EPICS XL-MCL Flow Cytometer (Beckman Coulter, Fullerton, CA) and analyzed using EXPO 32 ADC software. The collection protocol was designed to sort cell populations on the distribution of forward light scatter versus side light scatter adjusted to detect fluorochrome emission in the range of 680 (PE-Cy5) and 525 nm (fluorescein isothiocyanate). Cytometry data collection was terminated after 5000 CD15-positive cells were detected. The data were analyzed by calculating the mean CD11b-PE-Cy5 fluorescence detected on strongly forward and side light scatter cells (deemed granulocytes). The mean CD11b-PE-Cy5 fluorescence was calculated from two independent measurements of each hC5a concentration tested and plotted as a function of hC5a concentration to determine EC50. For NDT 9513727 data, percentage inhibition of C5a-stimulated response was calculated from two independent measurements of each drug concentration tested and plotted as a function of drug concentration to determine IC50.

Pharmacokinetics of NDT 9513727 in Rats and Monkeys. NDT 9513727 was formulated in PEG 400/water [50/50 (v/v)] for i.v. studies and in 0.5% methylcellulose/0.1% triacetin/water for oral studies. Drug solution was administered to male Sprague-Dawley rats or male cynomolgus macaque monkeys by bolus intravenous injection or oral gavage. Blood samples (~0.4 ml each for rat, ~1.0 ml for monkey) were collected in 1.5-ml tubes containing heparin from rats via an indwelling jugular catheter or from monkeys via the saphenous or femoral vein into tubes containing potassium EDTA. Blood samples were kept on ice and centrifuged within 30 min of collection. The plasma fractions were frozen at −20°C. NDT 9513727 was extracted from plasma by protein precipitation with acetonitrile and analyzed by a validated liquid chromatography/tandem mass spectrometry method. Pharmacokinetic parameters were determined for each individual animal using the WinNonlin program (Pharsight, Mountain View, CA) and then averaged.

Human C5a-Induced Neutropenia in Gerbils. All experiments in Mongolian gerbils were performed in accordance with the approval of the Animal Care and Use Committees of Wesleyan University and Neurogen Corporation and in accordance with the Guide for the Care and Use of Laboratory Animals (1996). Six-week-old Mongolian gerbils (Charles River Laboratories, Inc., Wilmington, MA) were housed for 2 days before experimentation. The method of Sumichika et al. (2002) was used with a few modifications. In brief, Mongolian gerbils were anesthetized with 60 mg/kg i.m. Telazol (Webster Veterinary Supply, Sterling, MA) and were placed on a heating pad to keep body temperature approximately 37°C throughout the experiment, and a catheter was placed in the jugular vein. At approximately 15 min after anesthesia administration, an initial 100-μl blood sample was collected via the jugular catheter into a sodium EDTA tube. This was defined as sample t = −5 min. Similarly, a second blood sample was collected 5 min later, t = 0 min. hC5a (diluted in 50 μl of saline) was administered immediately after the t = 0 min blood sample via the jugular catheter. Blood samples were collected as described at t = 1, 3, 5, and, in C5a dose-response experiments, 10 min. Blood samples were kept on ice and analyzed as rapidly as possible on a HemaVet 950 blood analyzer (Drew Scientific, Oxford, CT). For experiments with NDT 9513727, drug was weighed and added to the appropriate volume of 0.5% methylcellulose in water and probe sonicated. Triacetin was added to achieve 0.1% final concentration, and the drug suspension was stirred with a magnetic stir bar overnight. NDT 9513727 was stable in dosing solution up to 1 week after preparation. Food was removed from the animal cages for 1 h before drug administration. NDT 9513727 or 0.5% methylcellulose/0.1% triacetin control solution was administered to gerbils by oral gavage at a volume of 10 ml/kg. The observed concentration of neutrophils varied between subjects. NDT 9513727 was administered 2 h before hC5a challenge. The neutrophil concentrations observed in each of the blood samples were expressed as a percentage of baseline concentration, which was calculated as the average of the t = −5 min and t = 0 min blood neutrophil concentrations. Unpaired Student’s t test was used to determine significance versus vehicle.

Human C5a-Induced Neutropenia in Cynomolgus Macaque. All experiments performed in cynomolgus macaque (Macaca fascicularis) were performed at Covance Research Products with the approval of Covance Research Products Animal Care and Use Committee and in compliance with the Guide for the Care and Use of Laboratory Animals. Animals were housed in groups until the day before the experiment, when they were housed in individual cages in a climate controlled environment. Two males and two females weighing 3 to 4 kg were used.
in each experiment, except in the 75 mg/kg study (two males, one female). Animals were given ad libitum access to water during the experiments, but no food was provided during the period of 8 h before to approximately 5 h after drug administration. NDT 9513727 was suspended in 0.5% methylcellulose/0.1% triacetin in water and stirred overnight in the dark before administration. Drug solution or vehicle was administered by nasogastric gavage in a volume of 10 ml/kg body weight. Approximately 3 to 3.5 h postdrug administration, animals were anesthetized with ketamine (0.3 ml of ketamine and 0.2 ml of xylazine i.m.), and an intravenous catheter was introduced. While animals were anesthetized, a constant saline drip was maintained, and body temperature was kept at –37 °C with a warming pad. Fifteen minutes after intravenous placement, a blood sample was removed from the saphenous vein by venipuncture and collected in a tube containing sodium EDTA anticoagulant. Subsequent blood samples were collected in a similar fashion at 30, 31, 35, 40, and 60 min after intravenous placement. Approximately 4-h postdrug administration (30 min after intravenous placement), a solution of 30 µg/ml hC5a in sterile saline, was injected intravenously to achieve a final dose of 10 µg/kg. Blood samples were mixed gently, kept briefly on ice, and analyzed quickly on a HemaVet blood analyzer. Neutrophil levels were analyzed as described above. These experiments were a single crossover, paired design such that each subject was tested twice, once after oral NDT 9513727 and once after oral vehicle, such that data were analyzed by paired Student’s t test for statistical significance.

Results

Identification of NDT 9513727. The human brain C5a receptor cloned was identical to the neutrophil receptor reported by both Boulay et al. (1991) and Gerard and Gerard (1991). The hC5aR was either expressed by itself or coexpressed with the G-protein subunits consisting of rat Gt,12, human Gt,1, and bovine Gt,2. When assessed by saturation binding using [125I]C5a, membranes from S9 cells that were infected with hC5aR alone (i.e., in the absence of G-proteins) possessed two marked affinity states of 0.14 and 0.78 nM with receptor densities of 109 and 287 fmol/mg, respectively. On the other hand, when S9 cells were coinfected with hC5aR and Gt,12, Gt,1, and Gt,2, the two affinity states remained the same, but the measurable receptor density was 4–5 fold higher (455 versus 1541 fmol/mg, data not shown). The presence of NaCl in the radioligand binding assay buffer was critical to the identification of the antagonist lead series. The lead optimization strategy, highlighted by Hutchison and Krause (2004), identified the novel nonpeptide C5aR antagonist NDT 9513727, shown in Fig. 1.

Radioligand Binding Analysis of NDT 9513727 at C5aR. NDT 9513727 displaced [125I]C5a binding with an IC50 value of 11.6 ± 1.0 nM, n = 31 (Fig. 2). Although NDT 9513727 was found to have potent activity in both gerbil and monkey (cynomolgus macaque), lung membrane preparations (6.4 and 7.3 nM, respectively, Table 1), it displayed minimal activity in rat, mouse, and dog lung preparations (IC50 values greater than 10 µM, Table 1). Binding of NDT 9513727 to hC5aR was, like other reported small-molecule hC5aR antagonists, dependent on W213 in transmembrane domain V (Waters et al., 2005; data not shown).

In Vitro Functional Activity of NDT 9513727. Coexpression of C5aR with G-proteins in baculovirus-infected S9 cells was also critical for establishing the GTPγS functional assay. When the same two batches of membranes (S9.9/hC5aR and S9.9/hC5aR.I2β3y2 membranes) were assayed using hC5a-stimulated GTPγS binding, the EC50 remained unchanged, but the maximum agonist-stimulated response was increased roughly 6-fold in the S9.9/hC5aR.I2β3y2 membranes (data not shown). All subsequent GTPγS[35S] binding experiments were performed with membranes obtained from C5aR and G-protein coexpressed baculovirus-infected S9 cells.

In the presence of 10 nM hC5a (EC50 = 18 nM ± 1.7, n = 4), NDT 9513727 reversed the hC5a agonist effect in a dose-dependent fashion, with an IC50 = 9.2 ± 0.9 nM, n = 22 (Fig. 3A). Although NDT 9513727 consistently and dose-dependently lowered the GTPγS[35S] binding signal below the baseline, AcF[OPdChaWr] only decreased the hC5a-stimulated GTPγS[35S] binding response to baseline, with an 

![Image](image1.png)

Fig. 1. Structure of NDT 9513727.

![Image](image2.png)

Fig. 2. NDT 9513727 has affinity for the hC5aR. Both hC5a and NDT 9513727 (●) dose-dependently compete for [125I]C5a binding using S9 cell membranes expressing the recombinant hC5aR along with the G-proteins αt,1, βt, and γt. The data are presented as the percentage specific [125I]C5a binding as a function of ligand concentration. The ligand dose-responses yield an IC50 = 0.29 ± 0.1 nM for hC5a, with an nH = 0.6 ± 0.03, and 11.6 ± 1.0 nM for NDT 9513727, with an nH = 0.8 ± 0.6 nM. The data are representative of mean ± S.E.M., n = 6 for hC5a, and n = 31 for NDT 9513727.

![Image](image3.png)

Table 1. C5a receptor sequence identity across species and competition binding activity of NDT 9513727

<table>
<thead>
<tr>
<th>Species</th>
<th>Receptor</th>
<th>Identity to Human</th>
<th>NDT 9513727 IC50 (nM)</th>
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<tbody>
<tr>
<td>Human</td>
<td>100</td>
<td>11.6</td>
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</tr>
<tr>
<td>Macaque</td>
<td>95</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td>Canine</td>
<td>68</td>
<td>&gt;10 µM</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>64</td>
<td>&gt;10 µM</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>65</td>
<td>&gt;10 µM</td>
<td></td>
</tr>
<tr>
<td>Gerbil</td>
<td>66</td>
<td>6.4 µM</td>
<td></td>
</tr>
</tbody>
</table>

* [125I]hC5a radioligand binding used hC5a as the competing ligand for all tissue preparations.
GTP$_{H9253}$[35S] binding activity was dose-dependently decreased by NDT 9517011 in the absence of C5a stimulation. The baseline demonstrated significantly greater efficacy (maximal GTP$_{H9253}$[35S] binding assay. Compared with hC5a, NDT 9517011 was clearly more efficacious than the full peptide superagonist NDT 9513727 (EC$_{50}$ 18.0 nM, n = 4) and 2578 ± 698 nM (n = 3), respectively. NDT 9513727 dose-dependently decreases the baseline, with an IC$_{50}$ = 2.4 ± 0.8 nM (n = 4). The experimental results shown are representative of the several performed.

Species selectivity is evident for both peptide and nonpeptide C5aR ligands for the C5a receptor. The competition binding data presented in Table 1 demonstrates that NDT 9513727 has limited affinity for the rat receptor complicates the evaluation of NDT 9513727 affinity at the rC5aR (Fig. 6; data not shown). NDT 9513727 functions as an inverse agonist. The inverse agonist property of NDT 9513727 was demonstrated by the GTP$_{H9253}$[35S] binding activity of hC5aR (IC$_{50}$ 400% above basal compared with the rat receptor (Fig. 4). When the superagonist NDT 9513727 was reversed the hC5a-stimulated GTP$_{H9253}$[35S] binding signal below baseline, indicative of inverse agonism (Fig. 4). To more conclusively demonstrate the inverse agonist property of NDT 9513727, the GTP$_{H9253}$[35S] binding assay was modified to allow ligand testing in the absence of hC5a (inverse agonist mode; Fig. 3B). Although both NDT 9513727 and AcF[OPdChaWr] dose-dependently reversed the hC5a-stimulated GTP$_{H9253}$[35S] binding response (Fig. 3A), only NDT 9513727 dose-dependently reduced the constitutive GTP$_{H9253}$[35S] activity of hC5aR (IC$_{50}$ = 5.7 ± 1.6, n = 5, Fig. 3B).

To determine whether NDT 9513727 was a competitive antagonist, multiple hC5a dose responses were run using the agonist mode GTP$_{H9253}$[35S] binding assay in the presence of increasing concentrations of NDT 9513727 (0, 3, 10, 33, and 100 nM, Fig. 5). The data illustrate that at doses of at least 100 nM, the effect of NDT 9513727 on hC5aR responses was surmountable by high doses of hC5a. In addition, a parallel rightward shift in hC5a dose-response curves indicates that the NDT 9513727 mechanism of action is competitive in nature. A Schild plot of the data from Fig. 5 yielded a slope of 1.09, K$_{d}$ = 3.7 nM (r$^2$ = 0.96), consistent with competitive antagonism (this experiment was repeated once with similar results). When AcF[OPdChaWr] was similarly assessed, a rightward parallel shift in hC5a dose-response activity was seen, suggesting that the peptide antagonist also functions as a surmountable, competitive antagonist (data not shown).

222.7 ± 41.7 nM, n = 7. The GTP$_{H9253}$[35S] binding assay can be run three different modes: agonist mode, antagonist mode as described above, and inverse agonist mode. In agonist mode, ligands can be assessed for their ability to functionally activate the hC5aR. Screening compounds with the affinity assay described above resulted in the discovery of both nonpeptide agonists and antagonists as determined by the agonist mode GTP$_{H9253}$[35S] binding assay. Compared with hC5a, NDT 9517011 demonstrated significantly greater efficacy (maximal efficacy ∼400% above basal compared with the −100% above basal for hC5a; Fig. 4). Although the superagonist NDT 9517011 was clearly more efficacious than the full peptide agonist hC5a, its potency was significantly lower (EC$_{50}$ = 2580 versus 18 nM for hC5a; Fig. 4). When the hC5aR was activated by the nonpeptide superagonist NDT 9517011, the nonpeptide antagonist NDT 9513727 had nearly the same functional potency as it had for hC5a function in the GTP$_{H9253}$[35S] assay (data not shown). Agonist mode testing of NDT 9513727 indicated that not only did it not possess any detectable agonist activity, it dose-dependently lowered the hC5aR GTP$_{H9253}$[35S] binding signal below baseline, indicative of inverse agonism (Fig. 4).
shown). Sf9 membranes expressing rC5aR along with the G-proteins \( \alpha_{i2}, \beta_1, \) and \( \gamma_2 \) were used in an agonist mode GTP\(^{35}S\) binding assay to determine potency and efficacy of both rC5a and hC5a. Although rC5a is a potent \((8.3 \pm 1.0 \text{ nM; } n = 5)\) and efficacious peptide at the rat receptor, the human peptide has limited activity at the rat receptor (Fig. 6A). Based on the aforementioned peptide species selectivity, to directly determine the potency of NDT 9513727 at the rC5aR, 10 nM rC5a was used to stimulate GTP\(^{35}S\) binding that was subsequently challenged with NDT 9513727. NDT 9513727 was found to have no significant activity at the rC5aR up to 10 \( \mu \text{M} \) (Fig. 6B). The hC5L2R displays 49% identity with the hC5aR (Cain and Monk, 2002; Okinaga et al., 2003) and binds both hC5a and hC5a desArg with high affinity (Table 2). No binding activity, however, was observed for NDT 9513727 at the C5L2R (Table 2).

hC5a dose-dependently stimulated increases of intracellular calcium mobilization in dibutyryl cAMP-differentiated U937 cells, which endogenously express the C5a receptor. In this whole-cell assay, hC5a functioned with an EC\(_{50}\) of 0.48 \pm 0.05 nM, \( n = 13 \) (Fig. 7A). NDT 9513727 dose-dependently reversed an EC\(_{50}\) dose of hC5a, with an IC\(_{50}\) = 1.9 \pm 0.11 nM, \( n = 46 \) (Fig. 7B). Similar to the results shown for the GTP\(^{35}S\) assay in Fig. 5, the evaluation of NDT 9513727 in a Schild-type analysis in the calcium mobilization assay was also consistent with that of a competitive antagonist (data not shown).

C5a activates several proinflammatory functions of neutrophils. In these studies, hC5a stimulated a chemotactic response in both freshly prepared human polymorphonuclear neutrophils and human U937 cells with an EC\(_{50}\) maximal chemotaxis response of 0.5 and 0.4 nM, respectively. As shown in Fig. 8A, NDT 9513727 blocked hC5a-stimulated chemotaxis activity in human polymorphonuclear neutrophils and U937 cells with an IC\(_{50}\) of 4.7 \pm 0.8 nM \( (n = 8) \) and 4.3 \pm 0.3 nM \( (n = 42) \), respectively. HL-60 cells were stimulated by hC5a to release

![Fig. 5. NDT 9513727 is a competitive antagonist of the hC5aR. Antagonism by NDT 9513727 (●, 3 nM; □, 10 nM; □, 35 nM; x, 100 nM) of GTP\(^{35}S\) binding stimulated by hC5a (●, control) in membranes from Sf9 cells recombinantly expressing hC5aR and \( \alpha_{i2}, \beta_1, \) and \( \gamma_2 \). Note that NDT 9513727 is a surmountable antagonist because the highest concentrations of NDT 9513727 resulted in responses up to 310 and 340% above baseline at the maximal hC5a concentration.](#)

![Fig. 6. Species selectivity is evident with both peptide and nonpeptide ligands for the C5aR. The GTP\(^{35}S\) binding assay run in "agonist mode" was used to assess peptide potency and efficacy at the rC5aR. A, Sf9 cell membranes expressing recombinant rC5aR along with the G-proteins \( \alpha_{i2}, \beta_1, \) and \( \gamma_2 \) were stimulated with either recombinant hC5a (●) or rC5a-His (○), and data are shown as changes in GTP\(^{35}S\) binding relative to basal activity as a function of ligand concentration. rrC5a-His functions as an agonist at the rC5aR, with an EC\(_{50}\) of 8.3 \( \pm \) 1.0 \( \text{nM; } n = 5 \)), but hC5a has no agonist activity at the rat receptor. B, to directly test the species selectivity of NDT 9513727, rC5aR was stimulated with 10 nM rC5a, and the GTP\(^{35}S\) binding signal was challenged with increasing concentrations of NDT 9513727. NDT 9513727 had no activity at the rC5aR up to 10 \( \mu \text{M} \). Representative experiments are shown.](#)

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Recombinantly Expressed Receptor*</th>
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<tbody>
<tr>
<td>hC5aR</td>
<td></td>
</tr>
<tr>
<td>hC5a**</td>
<td>0.05 ± 0.01 nM</td>
</tr>
<tr>
<td>hC5a desArg**</td>
<td>14.1 ± 1.7 nM</td>
</tr>
<tr>
<td>NDT 9513727</td>
<td>11.6 ± 5.3 nM</td>
</tr>
<tr>
<td>hC5L2R</td>
<td></td>
</tr>
<tr>
<td>hC5aR</td>
<td></td>
</tr>
<tr>
<td>hC5a**</td>
<td>0.85 ± 0.07 nM</td>
</tr>
<tr>
<td>hC5a desArg**</td>
<td>16.2 ± 3.0 nM</td>
</tr>
</tbody>
</table>

* The CHO cell expression system was used for all examples except NDT 9513727 activity at the hC5aR, which used baculovirus-expressed C5aR. All values shown are IC\(_{50}\) values.

** Synthesized and purified using His-tag methodologies (see Materials and Methods).

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TABLE 2
Ligand potencies at hC5a and hC5L2 receptor as measured by radioligand binding with \( [^{125}\text{I}]\)hC5a.

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Fig. 5. NDT 9513727 is a competitive antagonist of the hC5aR. Antagonism by NDT 9513727 (●, 3 nM; □, 10 nM; □, 35 nM; x, 100 nM) of GTP\(^{35}S\) binding stimulated by hC5a (●, control) in membranes from Sf9 cells recombinantly expressing hC5aR and \( \alpha_{i2}, \beta_1, \) and \( \gamma_2 \). Note that NDT 9513727 is a surmountable antagonist because the highest concentrations of NDT 9513727 resulted in responses up to 310 and 340% above baseline at the maximal hC5a concentration.
The IC50 values for NDT 9513727 in these binding assays performed at Cerep (Paris, France) on a radiolabeled C5a from C5L2-expressing cells. Radioligand inhibition response was blocked by NDT 9513727, with an IC50 of 1.1 nM. ROS inhibition of C5a-stimulated intracellular Ca2+ mobilization from U937 cells was dose-dependently antagonized by NDT 9513727 with an IC50 of 1.1 nM. 

**Pharmacokinetics of NDT 9513727 in Rat and Monkey.** The pharmacokinetic profile of NDT 9513727 in rat was characterized by low plasma clearance (1.4 l/h/kg), moderate volume of distribution (3.6 l/kg), and moderate plasma elimination half-life (t1/2 = 4.8 h). Dosed orally at 50 mg/kg in male rats, NDT 9513727 (in 0.5% methylcellulose/0.1% triacetin water) exhibited a maximal plasma concentration of 280 nM at 2 h post-dose, with a terminal half-life of 80% at 10 μM NDT 9513727, with an IC50 of 0.6 μM.

**C5a-Induced Neutropenia in Mongolian Gerbils.** Circulating blood neutrophil levels decreased after i.v. administration of hC5a to Mongolian gerbils (Fig. 10A). This neutropenia effect was hC5a dose-dependent and transient with neutrophil levels returning to pre-hC5a levels 10 min post-hC5a dose. The maximum reduction in circulating neutrophil concentration was observed at an hC5a dose of 300 μg/kg. Oral administration of NDT 9513727 at a dose of 25.2 mg/kg to male cynomolgus monkeys (n = 3) resulted in a Cmax of 830 ± 198 nM, with a Tmax of 4 h and moderate oral bioavailability (%F = 26).

**C5a-Induced Neutropenia in Cynomolgus Macaque.** As observed in Mongolian gerbil, i.v. administration of hC5a to cynomolgus macaque resulted in a transient decrease in circulating neutrophil levels. Initial experiments indicated that a dose of 10 μg/kg i.v. hC5a was sufficient to produce a 60 to 70% decrease in circulating neutrophils 1 min post-administration (data not shown). NDT 9513727, given 4 h before the hC5a challenge, exhibited an oral, dose-dependent inhibition of hC5a-induced neutropenia (Fig. 11), with an MED of 5 mg/kg p.o., from which plasma drug concentrations were measured as 148 ± 58 nM (n = 4). The plasma concentrations required to achieve a 66% inhibition of hC5a-induced

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**Fig. 7.** Functional activity of NDT 9513727 at the hC5aR as measured by inhibition of C5a-stimulated intracellular Ca2+ mobilization from U937 cells. A, rhC5a-stimulated intracellular Ca2+ mobilization (■) in dibutyryl cAMP differentiated U937 cells. Mean EC50 = 0.48 ± 0.05 nM, n = 13. B, functional antagonism by NDT 9513727 of C5a-stimulated intracellular Ca2+ mobilization (■) in dibutyryl cAMP differentiated U937 cells expressing hC5aR. Mean IC50 = 1.9 ± 0.11 nM, n = 46. Representative experiments are shown.
neutropenia (25 mg/kg p.o. dose) were 410/11006 nM (n = 4). The inhibition of neutropenia observed in the 75 mg/kg dose group (plasma exposure/11005 832/11006 370 nM, n = 3) did not reach statistical significance (p = 0.088).

**Discussion**

NDT 9513727 is an orally active, potent, and selective antagonist of the hC5a receptor. In recombinant or natively expressed hC5aR radioligand binding or functional assay systems, NDT 9513727 displays subnanomolar to single-digit nanomolar antagonist activity. The in vitro functional assays ranged from a direct assessment of GTP for GDP exchange at a G-protein subunit coupled to the C5aR, to downstream receptor-mediated events including intracellular calcium mobilization, and functional cellular responses including degranulation, oxidative burst, and chemotaxis. The GTP-$\gamma^{35}$S
binding assay allowed for the simultaneous discovery of small-molecule agonists and inverse agonists, as shown in Fig. 4. A representative small-molecule agonist, 4-(2-[cyclohexyl(phenyl)methyl]amino)-2-oxoethyl)-N-(diphenylmethyl)-2-piperazinecarboxamide, although clearly weak with regard to potency, demonstrates superagonism as compared with hC5a. Importantly, the effect of this superagonist in the GTPγS binding assay was antagonized by NDT 9513727, with virtually identical pharmacology as compared with hC5a.

Studies on the pharmacologic mechanism of action of NDT 9513727 indicate that it functions in a manner consistent with competitive antagonism and is surmountable by hC5a. The fact that NDT 9513727 is an inverse agonist at the hC5aR complicates the further analysis of these data with a Schild regression because the curves do not conform to the strict requirements of Schild analysis. Examination of data for two peptidic antagonists, AcF[OPdChaWr] and F[OPd-ChaWr], reveals more easily interpreted profiles; neither peptidic antagonist demonstrated any detectable inverse agonism (Fig. 3; data not shown), and Schild analyses clearly indicated that both peptides were surmountable antagonists with parallel, right-shifted dose-response curves. Previously, F[OPdChaWr] was assessed in a Schild analysis using granulocytes and umbilical artery cell preparations (Paczkowski et al., 1999). In these whole-cell functional assays evaluating myeloperoxidase release or spasmogenic responses, the peptide antagonist appeared insurmountable in nature. It is possible that differences in the receptor systems used and endpoints assessed in these studies explain the apparent divergent results.

NDT 9513727 acts as an inverse agonist, in contrast to F[OPdChaWr] (Paczkowski et al., 1999), which functions as a neutral antagonist. Theoretically, inverse agonists may provide therapeutic advantages over neutral antagonists. For clinical situations in which GPCR mutations cause ailments because of excessively high constitutive activity, the potential utility of inverse agonists is clear (de Ligt et al., 2000; Seifert and Wenzel-Seifert, 2002). Beyond these orphan indications, however, the benefit of inverse agonists over neutral antagonists is more speculative. One analysis shows that inverse agonists predominate in the current world of GPCR modulators in accord with the theoretical predictions (Kenakin, 2004). One might suspect that the greatest likelihood for neutral antagonism might be with peptide antagonists that would be predicted to interact with the receptor as the peptide agonist does, thereby merely blocking its effect as...
opposed to having intrinsic activity. The data shown in Fig. 3, which compare the relative inverse agonism of NDT 9513727 with the peptide antagonist AcF[OPdChaWr], support the above-stated assumption.

NDT 9513727 displays species selectivity as shown in Table 1. Although the gerbil receptor is only 66% identical to the hC5aR (Waters et al., 2005), NDT 9513727 maintains high potency at the gerbil receptor (6.4 versus 11.6 nM at the hC5aR). The rodent receptors (mouse and rat) and the canine receptor, although also having roughly 65% identity to the hC5aR, demonstrate no detectable affinity. Although species selectivity issues are not uncommon with small-molecule GPCR antagonists, particularly with chemokine and related receptors, the situation is further complicated by the use of a single C5a agonist species selectivity. Both rC5a and hC5a have comparable binding affinity at the hC5aR (0.05 ± 0.01 and 0.14 ± 0.01 nM, respectively) and in other hC5aR in vitro tests. However, although rC5a has high potency and functional activity at the rC5aR, it has at least 3 orders of magnitude weaker activity at the hC5aR. NDT 9513727, in addition to having no detectable activity for rat, mouse, or canine C5aR in the competition assay (Table 1), exhibited no detectable functional GTPγS binding activity at the rC5aR when the rC5a was used (Fig. 3).

A two-site model of C5a agonist binding to the C5aR has been strongly supported by experimental data (Chenoweth and Hugli, 1980; Siciliano et al., 1994; Chen et al., 1998; Gerber et al., 2001), in which the relatively large C5a 4-helix bundle ligand has a strong ionic interaction with N-terminal receptor domain site one (residues 21–30), which accounts for about 50% of the binding energy. Subsequently, the four-helix bundle-N-terminal receptor domain complex positions the C5a carboxyl-terminal domain in proximity with interhelical regions of the receptor for site two binding, resulting in receptor and G-protein activation. The intramolecular site of NDT 9513727 action on C5aR has not been established. The similar potency of NDT 9513727 on functional responses and binding supports the notion that the small-molecule antagonist may prevent receptor activation by interacting either directly or allosterically with site two of the C5aR. This model may explain the observations that some small-molecule C5a antagonist compounds demonstrated incomplete displacement in affinity assays and that a solid correlation between functional activity and affinity is not observed with all test compounds identified in the discovery program (data not shown).

NDT 9513727 was also evaluated in a battery of more than 50 neurotransmitter and hormone receptor binding assays and was found to display little to no activity. The most significant was at the A3 adenosine receptor, where an IC50 of 165 nM was observed in a binding assay, but no A3 activity was observed in a functional calcium mobilization assay at concentrations up to 10 μM. Functional antagonism of the L-type calcium channel was observed at 2 to 3 μM, which was some 250 to 2200-fold weaker than the range of functional C5a antagonist activity (at GTPγS and ROS generation, respectively). No binding activity was observed at C5L2 (Table 2), a C5aR homolog (originally named gpr77) isolated from human and other species that displays 49% identity with the hC5aR (Cain and Monk, 2002; Okinaga et al., 2003).

Both in vivo and ex vivo approaches were utilized to investigate NDT 9513727 under physiological conditions. Acute neutropenia has been used to demonstrate the in vivo efficacy of C5aR antagonists in rodents (Pellas et al., 1998). This approach takes advantage of the fact that C5aR activation mediates increased neutrophil adhesion via increased cell surface integrin receptor expression (Crowell and Van Epps, 1990), specifically the CD11b/CD18 complex, Mac1. Because of the species selectivity of NDT 9513727, gerbil and primate models were used to investigate neutropenia in vivo. In gerbil, NDT 951237 blocked C5a-stimulated neutrophilia after oral dosing, displaying an ED50 of 2.2 mg/kg and 92% inhibition at 10 mg/kg. In cynomolgus macaque, NDT 9513727 exhibited 66% inhibition of C5a-stimulated neutropenia at 25 mg/kg p.o. Efficacy in animals given 75 mg/kg p.o. NDT 9513727 was not substantially better than 25 mg/kg p.o., which may be attributed to the lower number of subjects in the higher dose group, lack of dose-proportional increase in plasma exposure, and/or variability in plasma exposure within dose groups. In monkey and rat, NDT 9513727 exhibited low plasma clearance and low volume of distribution and variable oral bioavailability. The compound also exhibits very high human plasma protein binding (>99%; data not shown). Hence, the lack of dose-effect correlation between the gerbil and monkey models may be related to species differences in plasma protein binding and/or oral bioavailability.

C5a-mediated up-regulation of CD11b on human blood granulocytes ex vivo can be directly measured in undiluted human blood by flow cytometry. In this test, NDT 9513727 displayed concentration-dependent activity, with an IC50 of 0.6 μM. In a similar vein, reduced activity of NDT 9513727 was observed in the FLIPR assay in the presence of added human serum (data not shown). The modest potency of NDT 9513727 in vivo and ex vivo compared with the serum-free in vitro whole-cell assays is likely because of the high plasma protein binding exhibited by the compound (as noted above), although other factors cannot be completely ruled out. In any case, the concentration required to achieve approximately 50% inhibition is similar in both the cynomolgus macaque neutropenia and human granulocyte CD11b expression assays, suggesting that these factors are similar in both species.

NDT 9513727 is a novel, potent, small-molecule C5a receptor antagonist that exhibits characteristics of an inverse agonist. It is highly selective for the primate and gerbil C5a receptor subtypes and is otherwise selective versus all other targets against which it has been tested. NDT 9513727 is orally bioavailable, with desirable pharmacokinetics in multiple species, and it effectively inhibits C5a-induced activity in vivo and in human whole blood. NDT 9513727 is a promising experimental therapeutic for the treatment of human inflammatory disease.

Acknowledgments

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References


