
# Development of a Novel TGR5 Agonist for the Treatment of Ulcerative Colitis

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**Abstract**

The G protein-coupled receptor known as TGR5 (Takeda G protein-coupled receptor 5) or GPBAR1 (G protein-coupled bile acid receptor 1) has been shown to actively modulate cellular metabolism and inflammation. TGR5 is a promising target for drug development for chronic inflammatory diseases based on the inhibition of cytokine production and inflammatory response that occurs through the activation of TGR5. Ulcerative Colitis (UC), a chronic inflammatory bowel disease (IBD), is a candidate for treatment with a TGR5 agonist. UC is characterized by mild to severe mucosal inflammation in the colon and rectum leading to weight loss, cramping, loose stools and bloody stools. Currently, patients are treated based on severity using corticosteroids, immunosupressants, anti-inflammatories and/or injectable antibody treatments.

This report details the steps in the development of an orally administered TGR5 agonist to treat UC as an anti-inflammatory and immunosupressant through receptor activation on macrophages. The flow scheme of decisions described begins with virtual and *in vitro* screening techniques, continues to the methods for *in vivo* validation, pharmacodynamics, pharmacokinetics and toxicology, and ends with a brief overview of the structure of clinical trials. This document presents the justification of the flow scheme based on evidence presented in the scientific literature.

# Contents

# 1 Introduction

Takeda G protein-coupled receptor 5 (TGR5) is a G protein-coupled receptor that is naturally activated by bile acids (BAs) and is expressed in varying levels throughout the body. TGR5, also known as G protein-coupled receptor 131 (GPR131), membrane-type receptor for bile acids (M-BAR) and G protein-coupled bile acid receptor 1 (GPBAR-1), has been shown to play an important role in the regulation of metabolism, energy expenditure, weight gain, glucose homeostasis, gall bladder filling, bile composition and the regulation of the BA pool [1, 2]. Importantly, there is a strong body of evidence that TGR5 signaling is involved in regulating immune responses and inflammation response. The expression of TGR5 throughout the gastrointestinal tract and in inflammatory cells, specifically macrophages, has highlighted TGR5 as a potential target for intervention in inflammatory bowel diseases such as Crohn's disease and Ulcerative Colitis.

## 1.1 Bile Acid Synthesis and Metabolism

In the body, TGR5 is naturally responsive to several BAs, namely lithocholic acid (LCA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA) and cholic acid (CA). The bile acid pool differs between mice and humans. BAs are synthesized in the liver through either the classical/neutral pathway or the alternative/acidic pathway (roughly 25%) [3, 4]. The synthesis follows several steps: initiation with cholesterol 7$\alpha$-hydoxylase, modification of the sterol ring and shortening of the side chains into the primary BAs of CA and CDCA. The BAs are then conjugated with either glycine or taurine in humans and mice respectively [3]. Once synthesized, BAs are metabolized in the intestine and liver and stored in the gall bladder. BAs are released to aid with digestion of lipids and then the majority are reabsorbed in the ileum or in the kidney.

## 1.2 TGR5 Signaling

There are several proposed mechanisms for the role of TGR5 in inflammation. Three main mechanisms of TGR5 mediated inhibition of cytokine production are illustrated in Figure 1.1. Each of the mechanisms interferes in some way with pro-inflammatory cytokine transcription from the NF-$\kappa$B pathway. Common to all three mechanisms, the activation of TGR5 triggers an upregulation of cyclic adenosine monophosphate (cAMP). In the first mechanism (Figure 1.1A), the increase in cAMP inhibits NF-$\kappa$B by blocking I$\kappa$B$\alpha$ Kinase (IKK) stimulated I$\kappa$B$\alpha$ phosphorilation [5]. In the second mechanism (Figure 1.1B), increased cAMP stimulates Protein Kinase A (PKA). PKA then upregulates the proto-oncogene cFos which binds to the p65 subunit of NF-$\kappa$B and blocks the production of inflammatory cytokines [5]. The stimulation of PKA by increased cAMP also plays a role in the third mechanism (Figure 1.1C). In this mechanism, PKA generates cAMP responsive element binding protein (CREB). CREB actively competes with NF-$\kappa$B to bind to CREB binding protein (CBP). When NF-$\kappa$B is not bound to CBP fewer pro-inflammatory cytokines are produced. Additionally, CREB bound to CBP induces the transcription of the anti-inflammatory cytokine IL-10 [5].

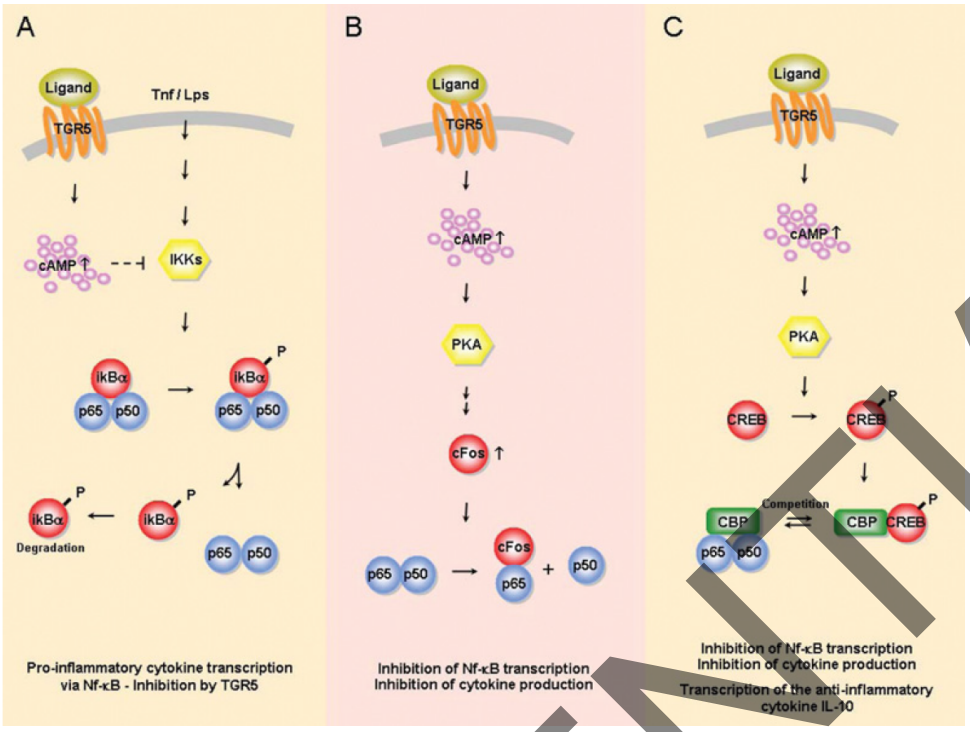


**Figure 1.1:** Proposed mechanisms of action for TGR5 receptor in macrophages. Image from Pols et al, 2014. Hexagons are kinases, circles are transcription factors, and squares are cofactors [5]. TGR5 activation upregulates cAMP production which decreases inflammation through interference with the two subunits (p65 and p50) of NF-$\kappa$B by three mechanisms. **(A)** cAMP inhibits i$\kappa$B$\alpha$ phosphorylation inhibiting the translocation of NF-$\kappa$B and subsequent activation of macrophages. **(B)** cAMP stimulates PKA up-regulation of cFos which binds to and inhibits the transcription of the p65 subunit. **(C)** cAMP activated PKA phosphorylates CREB which not only competitively binds to CPB, the same cofactor needed for NF-$\kappa$B transcription, but also induces transcription of the anti-inflammatory cytokine IL-10 [5]

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## 1.3 TGR5 in Intestinal Inflammation

TGR5 plays a multifaceted role in chronic intestinal inflammation. It is expressed throughout the intestinal epithelium as well as in important inflammatory cells including monocytes and macrophages. The primary role of TGR5 in intestinal homeostasis is believed to be the attenuation of the inflammatory response [6]. Through the signaling pathways in Figure 1.1 BA activation of TGR5 under inflammatory conditions decreases the release of pro-inflammatory cytokines and the synthesis of NF-$\kappa$B by macrophages. Baars et al. tested the activation of TGR5 signaling on differentiating intestinal cells from patients with Crohn's disease in vitro. TGR5 activation by both BAs and TGR5 agonists greatly inhibited the TNF-$\alpha$ production in response to bacteria for differentiated macrophages and for peripheral CD14+ monocytes [6]. Coupled with this influence on immunity and inflammatory response, TGR5 expression also plays a role in the structural homeostasis of the intestinal epithelium. At 12 months, TGR5$^{-/-}$ mice show significant changes in crypt morphology and reduction of mucous cells [7]. Additionally, these TGR5$^{-/-}$ mice had increased severity of induced colitis compared to wild type mice. Oral administration of a TGR5 agonist in mice with colitis showed a dose dependent attenuation of colitis symptoms and expression of inflammatory cytokines [7].

## 1.4 Ulcerative Colitis

Ulcerative Colitis (UC) is a chronic inflammatory bowel disease (IBD) characterized by the mucosal inflammation in the rectum and colon. In IBD, chronic inflammation is caused by an abnormal immune response to luminal antigens [8]. UC can present itself at any age, peak incidence is between 30-40 years of age with incidence rates ranging between 2.2 to 19.2 cases per 100,000 people in North America and from 1.5 to 24.3 cases per 100,000 people in Europe [9, 10]. Though the incidence rates have stabilized in high incidence areas, they continue to grow in lower incidence areas and developing countries. UC can vary in severity from mild, where patients present intermittent rectal bleeding, mild cramping and diarrhea, to severe, where patients present greater than 10 loose stools per day, severe cramping, high fever, rapid weight loss and severe bleeding.

Traditional treatment for UC varies with severity. For low severity cases, aminosalicylates are used to control the disease while steroids and immunosuppressants are taken to control flares and are discontinued throughout times of remission. However, in medium to severe cases these measures are often insufficient and the condition must be controlled with biological treatments and immunosuppressants, while colorectomy is needed in up to 15% of patients as a final resort [9, 11]. Patients have benefited significantly from the recent introduction of anti-TNF-$\alpha$ biologic treatments (monoclonal antibody therapy). These treatments, including adalimumab, certoluzymab, and infliximab, must be administered through regular self injection or through IV administration at a treatment center. The antibody treatments have also shown loss of efficacy over 6-12 month studies as well as occurrences of infection, demyelinating disorders and even lymphoma [12]. Many patients require treatment with corticosteroids during flare ups. Corticosteroids can result in acute toxicity and frequent use of corticosteroids can create serious side effects including hypertension, osteoporosis, diabetes, psychosis and cateracts [13]. The development of a novel oral anti-inflammatory and immunosuppressant through the activation of TGR5 could decrease the risk of severe side effects faced by patients who may otherwise require corticosteroids or antibody treatments. A TGR5 agonist could also potentially increase patient medication compliance by simplifying administration.

## 1.5 Drug Development Overview

This report details the drug development process for a TGR5 agonist to treat Ulcerative Colitis. The agonist will be developed as a standalone treatment for therapy with naive patients; however, if successful a second clinical trial will test the efficacy as an add-on treatment for patients with severe UC currently receiving anti-TNF-$\alpha$ antibodies. TGR5 can be activated by natural BAs, semi-synthetic BAs (INT777) or by chemical synthetic small molecules [14]. BA mimicking drugs are cycled through the body through the BA transport recycling mechanisms and may build up in the kidneys, gallbladder and liver. To avoid the cycling of the drug, the discovery and development will focus only on synthetic small molecules. The chart in Figure 1.2 is a broad overview of this process. A virtual screen using a G protein-coupled receptor homologue scanned thousands of potential compounds for an initial overview. The virtual screen will be expanded to $\tilde{1}$,000,000 compounds in order to identify 10,000 hits that advance to *in vitro* screening. Primary and secondary *in vitro* screenings, an *ex vivo* screening, and *in vivo* validations including pharmacology and toxicity tests further filter the hits until the final optimized

compound enters clinical trial.

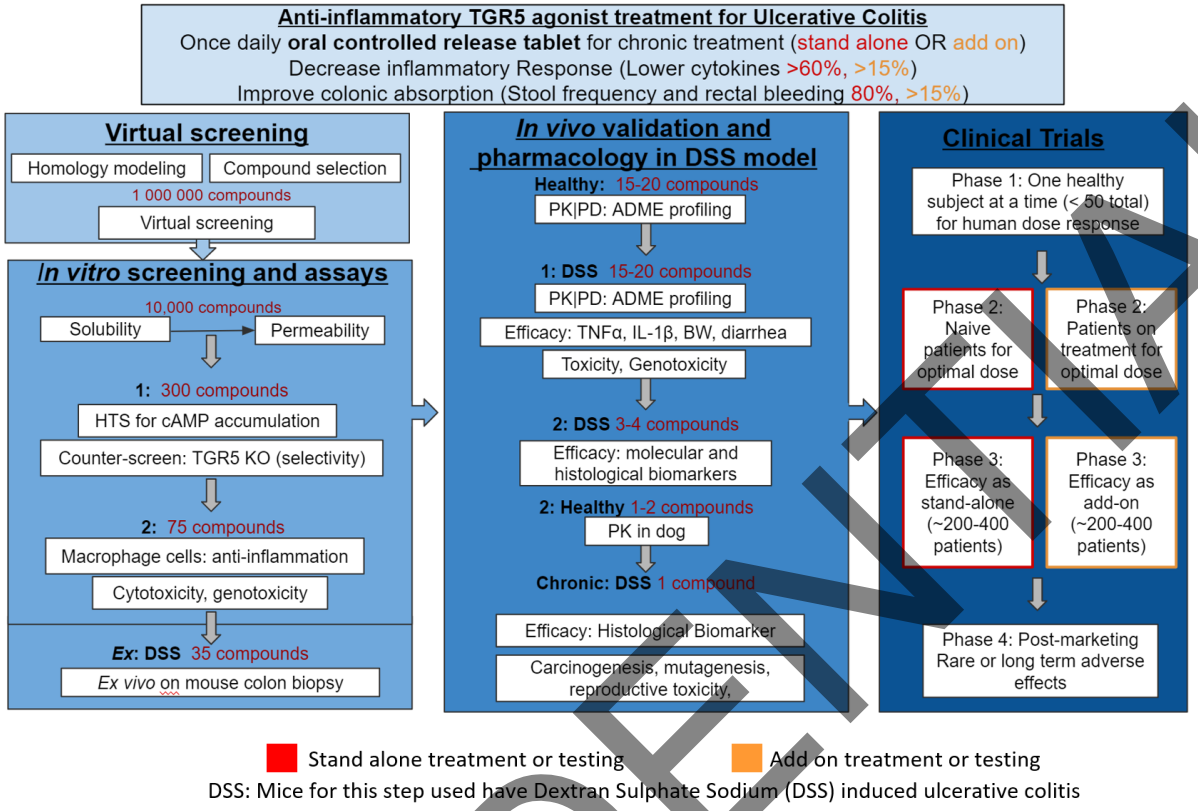


**Figure 1.2:** Drug development pipeline for the development of a TGR5 agonist to treat ulcerative colitis. Sections in/outlined in orange are the optional tests and conditions for assessment as an add-on medication.

The final success of the drug candidate will be evaluated by two criteria. First, the ability to induce complete remission during a UC flare. This will be judged based on the self reported and physician assessed symptoms, such as rectal bleeding and stool frequency. A drug that is able to induce complete remission in 80% of treated patients will be considered successful. The second criteria is a 60% decrease in cytokine levels, TNF-$\alpha$ and IL-$\beta$1, and is reflective of the drugs ability to decrease the chronic inflammation that perpetuates UC.

# 2 Virtual Screening

As a membrane protein, TGR5 is difficult to isolate for crystallization. Due to the lack of described structure, a homology model of the structure was developed for the virtual screen. The structure was modeled using the SWISS-MODEL homology modeling server [15]. The two highest scored structures were built using a human adenosine receptor (Protein Databank Identifier (PDB): 5mzj) and a human muscarinic acetylcholine receptor (PDB: 5cxv) as templates.

A structure based on the human adenosine receptor was selected (Figure 2.3) for the virtual screen. It was selected due to its high local quality, both in general and in residues that could be important for the binding (Figure 2.4).

The virtual screening was performed using AutoDock Vina [16]. A box with a volume of 10,982 Å$^3$ was defined at the top of the structure as a potential docking site. Keeping

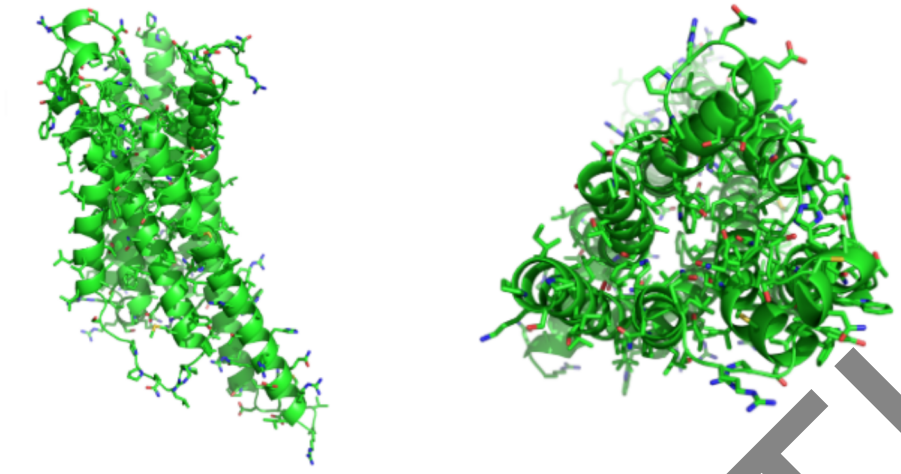

**Figure 2.3:** Side (left) and top (right) views of the TGR5 homology structure based on the human adenosine receptor. The protein backbone is drawn as a ribbon diagram with sidechains represented as sticks. Oxygen atoms are in red and nitrogen atoms in blue.

in mind Lipinski's rule of five[1], compounds from the ZINC 15 database with molecular weight between 375 and 425 Da and partition coefficient between 4 and 5 were selected [17]. This selection resulted in 388,000 compounds. Of these compounds, 2523 were randomly selected for virtual screening. The results are illustrated in Figure 2.5. Moving forward, a total of roughly 1,000,000 molecules will be virtually screened. The top 1% of these molecules (for a total of 10,000 compounds) will be selected for the use in *in vitro* screening. A corresponding threshold for the already screened molecules is illustrated in Figure 2.5.

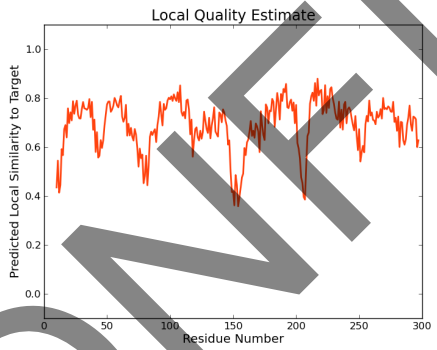


**Figure 2.4:** Local quality estimate for the homology structure. For each residue the expected similarity to the native structure is shown. Scores below 0.6 indicate residues with low quality. In the homology model used for analysis, residues with a low score are located in loop regions with less defined structures.

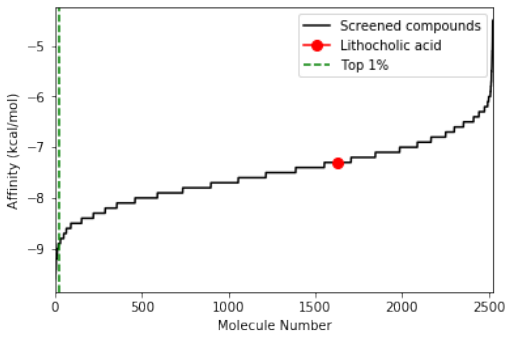


**Figure 2.5:** Affinity of the virtually screened molecules. The molecules were sorted based on affinity (lower binding energy results in better binding). The figure also shows the value for lithocholic acid, a native ligand of the structure and a threshold that denotes the molecules (top 1%) with the best binding.

[1] Drug candidates should have: less than hydrogen donors, less than 10 hydrogen acceptors, molecular weight (MW) < 500 Da, octonal water partition coefficient <5 ClogP

# 3 *In vitro* Screening

*In vitro* screening will be used to decrease the overall number of hits found in the virtual screen. The *in vitro* screening will evaluate the physical properties, efficacy, *in vitro* pharmacodynamics (PD) and toxicity to determine which compounds will move forward to *in vivo* validation.

## 3.1 Physical properties measurement

### 3.1.1 Solubility

10,000 representative compounds from the virtual screen will undergo solubility testing. Solubility describes the amount of substance required to saturate a solution at constant temperature and pressure. It is an important limiting parameter for orally administered drugs to achieve a pharmacological response. Drugs that are poorly soluble in water have inadequate and variable bioavailability and may result in gastrointestinal mucosal toxicity [18].

Solubility will be determined using the saturation shake-flask solubility method which consists of continuously adding a solute to an aqueous solution until saturation. UV spectrophotometry will measure the compound concentration in the solution. Measurements will be conducted at different pH values (pH 1-7.5), to ensure physiological relevance [19]. Only compounds that are soluble, freely soluble or very soluble (30:1 solvent:solute ratio [20]) over the physiological range of pH 1 to pH 7.5 will be kept for permeability testing.

### 3.1.2 Permeability

Approximately 1,000-2,000 compounds from the solubility screen will undergo permeability testing. A membrane's ability to allow a substance to pass through it, known as the permeability, is a key factor in the absorption and distribution of drugs.

The Caco-2 permeability assay (Figure 3.6) will be used to determine how well the compound diffuses across an epithelial membrane. The epithelial monolayer of the assay mimics the intestinal epithelium, therefore the results from this assay can predict the permeability of the compound in the human intestine and investigate drug efflux [21]. The concentration of the compound on both sides of the monolayer will be measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The 300 compounds that best diffuse across the monolayer will have the highest concentration in the basolateral liquid and will proceed to primary *in vitro* screening.

## 3.2 Primary *in vitro* Screening: Pathway Activation

Following solubility and permeability testing, appropriate *in vitro* assays must be developed. It has been shown that some synthetic TGR5 agonists activate the human and mouse TGR5 receptors very differently [22]. In order to address this discrepancy the tests will use a cell line which overexpresses human TGR5 (hTGR5). The primary *in vitro* screening will be prepared with Human Embryonic Kidney cells 293 (HEK293) that are easy to control and maintain. The cell lines will be transfected with a plasmid containing a hTGR5 receptor.

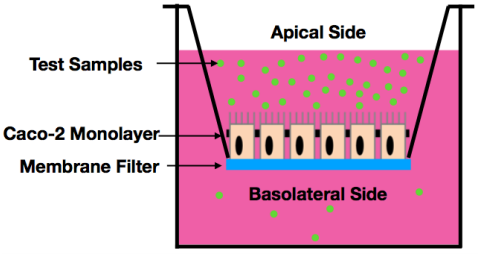


**Figure 3.6: Caco-2 assay**: Caco-2 cell monolayers will be cultured on semipermeable plastic. Test compounds will then be added to the apical side of the monolayer. After incubation for various lengths of time, aliquots of the buffer in opposite chambers will be removed for the determination of the compounds' concentration and the computation of the rates of permeability for each compound. LC-MS/MS will be used to measure compound concentration in the liquid.

The primary screen will investigate hTGR5 activation through the detection of cAMP levels. Two viable approaches for screening cAMP include accumulation assays and reporter gene assays. In an accumulation assay, labeled cAMP competes with intracellular cAMP following lysis for binding to a labeled anti-cAMP antibody. Alternatively, reporter gene assays rely on the detection of expression levels of a reporter gene regulated by a cAMP activated transcription factor. The screen will use an accumulation assay due to its simplicity.

The compounds will be tested using the Homogeneous Time Resolved Fluorescence (HTRF®) Assay from Cisbio international (Figure 3.7). This method uses the principle of Fluorescence Resonance Energy Transfer (FRET) (Figure 3.8) in which energy is transfered from one fluorescent marker to another. It is a simple method, requiring only labeled cAMP and labeled anti-cAMP antibody solutions, and offers easily quantifiable results. The presence of cAMP is quantified by a HTRF compatible analyzer. The results will be given as a ratio between luminosity at 665 nm and 620 nm, which is referred to as the HTRF ratio. A lower ratio is therefore desirable, as it indicates higher cAMP accumulation, leading to higher luminosity at 620 nm.

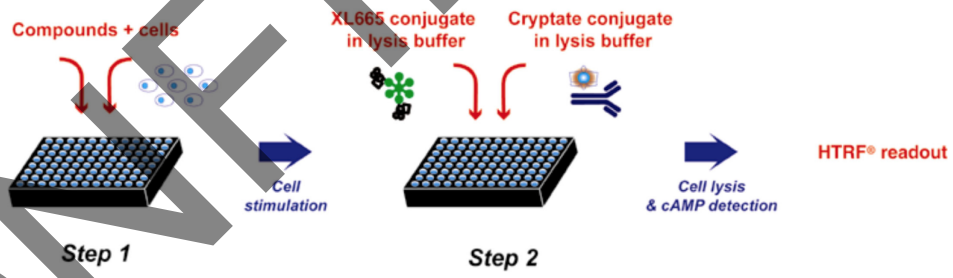


**Figure 3.7:** Experimental steps in the HTRF® assay (Degorce,2003) [23]: The HTRF® assay is specifically designed for detecting the concentration of cAMP produced after TGR5 activation. It uses Fluorescence Resonance Energy Transfer (FRET) technology: labeled anti-cAMP antibodies and cAMP-XL665 emit long lifetime fluorescence at 665 nm when bound together in the absence of cellular cAMP (Figure 3.8). Production of cAMP resulting from TGR5 activation induces a competition between cellular cAMP and labeled cAMP-XL665 for binding to anti-cAMP antibodies. When labeled antibodies are bound to unlabeled (cellular) cAMP, FRET does not occur and fluorescence is only seen at 620 nm.


**Figure 3.8:** FRET mechanism during labeled and natural cAMP competition (Williams, 2003) [24]

Compounds will be screened in a 384-well plate containing HEK293 cells overexpressing hTGR5. Two positive controls will be used, the natural agonist LCA and the synthetic agonist INT-777. A blank condition, will serve as the negative control. The HTRF® assay reagents and lysis buffer will be added after the cells have been exposed to the compounds.

At the end of the screening, compounds resulting in an HTRF ratio below 2,000 will be retested. Those hits with HTRF ratios confirmed in the retest will be subject to a counter-screen with HEK293 with a loss-of-function (LoF) mutation of TGR5. This will confirm that the compounds identified in the primary screen act through TGR5.

Successful compounds will be sent to the company DiscoverX to check for potential non-specific GPCR, kinase, and nuclear receptor activation using the gpcrMAX$^{SM}$ [25], the scanMAX$^{SM}$ [26] and the NHRscan$^{SM}$ [27] assay panels, respectively. Only compounds without off-target effects will be kept.

Finally, EC50 values for the remaining hits will be determined by establishing a dose-response relationship between different concentrations (e.g. 0, 2, 4, 6... 20 $\mu$M [8]) of each compound and the subsequent cAMP increase. The cells will be incubated at each concentration for the same time period and will subsequently be lysed and analyzed for cAMP increase. The 75 agonists with lowest the EC50 values will be kept for secondary screening.

## 3.3 Secondary *In Vitro* Screening: Anti-Inflammatory Pathways

75 candidates will undergo a three-fold secondary screening (Figure 3.9) focusing on the induction of the anti-inflammatory properties associated with TGR5 activation. The level of TNF-$\alpha$ will be used as a screening marker, due to its role in the degradation of the colonic lining in UC [28].

Several models will be tested in order to best emulate the disease phenotype. The first *in vitro* model will look at differentiating macrophages [8]. Macrophages will be differentiated with both macrophage colony-stimulating factor (M-CSF) and interferon-$\gamma$ (IFN-$\gamma$) [8]. Once macrophages are differentiated, they will be activated with Lipopolysaccharide (LPS). The cells will then be incubated with different compound concentrations in duplicates (2, 4, 6, 8 and 10 $\mu$M). These concentrations will be used as 10 $\mu$M of the TGR5 agonist known as 3-(2-chlorophenyl)-N-(4-chlorophenyl)-N, 5-dimethylisoxazole-4-carboxamide, has been shown to lower TNF-$\alpha$ expression by 50% [8]. Negative and positive controls will also be included on the plate. TNF-$\alpha$ levels will be visualized and quantified with ELISA and a spectrophotometer (Figure 3.9A). To identify compounds that activate the TGR5 pathway, an HTRF-compatible analyzer will test for the accumulation of cAMP (Figure 3.9B). A third test will assess the inflammatory gene expression levels using Nanostring's nCounter® Human Inflammation v2 Panel (Figure

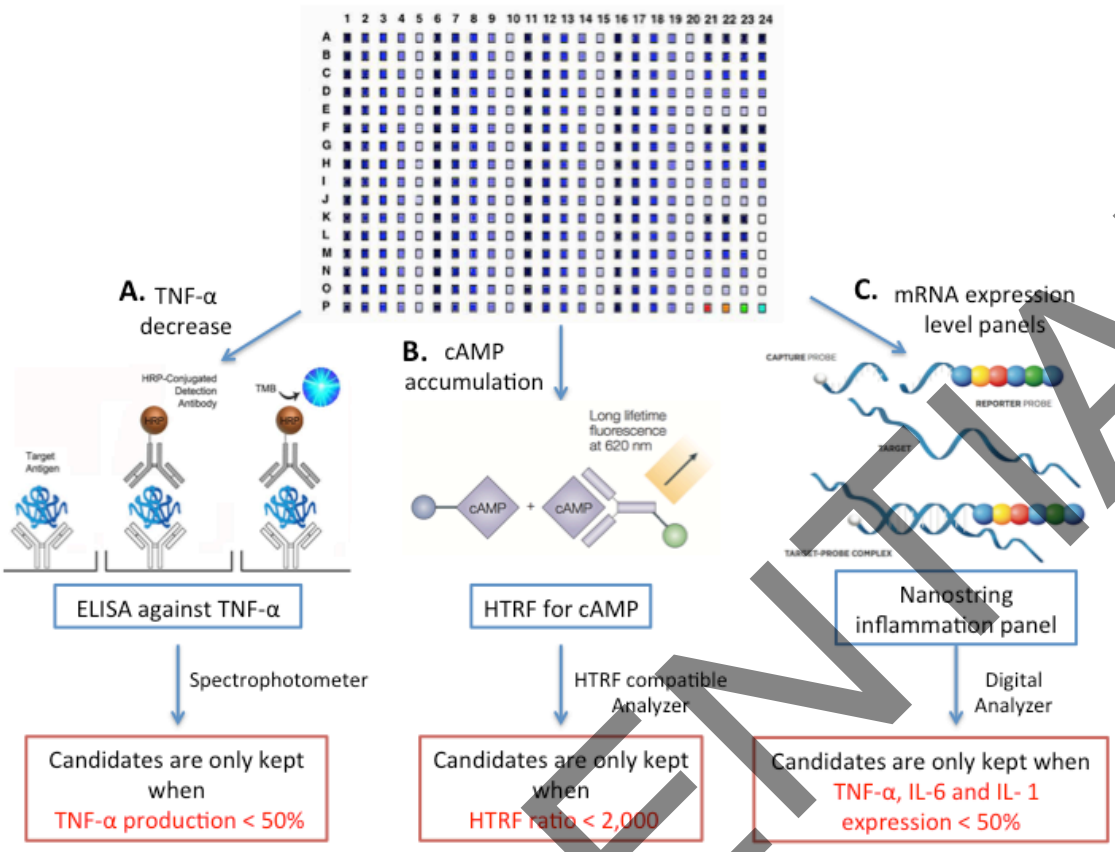


**Figure 3.9: Analysis of the secondary screening:** 75 candidate compounds will be added at concentrations 2, 4, 6, 8 and 10 $\mu$M to activated and differentiated macrophages in three different 384-well plates. Both positive and negative controls will be tested. The samples will be incubated for an hour and each plate will then be analyzed with different markers. **Note that the representation of the plate is a schematic and not a true layout**. **(A)** The first plate will be analyzed with ELISA in order to look for a decrease in TNF-$\alpha$ production (Figure from http://www.mybiosource.com/learn/ELISA). The optical density of ELISA will then be analyzed with a spectrophotometer and only candidates that show a 50% reduction of TNF-$\alpha$, compared to the blank condition, at 10 $\mu$M compound concentration will be kept. **(B)** The next plate will be used to monitor the accumulation of cAMP in the samples in order to verify the activation of the TGR5 pathway. This will be done using HTRF technology as explained above in the primary screening. Using an HTRF-compatible analyzer, only candidates with an HTRF ratio lower than 2,000 at 10 $\mu$M compound concentration will be kept (reference: [24] and « A guide to optimizing agonists for Galphas », Cisbio). **(C)** The final plate will be used to examine cytokine mRNA expression. The levels of their mRNA can be measured using the nCounter® Human Inflammation v2 Panel, from Nanostring. Digital analysis will establish the presence levels of inflammatory cytokine mRNA. TNF-$\alpha$, IL-1 and IL-6 mRNAs will be quantified specifically, for their role in UC [28]. (reference: « nCounter® Analysis System », nanoString technologies). A decrease of 50% in mRNA counts for each of the cytokines: TNF-$\alpha$, IL-1 and IL-6, compared to the blank conditions, at 10 $\mu$M compound concentration will be kept. The counts will be made using the nCounter® Analysis system.

3.9C). The gene expression level will be checked to determine if the gene transcription of inflammatory cytokines is lowered when a compound activates TGR5.

Following the secondary screening, the dose response curve for TNF-$\alpha$ inhibition will be directly compared with the dose response curve of cAMP production to establish a relationship between TNF-$\alpha$ inhibition and TGR5 activation. The experiment for TNF-$\alpha$ will use the same compound concentrations and incubation times as for the EC50 measurements in the primary screening.

## 3.4 *In vitro* Toxicology Assays

The majority (40%-50%) of compounds identified in drug discovery primary screens often fail because of toxicity [29, 30]. Therefore, fast and productive *in vitro* toxicity tests are needed to select the best drug candidates before beginning *in vivo* studies.

### 3.4.1 Cytotoxicity

Normally, cytotoxicity assays are conducted among the earliest toxicity assays. *In vitro*, compounds are considered to be cytotoxic if they interfere with cellular attachment, significantly alter morphology, adversely affect cell growth rate or cause cell death [31].

Digital Holographic Microscopy (DHM) will be used to assess cytotoxicity. DHM generates quantitative phase images without added labels for analysis of cell viability. The digital and label-free nature of DHM saves time and cells. With DHM cell viability is discerned based on the optical path difference (OPD) (Figure 3.10). As cells die they will become round and detach from the plate leading to larger OPD values. Only compounds showing good cell viability will move forward in the testing.

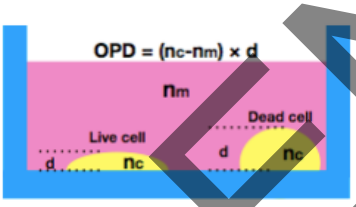


**Figure 3.10:** Schematic of mechanism of DHM assay measured OPD of cells: DHM measures a signal that is proportional to both the cell thickness and the intracellular refractive index [32, 20]. The readout is quantitatively measured by the optical path difference (OPD). Growing cells are normally attached to the plate, but the morphology of dead cells is changed from a flat to a round shape, therefore, the OPD of dead cells will be increased.

### 3.4.2 Genotoxicity

Genetic toxicology assays will be used to detect drug candidates that induce potential genetic damage. Three *in vitro* aspects of genetic toxicology, gene mutation and both structural and numerical chromosome aberrations, will be assessed. The Ames test will evaluate gene mutation and the *in vitro* micronucleus test will evaluate the two forms of chromosome abberations [33].

The Ames test, Figure 3.11, is a bacterial reverse mutation assay which is commonly used as an initial screen to determine gene mutation caused by new chemicals and drugs [33]. Ames testing uses a number of *Salmonella* strains which carry a defective (mutant) gene that renders them unable to synthesize the essential amino acid histidine and grow to form colonies. However, new mutations caused by chemical compounds can restore the gene's ability to synthesize histidine. These mutations enable the bacteria to grow in the absence of histidine. Based on the Ames test results all mutagenic compounds will be eliminated as candidates for the final drug.

The *in vitro* micronucleus assay, Figure 3.12, is used to detect chemical compounds that induce chromosome aberration [34]. This assay is an important component of a genotoxicity screening as it will identify chemicals that could produce chromosomal damage. This chromosomal damage can generate small extra-nuclear bodies, known as micronuclei, that contain whole or fragmented chromosomes that were not incorporated into the daughter nuclei during mitosis [35]. Chinese Hamster Ovary (CHO) cells

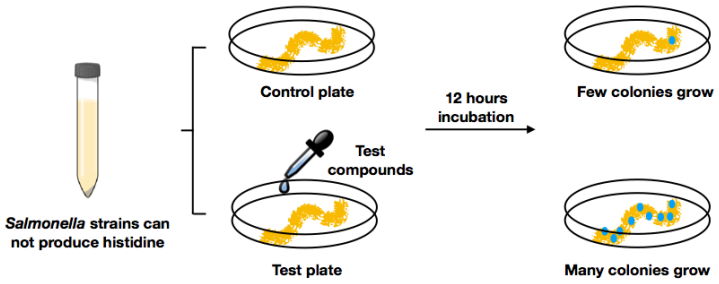


**Figure 3.11: Schematic representation of Ames test to detect mutagenic potential of a compound**. Two plates are prepared. One plate includes the testing compounds for detecting mutagenicity. Another control plate contains no chemical. After incubation for 12 hours, only mutated bacteria will be able to grow and form more colonies compared with the control. It should be noted that a few colonies can develop on control plates due to a natural back-mutation.

are commonly used to perform the *in vitro* micronucleus assay due to their stable and well-characterized karyotype. The cells will be treated with varying concentrations of the compounds, fixed and scored by a software to score for the presence of micronuclei. This score translates to the concentration dependent toxicity of the compound. This test is fast and allows for the scoring of more cells, giving it a statistical advantage. The assay will be run using 8 concentrations in duplicate, the 35 compounds that are the least cytotoxic will continue in the drug development process.

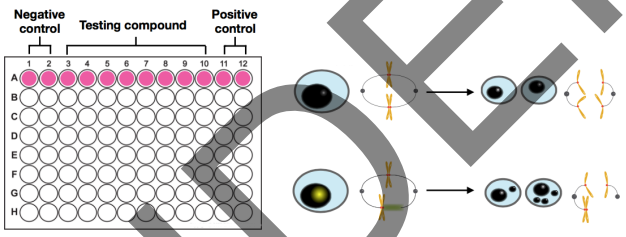


**Figure 3.12: Schematic of *in vitro* micronucleus assay**. The cells are seeded in 96-well plates and treated with the test compounds. The number of micronucleus will be scored. Compounds that induce chromosome damage will lead to the induction of micronuclei in interphase cells.

# 4 Mouse Models of Ulcerative Colitis

After effect confirmation using *in vitro* screening, a final *ex vivo* screen will further focus the potential compounds before the complexities of treatment are investigated *in vivo*. For the *ex vivo* and *in vivo* evaluations there are several potential mouse models of colitis - chemically induced, bacterially induced and transgenic models. The pros and cons of each, described briefly in Table 4.1, are weighed in the decision of the model.

*Ex vivo* screening and *in vivo* validation will use a Dextran Sulfate Sodium (DSS) chemically-induced mouse model of UC. All tests will be done on transgenic BALB/C mice with a human TGR5 receptor (hTGR5). DSS is widely used (often in BALB/C mice) due to its ease of administration, ability to mimic both chronic or acute human UC and for the ability to guide the severity with design [37, 38, 39]. The use of a chemically-induced model avoids the cross-breeding step needed to create transgenic UC mice with hTGR5, improving time and cost efficiency. A noteworthy downside to the DSS model is a lack of control of severity from mouse to mouse. To address this downfall the protocol for induction of colitis with DSS calls for the use of 5-10 mice per

**Table 4.1:** Comparison of benefits and drawbacks of various chemical, bacterial or transgenic mouse models of ulcerative colitis. Summary of information from Low et al. 2013, Manicassamy and Manoharan, 2014, and Randhawa et al. 2014.

| Model | Pros | Cons | Notes |
|---|---|---|---|
| | | **Chemical** | |
| Dextran Sulfate Sodium (DSS) | • Easy to administer<br>• Schedule chronic/acute<br>• Rough dosage control of severity<br>• Mimics human UC | • Variable severity | Good to study contributions of innate immune response [36] |
| Oxazolone | • Mimics human UC | • Lacks chronic phenotype<br>• Intrarectal administration | |
| 2, 4, 6-Trinitrobenzene sulfonic acid (TNBS) | • Chronic phenotype<br>• Mimics human UC | • Intrarectal administration | Good to study T helper cell-dependent mucosal immune responses [37] |
| | | **Bacterial** | |
| Salmonella | • Mimics crypt loss and erosion | • Risk of systemic infection[38] | Good for acute study only |
| Adherent-invasive E.Coli | • Mimics human UC | • Simultaneous DSS treatment | |
| | | **Transgenic** | |
| IL-7 Tg Mice | • Early UC development<br>• Mimics human UC | • Later UC progression | Upregulated IL-7 maintains cD4+ T cells<br>Good for T cell function interventions |
| TCR$\alpha$ KO | • Mimics chronic human UC | • SPF facility<br>• Long development time | Previous interventions tested |
| Wiskott-Aldrich Syndrome (WASP KO) | • Mimics human disease<br>• Some WASP- humans dev. UC | • Long development time | Previous interventions tested |
| Mdr1a KO | • Mimic ulcers, crypt and goblet loss | • 25% develop colitis<br>• Long development time<br>• SPF facility | Good for drugs targeting intestinal epithelial barrier dysfunction |
| IL-2 KO | • Strongly Mimics human UC<br>• Conventional environment | • 50% Mortality | Previous targets for UC severity control identified with IL-2 KO |
| G$\alpha$i2 KO | • Distinct lethal UC phenotype | | Good for study of B cell targeting |

treatment group [40]. *Ex vivo* screening and *in vivo* validation will be conducted using the protocols in Table 4.2. Primary and secondary testing will use male BALB/C hTGR5 mice between 7-9 weeks old and chronic testing will use both males and females beginning at the same age.

**Table 4.2:** Overview of DSS treatment for various stages of the ex vivo screening and in vitro validation

| Stage | Nb Compounds | Mice/Compound | Acute/Chronic | Severity | Protocol |
|---|---|---|---|---|---|
| *Ex vivo* | 30-40 | 0.5 | Acute | Mild-Mod | 5% DSS in drinking water days 0-7, water day 8, sacrifice[38, 37, 41]. |
| 1° *in vivo* | 15-20 | 5 | Acute | Mild-Mod | 5% DSS in drinking water days 0-7 water day 8, sacrifice [38, 37, 41]. |
| 2° *in vivo* | 3-4 | 10 | Chronic | Mod | 3% DSS in drinking water days 0-7, water day 8-21, 3% DSS days 22-28, water day 29, sacrifice [37]. |
| Chronic Efficacy | 1 | 20 | Chronic | Mod | 4 cycles of 5% DSS for 7 days, followed by water for 12 days[37]. |

# 5 *Ex vivo* Screening

The addition of an *ex vivo* screen will test hits in a more complete tissue setting to decrease the number of compounds that will move to the expensive *in vivo* testing. One excised colon generates approximately 10 cultures therefore with only 20-25 mice the *ex vivo* assay will decrease the hits from 30-40 to 15-20 [41]. Once the mice will be sacrificed the colons will be removed, washed, inspected for inflammation and prepared for culture. Colon tissue will be cut into cross sectional pieces and cultured at 37°C in 5% $CO_2$ for 24 hours in a medium containing 10$\mu$M of the compound [41]. The top 15-20 candidates, those with the lowest TNF$\alpha$ production and the most cAMP production measured using ELISA and HTRF, will be retained for *in vivo* validation.

# 6 *In vivo* Validation

After the selection of the top 15-20 compounds through the *in vitro* and *ex vivo* screenings the hit-to-lead phase will continue with *in vivo* pharmacokinetic (PK), efficacy and pharmacodynamic (PD) testing. Initial tests with healthy mice will focus on understanding the PK/PD of each compound. After these initial tests, the remaining *in vivo* testing will use the DSS model described above on mice expressing hTGR5.

## 6.1 Pharmacokinetics (PK)

The four components of PK - absorption, distribution, metabolism and excretion (ADME) - must be assessed in a healthy hTGR5 murine model. A thorough understanding of these properties will determine the appropriate dosages for *in vivo* tests by identifying saturation endpoints.

The 15-20 compounds will undergo an initial rapid PK study to determine the ADME properties. Each compound will be tested on 3 animals with oral gavage. This early PK study will determine which compounds are able to be absorbed through the intestine, overcome the ”first passage effect” and be detected in the bloodstream. Based on previous investigations for TGR5 agonists the dose determining study will test 5mg/kg, 20 mg/kg, 50 mg/kg and 100 mg/kg [14, 42, 43, 44, 45]. The dose that results in plasma saturation will be the dose used for future testing.

Blood samples will be collected from the tail vein over a 24 hr period at 7 times following the power of 2 rule (30 mins, 1hr, 2hr, 4hr, 8hr, 16hr, and 24hr) [46]. Urine and feces will be collected for up to 72hrs. The rapid PK approach will pool the samples of the three mice at each time point. Though inter-sample variation is lost, this method decreases the amount of analytic testing and will help minimize time and cost at this stage. In contrast, a conventional PK study does not pool the samples and requires more analytic testing. A comparison of the results from rapid and conventional PK studies show high correlations, with correlation coefficients of 0.79 for clearance ($C_L$ [mL/min/kg], 0.95 for volume of distribution ($V_{ss}$ [L/kg]), 0.90 for area under the curve (AUC [h*nM/mg/kg]) and 0.88 for maximum concentration ($C_{max}$ [nM/mg/kg]) [46]. These results support the use of this rapid PK study specifically in the hit-to-lead stage of drug development.

Absorption of the compound and subsequent bioavailability play a key role in the duration and intensity of the drug response. The blood drawn will be treated and spun down and the plasma tested by LC-MS/MS. The concentration of compound in the plasma over time will generate a concentration curve that describes the bioavailability of the compound. This analysis provides $C_{max}$, time at maximum concentration ($T_{max}$) and the remaining parameters including the AUC, half life ($t_{1/2}$) and $C_L$ (elimination) will be calculated with WinNonLin [14].

The tissue distribution of compounds that successfully enter the bloodstream will be investigated. Mice will be sacrificed at the time of $C_{max}$ and key organs - the small and large intestines, liver, gallbladder, and heart - will be harvested and tissue analyzed with mass spectrometry. Tissue absorption in the intestines is desired, where as high absorption in the gallbladder and heart may generate negative side effects [14]. The distribution detected in the tissues combined with the apparent volume of distribution

($V_d$), calculated as:

$$V_d = \frac{\text{Amount in body}[\mu g]}{\text{Plasma Concentration}[\mu g/mL]}$$

will be used to screen potential compounds. A small $V_d$ may indicate strong binding with blood plasma or a lack of distribution to tissues whereas a large $V_d$ may indicate good distribution to tissues or simply a high concentration in specific tissues.

Compounds that have successfully reached the bloodstream will undergo a full mass balance study (MBS) to understand the metabolism of the compound. The MBS will be conducted on $^{14}$C radiolabelled compounds with a radioactivity range of 1.5-199 $\mu$Ci/kg [47]. Plasma will be collected following the power of two rule up until 5X the plasma half life for the compound or for those with long half-lives the collection can stop when <1% of the total dose is excreted in the urine or feces [47]. In plasma, the total radioactivity will be compared to the amount of parent drug measured with LC-MS/MS. Finally, the mass balance of metabolites in feces and urine will generate insight on the major routes of excretion and metabolism in the healthy model.

This preliminary PK study will either confirm or eliminate compounds for the primary validation study. Compounds with poor bioavailability and absorption may be chemically optimized and retested. If absorption remains low, the combination of $V_d$ and tissue samples describe a compound that has very low tissue distribution or describe a compound that is highly concentrated in tissue excluding the colon the compound will not proceed.

## 6.2 Pharmacodynamics (PD)

A preliminary PD study, in conjunction with the preliminary PK study, will be conducted in healthy mice to investigate the effects of each compound on the body. This study will administer each compound over a 5 day period to 5 healthy mice in order to investigate the effects on metabolism, the heart, the gallbladder, the intestine and itching. Itching effects will be determined by monitoring mouse behaviour over the 5 days of treatment. Table 6.3 lists the key measurements that will be monitored for the PD study.

**Table 6.3:** Pharmacodynamic parameters for study

| Metabolism | Cardiac Function | Gallbladder | Intestine |
|---|---|---|---|
| • GLP-1 | • Standard blood pressure (mmHg) | • Overall Size | • Bile acids diarrhea |
| • Glucose homeostasis | • Heart Rate (bpm) | • Total BAs ($\mu$mol/l) | • Feces weight ratio (wet/dry) |
| • Weight loss | • LVFS* (%) & LVEF* (%) | | • Energy (ingested/excreted) |

*LVFS (left ventricular fractional shortening), LVEF (left ventricular ejection fraction)

Monitoring each compound's effects on metabolism is especially important in the search for a TGR5 agonist for treatment of UC. TGR5 activation has previously been linked to increased metabolism, weight loss and increased insulin sensitivity [48]. Though these may be positive effects of the compound, it is important that the agonist does not cause significant weight loss as this is a primary symptom of UC. These same PD measures will be retested in the DSS-induced UC murine model for the compounds remaining in secondary *in vivo* validation.

## 6.3 Primary Validation

The primary *in vivo* validation will be conducted in hTGR5 BALB/C mice with DSS induced UC and will rank the compounds in terms of efficacy. A second rapid PK

study with full MBS will be conducted in the colitis mouse model to observe the effects of the disease on the ADME of the compounds. At this stage, drug efficacy will be estimated by measuring plasma levels of the inflammatory cytokines TNF$\alpha$ and IL-1$\beta$ and by observing body weight and diarrhea score [9, 10]. A toxicity study will also be performed to assess the safety of the candidate compounds *in vivo*.

### 6.3.1 Efficacy

Following 7 days of 5% DSS in drinking water, mice will be treated by oral gavage based on the PK dosage study of the compounds for 5 days. At this scale, each compound will be tested on a small group of 5 mice and one group will remain untreated as a negative control. Whole blood will be collected prior to and after 1, 3 and 5 days of treatment. ELISA assays for both cytokines will be conducted on the plasma following the manufacturer's recommended protocol. The diarrhea score will be assessed every day based on stool characteristics (consistency and presence of occult blood) and the mice will be weighed every day to assess weight loss [40, 49]. This primary validation will not look at histological markers and will not characterize organ tissue to optimize time and cost. Histological tests on tissue will be conducted in the secondary validation.

### 6.3.2 Toxicology and Genotoxicity

A toxicity study will be performed only for the compounds showing effect in the previous study. Four doses, i.e. a placebo, an active dose (based on previous PK and PD studies), an intermediate dose (5x the active dose) and a toxic dose (10x the active dose), will be administered daily to 5 healthy male mice per dose level over 5 days, and the mice will be observed for 14 days. Mortality and morbidity will be assessed. Special toxicity studies will investigate effects on the nervous, circulatory, respiratory and immune systems [50]. Neurotoxicity will be infered from neurofunctional and behavioural signs. For each dead animal, morphological, biochemical and histological changes will be investigated [51]. Blood will be collected from each animal and erythropoietic cells will be scored for micronuclei as described previously (see section 3.4.2) to assess structural and numerical chromosome aberrations [52]. The top 3-4 compounds will be selected as those inducing the greatest decrease in the inflammatory and UC biomarkers without toxic effects.

## 6.4 Secondary Validation

The final candidate will be chosen from the top 3-4 through secondary validation, that will include detailed efficacy markers as well as a full conventional PK study. The goal of the secondary validation is to select the candidate with the best overall efficacy. To achieve this, the primary validation efficacy markers will be retested and expanded to include levels of inflammatory cytokines (TNF$\alpha$ and IL-1$\beta$) in the intestinal tissue, neutrophilic infiltration and intestinal epithelial damage. All of the measurements of efficacy were chosen as they are well-known markers of UC/UC progression or well-known symptoms of UC [10, 9]. Each compound will be administered by oral gavage at concentrations identified in PK dosing studies to 10 mice with DSS induced colitis.

The expression of inflammatory cytokines in the intestinal tissue will be assessed by RT-qPCR (reverse transcription quantitative polymerase chain reaction) after RNA

extraction from sections of colon of the sacrificed mice [40]. Each target mRNA measured level has to be normalized to the expression of an endogenous reference gene or to the total cellular RNA content to correct for errors in sample quantification, variation in RT-qPCR efficiency or in RNA integrity and variations arising from animals. After normalization, cytokine levels will be reported as relative mRNA expression. Plasma levels of the same cytokines will be measured by ELISA. The diarrhea score will be assessed every day based on stool characteristics (consistency and presence of occult blood) [40, 49]. The mice will also be weighed every day to assess weight loss. Neutrophilic infiltration will be assessed by Myeloperoxidase (MPO) assay on colonic tissue [40], since the concentration of this enzyme in tissue is correlated to the extent of neutrophilic infiltration [53]. Furthermore, blinded histologic scoring will be performed on Hematoxylin & Eosin (H&E) stained colonic tissue to measure the degree of epithelial damage and inflammation [40].

If two compounds have similar results in the above studies, both will be subject to a single administration, full, conventional *in vivo* PK evaluation in a large animal and a second rodent. Dogs and rats will be used for this PK study to stay consistent with the animals used in the chronic testing. If one clear lead has already emerged, this additional assessment will only be conducted on the final compound. If two compounds are tested, the absorption from the large animal PK will be taken into account in the final decision as it may be indicative of absorption in humans. A full MBS with radiolabelled compound in dogs and rats will only be conducted on the final compound.

The lead compound will be chosen as the candidate with the best combination of desirable PK parameters and significant decreases in the inflammatory and UC biomarkers compared to the untreated animals.

## 6.5 Chronic Treatment

After selection of the lead compound, a chronic study will determine the long term efficacy of treatment. Known biological activities of TGR5 agonists and chronic toxicity will be assessed with longer chronic studies. Since chronic toxicity has to be assessed on a rodent as well as on a non-rodent model, the studies will be performed on healthy rats and dogs [51].

### 6.5.1 Efficacy

Chronic UC will be mimicked by 4 cycles of 5% DSS in drinking water for 1 week followed by water for 10-14 days in BALB/C mice with hTGR5. After induction of UC, a group of 20 mice (10 female, 10 male) will be treated with the compound once daily by oral gavage for 28 days. 20 untreated mice will serve as the negative control. The secondary *in vivo* validation markers - diarrhea score, weight loss, plasma and intestine levels of inflammatory cytokines, neutrophilic infiltration and intestinal epithelial damage - will be reassessed for this chronic condition and treatment. Assuming satisfactory chronic efficacy the compound will continue chronic evaluation.

### 6.5.2 Chronic Toxicity

The chronic evaluation of the lead compound will continue with a battery of chronic toxicity tests to assess potential negative long-term effects. Four drug doses, i.e. a placebo, an active dose (based on previous PK and PD studies), an intermediate dose

(5x the active dose) and a toxic dose (10x the active dose) will be administered to 20 healthy rats and 8 healthy dogs of each sex per dose level over 9 months. The biological activities of TGR5 agonists assessed previously in healthy mice after acute treatment (see section 6.2) will be re-evaluated after chronic administration. These include testing effects on metabolism, cardiac function, gallbladder size, total BA pool size and itching as these are known potential liabilities of TGR5 compounds. Mortality and morbidity, neurotoxicity (based on neurofunctional and behavioural signs), hematologic toxicity and the effects on the immune and the respiratory systems will also be assessed [50]. There will also be a histological analysis of all tissues, with particular attention to carcinogenicity and reproductive toxicity. The liver and kidney, organs involved in drug metabolism, will also receive particular attention.

Separate specific reproductive studies will be conducted on rats and dogs based on EMA guidelines [54]. If no treatment-related teratogenic and/or embryo-fetal lethal effects are observed in an embryo-fetal development (EFD) study, a combined fertility and early embryo development (FEED) and pre/postnatal development (PPND) study will be performed [54]. Combining the FEED and PPND studies will reduce the number of overall animals required.

To gain approval and complete an Investigational New Drug (IND) application the drug must not be carcinogenic. This will be tested with a 24 month carcinogenicity study using the same animal groups and dose levels as above. A subsection of randomly selected animals will be sacrificed at 12 months to assess the development of tumors. If tumors will be present, the remaining animals will be sacrificed and the test will be stopped. Otherwise, on successful completion of 24 months without tumors, the compound will be considered non carcinogenic.

## 6.6 Selectivity and Mechanism of Action

The following studies, run in parallel with the chronic treatment studies, will better characterize the drug selectivity and mechanism of action *in vivo*.

The same tests and conditions from the secondary *in vivo* validation study will be repeated for the drug in TGR5 KO and hTGR5 (wild-type) mice. A comparison of the results in TGR5 KO and wild-type will indicate if the drug is acting non-selectively. Repeating this with conditional TGR5 KO mice, TGR5 KO only in macrophages, will provide insight on the mechanism of action [55]. One of two macrophage TGR5 KO groups will receive treatment. The treatment/no treatment results in the conditional KO mice compared to results in the hTGR5 mice will make clear whether or not the activation of macrophage TGR5 is responsible for the treatment response [55].

# 7 Clinical Trials

The final lead compound, after thorough pre-clinical animal trials will move to the clinical trial phase of drug development. The objective of clinical trials is to demonstrate safety and efficacy in humans in order to establish the benefits and risks of the drug. The design of clinical trials for patients with UC must adhere to universally accepted ethical standards of human subject research. These ethical principles include beneficence, non-maleficence, respect for persons, and justice.

## 7.1 Phase I

Phase I clinical trials are the first administration of the drug to humans and will be conducted on a single subject basis. The objective of phase I study will be to assess safety and tolerability, to determine the appropriate doses (safe doses), to test the route of administration and to evaluate human PK.

Sequentially ascending dose studies will be conducted in healthy humans to determine safe dosage levels. The principle of a unique ascending dose study is based on a small group of healthy volunteers (< 50) receiving a single dose of study drug. Volunteers will be observed and tested for a period of time to confirm the safety and characterize the PK of the study drug. The ascending dose study will begin with a dose that is 10X less than the minimum anticipated biologic effect level (MABEL) [56]. Data from the animal efficacy studies will be used to estimate the MABEL, as well as the pharmacologically active dose (PAD) and the anticipated therapeutic dose range (ATD) in humans [57]. These can be estimated by scaling from animal studies (mg/kg) to human equivalent dose (HED in mg/kg) based on body surface area (rat dose/6.2, mouse dose/12.3 and dog dose/1.8) [58]. The dose at which there was no additional benefit for increased dose represents the saturation of the receptor. This value from *in vivo* studies will be used as the maximum dose for healthy volunteers [57]. The lower doses will require only one subject, and as the doses increase, 3-5 subjects will be tested per dose. Throughout the ascending dose study, blood samples collected from the subjects as well as urine and stool samples will be used to determine human pharmacokinetics.

## 7.2 Phase II

Phase II clinical trials will investigate the PK, efficacy, side effects and toxicity of the drug in patients with UC [59]. In the beginning, phase II will be conducted with naive patients presenting mild-moderate colitis as a standalone treatment. If the drug is efficacious as a stand alone treatment a second phase II trial may be conducted. This second trial would evaluate the drug as an add-on for patients suffering from moderate-severe colitis currently on antibody treatments. The off-label use of the drug as an add-on to antibody treatment will be evaluated only in the clinical trial phase and no additional pre-clinical tests will be performed. One of the objectives in Phase II is to find the optimal dose that maximizes the efficacy/safety ratio (drug shows biological activity with minimal side-effects). The symptoms of UC, primarily the diarrhea, may result in different PK parameters compared to healthy subjects. This investigation will help determine if dosages must be increased during flare ups or in patients who experience high levels of diarrhea. The primary endpoint for the trial will be complete remission judged as a Mayo score of 0 for stool frequency and rectal bleeding and 0-1 for the endoscopic assessment (normal mucosal appearance) (Table 7.4) [60, 61]. Stool frequency and rectal bleeding subscores will be assessed during patient screening, throughout treatment and at the end-of-treatment visit while histological assessment of endoscopically obtained biopsies will evaluate crypt structure and intestinal epithelial degradation during screening and at the end-of-treatment visit [61]. The Physicians Global Assessment (PGA) will be noted at visits, however following FDA suggestions it will not be included as an endpoint [61].

**Table 7.4:** The Mayo Score used for assessment of remission. Reproduced from the FDA's *Ulcerative Colitis: Clinical Trial Endpoints Guidance for Industry* originally from Schroeder, Tremaine et al. 1987.

| Score | Stool Frequency* | Rectal Bleeding | PGA** |
|---|---|---|---|
| 0 | Normal for this patient | No blood seen | Normal |
| 1 | 1-2 more than normal | Streaks of blood in stool <50% of the time | Mild disease |
| 2 | 3-4 more than normal | Obvious blood in stool most of the time | Moderate disease |
| 3 | 5+ more than normal | Blood only | Severe disease |

*Stool frequency requires a baseline ”normal” for each patient from healthy/remission periods.
**Physician’s Global Assessment

## 7.3 Phase III & Phase IV

The aim of phase III is to evaluate the efficacy and safety of a new drug compared to that of a placebo or a reference standard drug. Phase III will be conducted as a double blind randomized trial and will use the same endpoint, complete remission, as phase II. If the compound was also successful as an add-on medication in phase II the drug will be tested again in both naive and antibody treated patients. After completion of Phase III the drug will be introduced to the market.

The Phase IV trial is a post-marketing study. The aim is to identify rare or long-term adverse effects over a much larger patient population and longer time period than was possible during the Phase I-III clinical trials. The drug efficacy will continue to be monitored throughout the post marketing study. At least 2 years of Phase IV clinical trials will be conducted.

# 8 Conclusion

Current treatments for ulcerative colitis, a chronic inflammatory bowel disease, include anti-inflammatories, immunosuppressants, corticosteroids and antibody injections. Though these treatments are relatively effective for many patients, the side effects and the compliance of the drugs (route of administration) can be improved. The development of a TGR5 agonist drug could likely address some of these concerns and improve the efficacy of treatment for patients suffering from UC. TGR5 activation may play a dual role in treating ulcerative colitis, by regulating the structural maintenance of the intestinal epithelium and decreasing the inflammatory response from macrophages. The novel TGR5 agonist drug will decrease macrophage production of pro-inflammatory cytokines that can degrade the colon’s epithelium leading to the pathogen infestation and a cascade of immune responses that contribute to the pathogenesis of UC [28].

The drug development process to identify a TGR5 agonist began with a virtual screen. This screen used AutoDock Vina and the homology model, stabilized A2A adenosine receptor to screen compounds in several chemical libraries and identify preliminary hits. The solubility of these 10,000 representative compounds will be assessed by the shake flask method. All compounds that are soluble in water will advance to permeability testing with the CaCo-2 permeability assay. The 300 compounds with the greatest permeability will advance to the primary *in vitro* screening.

The primary screen will use hTGR5 transfected HEK293 cells to identify compounds that lead to increased cAMP production using the HTRF assay (CisBio) and FRET luminescence. The 75 compounds with the lowest EC50 will advance to secondary *in*

*vitro* screening that will look specifically at the effect of compounds on LPS activated macrophages. The screen will quantify a number of downstream effects of macrophage TGR5 activation with several methods. These include using ELISA to determine the reduction of TNF-$\alpha$ production, using HTRF to determine cAMP upregulation and using a Nanostring mRNA panel to determine the levels of inflammatory cytokines. The secondary screen will also evaluate compound cytotoxicity using digital holographic microscopy to assess cell viability through optimal path distance. Overall, the top 35 compounds from the *in vitro* screening will move forward in the drug development process.

Prior to *in vivo* validation, compounds will be tested *ex vivo* in colonic tissue culture from mice with DSS induced colitis and the top 15-20 compounds will advance to *in vivo* testing. The *in vivo* validation has several steps. First, a pharmacological study on healthy mice will eliminate compounds with poor distribution, bioavailabity and/or adverse effects on key organs. Next primary validation will include a PK/PD study in a disease model, followed by efficacy testing (TNF-$\alpha$ levels, IL-$\beta$1 levels, mouse bodyweight and diarrhea) and a toxicity and genotoxicity study. A secondary validation on the top 3-4 compounds will include an in-depth efficacy analysis that extensively measures histological biomarkers. The final step of secondary validation, on only the final or final two compounds will be a dog and rat PK study. The *in vivo* validation finishes when the lead candidate undergoes a chronic efficacy and chronic toxicity study.

Once identified and tested for chronic effect and toxicity, the drug will move forward to the clinical trial phase. Clinical trials will begin with phase I, which will evaluate safety and PK in one healthy subject at a time. Phase II will evaluate the safety and efficacy in patients with UC. First, a group of naive patients taking only the TGR5 agonist drug will complete the phase II trial. If the results in naive patients are positive, a second phase II trial will be conducted. This second trial will evaluate the drug as an add-on medication for patients with severe colitis currently treated with anti-TNF-$\alpha$ antibodies and will begin directly in humans without additional pre-clinical evaluation. Once efficacy and PK in patients is established, phase III will expand treatment groups and evaluate efficacy against a placebo. Assuming the drug proves efficient it can be marketed after phase III. A standard long term post marketing study (phase IV) will follow patients through years of treatment to watch for rare side effects or long term toxicity.

# References


[1] H. Duboc, Y. Tache, and A. F. Hofmann. “The bile acid TGR5 membrane receptor: from basic research to clinical application”. In: *Dig Liver Dis* 46.4 (Apr. 2014), pp. 302–312.

[2] A. Perino and K. Schoonjans. “TGR5 and Immunometabolism: Insights from Physiology and Pharmacology”. In: *Trends Pharmacol. Sci.* 36.12 (Dec. 2015), pp. 847–857.

[3] C. Thomas et al. “Targeting bile-acid signalling for metabolic diseases”. In: *Nat Rev Drug Discov* 7.8 (Aug. 2008), pp. 678–693.

[4] T. Li and J. Y. Chiang. “Bile acid signaling in metabolic disease and drug therapy”. In: *Pharmacol. Rev.* 66.4 (Oct. 2014), pp. 948–983.

[5] T. W. Pols. “TGR5 in inflammation and cardiovascular disease”. In: *Biochem. Soc. Trans.* 42.2 (Apr. 2014), pp. 244–249.

[6] A. Baars et al. “The Gut Microbiota as a Therapeutic Target in IBD and Metabolic Disease: A Role for the Bile Acid Receptors FXR and TGR5”. In: *Microorganisms* 3.4 (Oct. 2015), pp. 641–666.

[7] S. Cipriani et al. “The bile acid receptor GPBAR-1 (TGR5) modulates integrity of intestinal barrier and immune response to experimental colitis”. In: *PLoS ONE* 6.10 (2011), e25637.

[8] K. Yoneno et al. “TGR5 signalling inhibits the production of pro-inflammatory cytokines by in vitro differentiated inflammatory and intestinal macrophages in Crohn’s disease”. In: *Immunology* 139.1 (May 2013), pp. 19–29.

[9] Ryan Ungaro et al. “Ulcerative colitis”. In: *The Lancet* 389.10080 (2017), pp. 1756–1770. ISSN: 0140-6736. DOI: https://doi.org/10.1016/S0140-6736(16)32126-2. URL: http://www.sciencedirect.com/science/article/pii/S0140673616321262.

[10] S. Garud and M. A. Peppercorn. “Ulcerative colitis: current treatment strategies and future prospects”. In: *Therap Adv Gastroenterol* 2.2 (Mar. 2009), pp. 99–108.

[11] Brijen Shah and Lloyd Mayer. “Current status of monoclonal antibody therapy for the treatment of inflammatory bowel disease”. In: *Expert Review of Clinical Immunology* 6.4 (2010), pp. 607–620. DOI: 10.1586/eci.10.45.

[12] B. E. Sands. “Inflammatory bowel disease: past, present, and future”. In: *J. Gastroenterol.* 42.1 (Jan. 2007), pp. 16–25.

[13] Uma Mahadevan. “Medical treatment of ulcerative colitis”. In: *Clinics in colon and rectal surgery* 17.01 (2004), pp. 7–19.

[14] H. Duan et al. “Discovery of Intestinal Targeted TGR5 Agonists for the Treatment of Type 2 Diabetes”. In: *J. Med. Chem.* 58.8 (Apr. 2015), pp. 3315–3328.

[15] Konstantin Arnold et al. “The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling”. In: *Bioinformatics* 22.2 (2006), pp. 195–201.

[16] Garrett M Morris et al. “AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility”. In: *Journal of computational chemistry* 30.16 (2009), pp. 2785–2791.

[17] Teague Sterling and John J Irwin. “ZINC 15–ligand discovery for everyone”. In: *J. Chem. Inf. Model* 55.11 (2015), pp. 2324–2337.

[18] Daisy Sharma et al. “Solubility Enhancement – Eminent Role in Poorly Soluble Drugs”. In: 2 (Nov. 2008).

[19] P. Sieger, Y. Cui, and S. Scheuerer. “pH-dependent solubility and permeability profiles: A useful tool for prediction of oral bioavailability”. In: *European Journal of Pharmaceutical Sciences* 105 (2017), pp. 82–90. DOI: 10.1016/j.ejps.2017.04.016.

[20] E. Baka, J. E. Comer, and K. Takacs-11ak. “Study of equilibrium solubility measurement by saturation shake-flask method using hydrochlorothiazide as model compound”. In: *J Pharm Biomed Anal* 46.2 (Jan. 2008), pp. 335–341.

[21] R. B. Van Breemen and Y. Li. “CaCo-2 cell permeability assays to measure drug absorption”. In: *Expert Opinion on Drug Metabplism & Toxicology* 1.2 (Aug. 2005), pp. 175–185.

[22] M. R. Herbert et al. “Synthesis and SAR of 2-aryl-3-aminomethylquinolines as agonists of the bile acid receptor TGR5”. In: *Bioorg. Med. Chem. Lett.* 20.19 (Oct. 2010), pp. 5718–5721.

[23] François Degorce. *Homogeneous Time Resolved Fluorescence (HTRF) – A Reference Technology Dedicated to Drug Screening*. 2003. URL: http://www.iptonline.com/articles/public/CISOLART.pdf.

[24] Christine Williams. “cAMP detection methods in HTS: selecting the best from the rest”. In: *Nature Reviews Drug Discovery* 3 (Feb. 2003), pp. 125–135.

[25] Discoverx.com. *gpcrMAX GPCR Assay Panel*. URL: https://www.discoverx.com/services/drug-discovery-development-services/gpcr-screening-profiling-services/gpcrscan-gpcr-profiling/gpcrmax.

[26] Discoverx.com. *scanMAX Kinase Assay Panel*. URL: https://www.discoverx.com/services/drug-discovery-development-services/kinase-profiling/kinomescan/scanmax.

[27] Discoverx.com. *NHRscan - Nuclear Hormone Receptor Panel*. URL: https://www.discoverx.com/services/drug-discovery-development-services/nhrscan-nuclear-receptor-profiling.

[28] Rodrigo Quera David Díaz-Jiménez Katya Carrillo and 03cela A. Hermoso. “Research of Immunology 03kers of UC”. In: Ulcerative Colitis from Genetics to Complications (Jan. 2012), pp. 21–40.

[29] “Rediscovering the sweet spot in drug discovery”. In: *Drug Discovery Today* 8 (2003), pp. 1067–1077.

[30] “Risks in new drug development: Approval success rates for investigational drugs”. In: *Clinical Pharmacology Therapeutics* 69 (2001), pp. 293–307.

[31] “Cytotoxicity of drugs and diverse chemical agents to cell culture”. In: *Toxicology* 16 (1980), pp. 59–66.

[32] B. Rappaz et al. “Noninvasive characterization of the fission yeast cell cycle by monitoring dry mass with digital holographic microscopy”. In: *J Biomed Opt* 14.3 (2009), p. 034049.

[33] K. Mortelmans and E. Zeiger. “The Ames Salmonella/microsome mutagenicity assay”. In: *Mutation Research – Fundamental and Molecular Mechanisms of Mutagenesis* 455.1-2 (Nov. 2000), pp. 29–60.

[34] M. L. Garriott, J. B. Phelps, and W. P. Hoffman. “A protocol for the in vitro micronucleus test”. In: *Mutation Research – Genetic Toxicology and Environmental Mutagenesis* 517.1-2 (May 2002), pp. 103–112.

[35] Lidiya Luzhna, Palak Kathiria, and Olga Kovalchuk. “Micronuclei in genotoxicity assessment: from genetics to epigenetics and beyond”. In: *Frontiers in genetics* 4 (2013).

[36] Stefan Wirtz and 03kus F. Neurath. “Mouse models of inflammatory bowel disease”. In: *Advanced Drug Delivery Reviews* 59.11 (2007). Prediction of Therapeutic and Drug Delivery Outcomes Using Animal Models, pp. 1073–1083. ISSN: 0169-409X. DOI: https://doi.org/10.1016/j.addr.2007.07.003. URL: http://www.sciencedirect.com/science/article/pii/S0169409X07001755.

[37] P. K. Randhawa et al. “A review on chemical-induced inflammatory bowel disease models in rodents”. In: *Korean J. Physiol. Pharmacol.* 18.4 (Aug. 2014), pp. 279–288.

[38] D. Low, D. D. Nguyen, and E. Mizoguchi. “Animal models of ulcerative colitis and their application in drug research”. In: *Drug Des Devel Ther* 7 (2013), pp. 1341–1357.

[39] S. Manicassamy and I. Manoharan. “Mouse models of acute and chronic colitis”. In: *Methods Mol. Biol.* 1194 (2014), pp. 437–448.

[40] B. Chassaing et al. “Dextran sulfate sodium (DSS)-induced colitis in mice”. In: *Curr Protoc Immunol* 104 (Feb. 2014), Unit 15.25.

[41] 03ia Fritsch Fredin et al. “The application and relevance of ex vivo culture systems for assessment of IBD treatment in murine models of colitis”. In: *Pharmacological Research* 58.3 (2008), pp. 222–231. ISSN: 1043-6618. DOI: https://doi.org/10.1016/j.phrs.2008.08.006. URL: http://www.sciencedirect.com/science/article/pii/S1043661808001400.

[42] N. D. Lewis et al. “A GPBAR1 (TGR5) small molecule agonist shows specific inhibitory effects on myeloid cell activation in vitro and reduces experimental autoimmune encephalitis (EAE) in vivo”. In: *PLoS ONE* 9.6 (2014), e100883.

[43] J. Zhu et al. “Design, synthesis and biological evaluation of a novel class of potent TGR5 agonists based on a 4-phenyl pyridine scaffold”. In: *Eur J Med Chem* 69 (Nov. 2013), pp. 55–68.

[44] Qingan Zou et al. “4-Benzofuranyloxynicotinamide derivatives are novel potent and orally available {TGR5} agonists”. In: *European Journal of Medicinal Chemistry* 82 (2014), pp. 1–15. ISSN: 0223-5234. DOI: https://doi.org/10.1016/j.ejmech.2014.05.031. URL: https://www.sciencedirect.com/science/article/pii/S0223523414004450.

[45] Dean P. Phillips et al. “Discovery of Trifluoromethyl(pyrimidin-2-yl)azetidine-2-carboxamides as Potent, Orally Bioavailable TGR5 (GPBAR1) Agonists: Structure–Activity Relationships, Lead Optimization, and Chronic In Vivo Efficacy”. In: *Journal of Medicinal Chemistry* 57.8 (2014). PMID: 24666203, pp. 3263–3282. DOI: 10.1021/jm401731q. URL: http://dx.doi.org/10.1021/jm401731q.

[46] Chun Li et al. “A modern in vivo pharmacokinetic paradigm: combining snapshot, rapid and full PK approaches to optimize and expedite early drug discovery”. In: *Drug Discovery Today* 18.1 (2013), pp. 71–78. ISSN: 1359-6446. DOI: https://doi.org/10.1016/j.drudis.2012.09.004. URL: http://www.sciencedirect.com/science/article/pii/S1359644612003078.

[47] Donglu Zhang et al. “Preclinical experimental models of drug metabolism and disposition in drug discovery and development”. In: *Acta Pharmaceutica Sinica B* 2.6 (2012), pp. 549–561. ISSN: 2211-3835. DOI: https://doi.org/10.1016/j.apsb.2012.10.004. URL: http://www.sciencedirect.com/science/article/pii/S2211383512001438.

[48] Mitsuhiro Watanabe et al. “Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation”. In: *Nature* 439.7075 (2006), pp. 484–489.

[49] A. Agus et al. “Western diet induces a shift in microbiota composition enhancing susceptibility to Adherent-Invasive E. coli infection and intestinal inflammation”. In: *Sci Rep* 6 (Jan. 2016), p. 19032.

[50] Susan A Elmore. “Enhanced histopathology of the immune system: a review and update”. In: *Toxicologic pathology* 40.2 (2012), pp. 148–156.

[51] S Parasuraman. “Toxicological screening”. In: *Journal of pharmacology & pharmacotherapeutics* 2.2 (2011), p. 74.

[52] David A Eastmond et al. “Mutagenicity testing for chemical risk assessment: update of the WHO/IPCS Harmonized Scheme”. In: *Mutagenesis* 24.4 (2009), pp. 341–349.

[53] M. Garrity-Park et al. “Myeloperoxidase immunohistochemistry as a measure of disease activity in ulcerative colitis: association with ulcerative colitis-colorectal cancer, tumor necrosis factor polymorphism and RUNX3 methylation”. In: *Inflamm. Bowel Dis.* 18.2 (Feb. 2012), pp. 275–283.

[54] European Medicines Agency. *EMA/CHMP/ICH/544278/1998 - ICH S5 (R3) guideline on reproductive toxicology: Detection of Toxicity to Reproduction for Human Pharmaceuticals*. [Date Accessed: 18.11.2017]. Aug. 2017. URL: http://www.ema.europa.eu/docs/en_GB/document_library/Annual_report/2010/05/WC500090712.pdf.

[55] Roland H. Friedel et al. “Generating Conditional Knockout Mice”. In: *Transgenic Mouse Methods and Protocols*. Ed. by Marten H. Hofker and Jan van Deursen. Totowa, NJ: Humana Press, 2011, pp. 205–231. ISBN: 978-1-60761-974-1. DOI: 10.1007/978-1-60761-974-1_12. URL: https://doi.org/10.1007/978-1-60761-974-1_12.

[56] Food and Drug Administration. *Guidance for industry: Estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers*. [Date Accessed: 04.12.2017]. July 2005. URL: https://www.fda.gov/downloads/drugs/guidances/ucm078932.pdf.

[57] European Medicines Agency. *Guideline on strategies to identify and mitigate risks for first-in-human and early clinical trials with investigational medicinal products*. [Date Accessed: 04.12.2017]. Sept. 2017. URL: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2017/07/WC500232186.pdf.

[58] A. B. Nair and S. Jacob. "A simple practice guide for dose conversion between animals and human". In: *J Basic Clin Pharm* 7.2 (Mar. 2016), pp. 27–31.

[59] Lawrence M. Friedman, Curt D. Furberg, and David L. Demets. "Fundamentals of Clinical Trials". In: (2010). DOI: 10.1007/978-1-4419-1586-3.

[60] Simon PL Travis et al. "Defining remission in ulcerative colitis". In: *Alimentary pharmacology & therapeutics* 34.2 (2011), pp. 113–124.

[61] Food and Drug Administration. *Ulcerative Colitis: Clinical Trial Endpoints Guidelines for Industry*. [Date Accessed: 20.11.2017]. Aug. 2016. URL: https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM515143.pdf.