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(54) Title: METHODS AND COMPOSITIONS FOR TREATING INFECTIONS

(57) Abstract: The invention relates, in part, to methods to identify compounds to treat an apicomplexan infection and/or reduce apicomplexan contamination, and to methods and compositions to treat apicomplexan infections and to reduce apicomplexan contamination. In some aspects the invention the apicomplexan infection or contamination is Cryptosporidium infection or contamination. In certain aspects the invention includes compositions useful to treat Cryptosporidium infection in a subject and/or to reduce Cryptosporidium contamination of a substrate such as a liquid or solid surface, object, or material.



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METHODS AND COMPOSITIONS FOR TREATING INFECTIONSRelated Applications

This application claims benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application
5 No. 61/566331, filed December 2, 2011, and U.S. Provisional Application No. 61/669822, filed July
10, 2012 the content of each of which is incorporated by reference herein in its entirety.

Government Interest

This invention was made with government support under ~~the~~ P20 ~~RR021905~~ grant awarded
by the National Institutes of Health. The Government has certain rights in the invention.

Field of the Invention

10 The invention relates, in part, to methods and compositions for treating apicomplexan
infections and infections by other organisms.

Background

15 Apicomplexan parasites are unicellular, eukaryotic, spore-forming (e.g., cyst or oocyte
forming) protists known to infect animals including humans. Infection by apicomplexan parasites
are often spread from contact with water sources, consumption of parasites in food or liquid, insect
transmission, and/or contact with contaminated surfaces and materials. Because these parasites
share many metabolic pathways with their animal hosts, it is extremely difficult to treat
20 apicomplexan infections or to identify effective therapeutic compounds because compounds that
inhibit or kill the parasite may also harm the infected host. In addition, apicomplexan parasites are
very difficult to culture and maintain which has impeded attempts to identify and screen potential
therapeutic compounds to treat apicomplexan infections and contamination.

De novo drug development is an expensive, long process and there may be a market/need
25 mismatch in the development of compounds. Some aspects of drug development may include
modification of existing drugs or drug repurposing such as the discovery of novel uses for already
approved compounds. Drug discovery and development approaches are complex and frequently
unsuccessful. Efficient, effective treatments are not currently available for most diseases caused by
the apicomplexan parasites. Despite its significant disease burden, treatment options for
30 apicomplexan parasitic infections are extremely limited. Niazoxanide (NTZ), a therapeutic agent
used to treat cryptosporidiosis, only exhibits moderate clinical efficacy in immunocompetent adults
and children [Amadi B., et al., (2002) Lancet 360: 1375-1380; Rossignol J.F., et al., (2001) J. Infect

Dis 184: 103-106] and its utility in AIDS patients continues to be debated [Amadi B., et al., (2002) Lancet 360: 1375-1380; Amadi B., et al., (2009) BMC Infect Dis 9: 195; Anderson VR & Curran MP (2007) Drugs 67: 1947-1967; Rossignol JF (2006) Aliment Pharmacol Ther 24: 887-894; **Rossignol J.F.**, et al., (1998) Trans R Soc Trop Med Hyg 92: 663-666; and Abubakar I., et al., (2007) Cochrane Libr: 531 CD004932].

Summary of the Invention

The invention in part, provides methods to identify candidate compounds to treat apicomplexan infections, and also provides methods to treat such infections and contamination by apicomplexan **parasites**. In certain aspects of the invention, a **cell-based** screening assay is provided that permits **identification** of lead compounds for the treatment of apicomplexan **infections** and contamination. The invention also provides in some aspects, *in vitro* methods to characterize lead compounds to determine whether or not they may be useful to treat an apicomplexan infection or to reduce or eliminate contamination by an apicomplexan parasite.

According to an aspect of the invention, methods of treating an apicomplexan infection in a subject are provided. The methods including administering to a subject having or at risk of having an apicomplexan infection an effective amount of at least one of a statin compound, a tetracycline compound, an anti-metabolite compound, a digoxin, docetaxol, idarubicin, daunorubicin, doxorubicin, homoharringtonine, oligomycin C, tegaserod, indomethacin, glipizide, or triptolide compound to treat the apicomplexan infection. In certain embodiments, the apicomplexan infection is a Cryptosporidiosis infection. In certain embodiments, the apicomplexan infection is a Babesia infection, a Plasmodium infection, a Cyclosporiasis infection, an Isosporiasis infection, an Eimeria infection, or a Theileria infection. In some embodiments, the statin compound is fluvastatin, cerivastatin, mevastatin, itavastatin, lovastatin, or simvastatin. In some embodiments, the tetracycline compound is doxycycline, demeclocycline, tetracycline, oxytetracycline, or minocycline. In certain embodiments, the anti-metabolite compound is floxuridine, 6-azauridine, carmofur, or 5-fluorouracil. In some embodiments, the subject is free of indications otherwise calling for treatment with the statin compound, the tetracycline compound, the anti-metabolite compound, or the digoxin, docetaxol, idarubicin, daunorubicin, doxorubicin, homoharringtonine, oligomycin C, tegaserod, indomethacin, glipizide, or triptolide compound, administered to treat the apicomplexan infection. In some embodiments, the subject is otherwise free of indications for treatment with a therapeutic agent comprising floxuridine, digoxin, chloroxine, docetaxel.

sdanibicin, daunorubicin, doxorubicin, homoharringtonme, hexachlorophene, 6~azauridine, oligomycin C, carmofur, tegaserod, 5-fluorouracil, indomethacin, glipizide, triptolide, tremilacin; doxycycline, minocycline, demeclocycline; tetracycline, oxytetracycline, fluvastatin, cerivastatin, mevastatin, itavastatin, lovastatin, or simvastatin. In certain **embodiments**, the compound is a statin and the subject is not undergoing treatment with the statin for a condition that is not the apicomplexan infection. In some embodiments, the compound is a tetracycline and the subject is not undergoing treatment with the **tetracycline** for a condition that is not the apicomplexan infection. In some embodiments, the compound is an anti-metabolite compound and the subject is not undergoing treatment with the anti-metabolite compound for a condition that is not the apicomplexan infection. In some embodiments, the compound is digoxin, docetaxol, idarubicin, daunorubicin, doxorubicin, homoharringtonme, oligomycin C, tegaserod, indomethacin, glipizide, or triptolide and the subject is not undergoing treatment with the compound for a condition that is not the apicomplexan infection. In certain embodiments, the apicomplexan infection includes the presence of one or more apicomplexan parasites in or on the subject. In some embodiments, the apicomplexan parasites comprise vegetative (e.g., sporozoites, merozoites, meronts, etc) and/or infectious apicomplexan parasites (e.g., oocysts). In some embodiments, the method also includes administering one or more additional anti-apicomplexan therapeutic agents to the subject. In some embodiments, the additional anti-apicomplexan therapeutic agent is NTZ. In certain embodiments, the compound is administered in a pharmaceutical composition. In some embodiments, the pharmaceutical composition further includes a pharmaceutically acceptable carrier. In some embodiments, the pharmaceutical composition further includes a targeting agent. In certain embodiments, the targeting agent is an agent that targets an epithelial tissue. In some embodiments, the pharmaceutical composition further includes an agent to reduce solubility of the compound in the subject's intestine. In some embodiments, the compound is administered orally, sublingually, intravenously, intramuscularly, subcutaneously, intrathecally, transdermally, intraperitoneally, transmucosally, or intranasally. In some embodiments, the compound is administered prophylactically. In certain embodiments, the method also includes selecting a subject at least in part on the basis that the subject has been exposed to an apicomplexan infection. In some embodiments, the subject has been diagnosed with an apicomplexan infection. In some embodiments, the Cryptosporidium infection of the subject includes infection by one or more of *C. parvum*, *C. hominis*, *C. amnis*, *C. felis*, *C. meleagridis*, or *C. muti*. In some embodiments, the subject is a vertebrate, and optionally is a mammal.

According to another aspect of the invention, articles of manufacture are provided. The articles including one or more containers housing a statin compound, a tetracycline compound, an anti-metabolite compound, a digoxin, docetaxol, idarubicin, daunorubicin, doxorubicin, homoharringtonine, oligomycin C, tegaserod, indomethacin, glipizide, triptolide, carvedilol, ketoconazole, mefloquine, imodium, or aripiprazole compound and instructions for administering the compound to a subject having or at risk of having an apicomplexan infection. In certain embodiments, the apicomplexan infection is a Cryptosporidiosis infection. In some embodiments, the apicomplexan infection is a Babesia infection, a Plasmodium infection, a Cvciosporiasis infection, an Isosporiasis infection, an Eimeria infection, or a Theileria infection. In some

embodiments, the statin compound is fluvastatin, cerivastatin, mevastatin, simvastatin, lovastatin, or simvastatin. In some embodiments, the anti-metabolite compound is floxuridine, 6-azauridine, carmofur, or 5-fluorouracil. In certain embodiments, the tetracycline compound is doxycycline, demeclocycline, tetracycline, oxytetracycline, or minocycline. In some embodiments, the articles of manufacture also include a container that includes an additional anti-apicomplexan therapeutic agent. In some embodiments, the additional anti-apicomplexan therapeutic agent is nitazoxanide (NTZ). In certain embodiments, the Cryptosporidium infection is an infection by one or more of *C. parvum*, *C. hominis*, *C. canis*, *C. felis*, *C. mekagridis*, or *C. maris*. In some **embodiments**, the apicomplexan infection includes the presence of one or more apicomplexan parasites in or on a subject. In some embodiments, the apicomplexan parasites are vegetative apicomplexan parasites (e.g., sporozoites, merozoites, meronts, etc) or infectious apicomplexan parasites (e.g., oocysts).

According to another aspect of the invention, pharmaceutical compositions for treatment of an apicomplexan infection are provided. The compositions including one or more of a statin compound, a tetracycline compound, an anti-metabolite compound, a digoxin, docetaxol, idarubicin, daunorubicin, doxorubicin, homoharringtonine, oligomycin C, tegaserod, indomethacin, glipizide, triptolide, carvedilol, ketoconazole, mefloquine, imodium, or aripiprazole compound. In some embodiments, the apicomplexan infection is a Cryptosporidiosis infection. In certain embodiments, the apicomplexan infection is a Babesia infection, a Plasmodium infection, a Cvciosporiasis infection, an isosporiasis infection, an Eimeria infection, or a Theileria infection. In some embodiments, the statin compound is fluvastatin, cerivastatin, mevastatin, itavastatin, lovastatin, or simvastatin. In some embodiments, the anti-metabolite compound is floxuridine, 6-azauridine, carmofur, or 5-fluorouracil. In certain embodiments, the tetracycline compound is doxycycline, demeclocycline, tetracycline, oxytetracycline, or minocycline. In some embodiments,

the pharmaceutical composition also includes one or more additional anti-apicomplexan therapeutic agents. In some embodiments, an additional anti-apicomplexan therapeutic agent is nitazoxanide (NTZ). In some embodiments, the pharmaceutical composition also includes a targeting agent. In certain embodiments, the targeting agent is an agent that targets an epithelial tissue. In some
 5 embodiments, the pharmaceutical composition also includes an agent that reduces solubility of the compound in the subject's intestine. In some embodiments, the composition is formulated oral, sublingual, intravenous, intramuscular, subcutaneous, cutaneous, dermal, intrathecal, **transdermal**, intraperitoneal, transmucosal, or intranasal delivery. In some embodiments, is formulated for prophylactic delivery. In certain embodiments, the Cryptosporidium infection includes infection by
 10 one or more of *C. parvum*, *C. hominis*, *C. catts*, *C. felis*, *C. meleagridis*, or *C. muris*.

According to yet another aspect of the invention, methods of decontaminating a substrate that includes or is at risk of including apicomplexan contamination are provided. The methods include contacting the substrate with an effective amount of at least one of a statin compound, a tetracycline compound, **an anti-metabolite** compound, a digoxin, docetaxol, idarubicin,
 15 daunorubicin, doxorubicin, homoharringtonine, oligomycin C, tegaserod, indomethacin, glipizide, triptolide, carvedilol, ketoconazole, mefloquine, imodium, or aripiprazole compound to reduce the apicomplexan contamination of or the risk of apicomplexan contamination of the substrate. In some embodiments, the apicomplexan contamination is Cryptosporidia contamination. In some embodiments, the apicomplexan contamination is Babesia contamination, Plasmodium
 20 contamination, Cyclosporiasis contamination, Isosporiasis contamination, Eimeria contamination, or Theileria contamination. In certain embodiments, the substrate is also contaminated with Clostridium, Entamoeba, Giardia, and wherein the method reduces the apicomplexan contamination and the Clostridium contamination. Entamoeba contamination, or Giardia contamination. In some embodiments, reducing the apicomplexan contamination of the substrate
 25 includes reducing the level of apicomplexan parasite on the substrate. In some embodiments, the statin compound is fluvastatin, cerivastatin, mevastatin, itavastatin, lovastatin, or simvastatin. In certain embodiments, the tetracycline **compound** is doxycycline, demeclocycline, tetracycline, oxytetracycline, or minocycline. In some embodiments, the anti-metabolite compound is floxuridine, 6-azauridine, capecitabine, or 5-fluorouracil. In some embodiments, the compound is
 30 chloroxine, hexachlorophene, or a combination thereof. In certain embodiments, the substrate includes a liquid. In some embodiments, the liquid includes water. In some embodiments, the substrate includes one or more of a metal, wood, plastic, glass, cork, fiber, a polymer, or a fabric. In

certain embodiments, the substrate includes a food substance. In some embodiments, the substrate is at least a portion of a tool, work surface, a medical device, body of water, clothing, skin, or an edible substance (e.g., a beverage, medication, food, etc.). In some embodiments, the contaminated substrate includes one or more apicomplexan parasites. In some embodiments, the apicomplexan parasite includes one or more of a vegetative (e.g., sporozoites, merozoites, meronts, etc) apicomplexan parasite or an infectious apicomplexan parasite (e.g., oocysts). In certain embodiments, the substrate is contacted by an aqueous composition comprising the compound. In some embodiments, the *Cryptosporidium* is *C. parvum*, *C. hominis*, *C. canis*, *C. felis*, *C. meleagridis*, or *C. baileyi*.

According to another aspect of the invention, compositions are provided. The compositions include a statin compound, a tetracycline compound, an anti-metabolite compound, a digoxin, doxorubicin, idarubicin, daunorubicin, doxorubicin, homoharringtonine, oligomycin C, tegaserod, indomethacin, glipizide, triptolide, carvedilol, ketoconazole, mefloquine, imodium, or aniprazole compound, and an additional anti-apicomplexan therapeutic agent. In some embodiments, the apicomplexan infection is a *Cryptosporidiosis* infection. In certain embodiments, the apicomplexan infection is a *Babesia* infection, a *Plasmodium* infection, a *Cyosporiasis* infection, an *Isosporiasis* infection, an *Eimeria* infection, or a *Theilera* infection. In some embodiments, the composition includes carvedilol, ketoconazole, mefloquine, Imodium, or aniprazole. In some embodiments, the statin compound is fluvastatin, cerivastatin, mevastatin, itavastatin, lovastatin, or simvastatin. In some embodiments, the anti-metabolite compound is floxuridine, 6-azauridine, carmofur, or 5-fluorouracil. In certain embodiments, the tetracycline compound is doxycycline, demeclocycline, tetracycline, oxytetracycline, or minocycline. In some embodiments, the composition also includes a detergent. In some embodiments, the composition also includes a pharmaceutically acceptable carrier. In some embodiments, the *Cryptosporidium* infection includes infection by one or more of *C. parvum*, *C. hominis*, *C. canis*, *C. felis*, *C. meleagridis*, or *C. muris*. In certain embodiments, the apicomplexan infection includes infection by one or more apicomplexan parasites. In some embodiments, the apicomplexan parasites includes a vegetative apicomplexan parasite (e.g., sporozoite, merozoite, meront, etc) and/or an infectious apicomplexan parasite (e.g., oocyst). In some embodiments, the additional anti-apicomplexan therapeutic agent is nitazoxanide (NTZ). In some embodiments, the composition includes carvedilol, ketoconazole, mefloquine, imodium, or aniprazole and NTZ.

According to another aspect of the invention, methods of decontaminating a substrate that includes or is at risk of including Clostridium contamination are provided. The methods including contacting the substrate with an effective amount of at least one of a chloroxine compound or a hexachlorophene compound to reduce the Clostridium contamination of or the risk of Clostridium contamination of the substrate. In certain embodiments, reducing the Clostridium contamination of the substrate includes reducing the level of Clostridium parasite on the substrate. In some embodiments, the substrate includes a liquid. In some embodiments, the liquid includes water. In some embodiments, the substrate includes one or more of a metal, wood, plastic, glass, cork, fiber, a polymer, or a fabric. In some embodiments, the substrate is at least a portion of a tool, work surface, a medical device, body of water, clothing, skin, or an edible substance (e.g., a beverage, medicine, or food, etc.). In certain embodiments, the Clostridium contamination includes contamination by Clostridium spores. In some embodiments, the substrate is contacted by an aqueous composition that includes the compound.

According to another aspect of the invention, methods of treating a Cryptosporidium infection in a subject are provided. The methods including: administering to a subject having or at risk of having a Cryptosporidium infection an effective amount of at least one of a compound that modifies cell cholesterol metabolism in the subject to treat the Cryptosporidium infection, wherein the compound is not a statin. In some embodiments, the subject is not undergoing treatment with a statin compound. In some embodiments, the Cryptosporidium infection includes the presence of one or more Cryptosporidium parasites in or on the subject. In some embodiments, the Cryptosporidium parasites include vegetative (e.g., sporozoites, merozoites, meronts, etc) and/or infectious Cryptosporidium parasites (e.g., oocysts). In some embodiments, the method also includes administering one or more additional anti-apieonipSexan therapeutic agents to the subject. In certain embodiments, the compound is administered in a pharmaceutical composition, in some embodiments, the pharmaceutical composition also includes a pharmaceutically acceptable carrier. In some embodiments, the pharmaceutical composition also includes a targeting agent. In some embodiments, the targeting agent is an agent that targets an epithelial tissue. In some embodiments, the pharmaceutical composition also includes an agent to reduce solubility of the compound in the subject's intestine. In certain embodiments, the compound is administered orally, sublingual ly, intravenously, intramuscularly, subcutaneously, mtrathically, transdermally, intraperitoneally, transmucosaliy, or iniranasaliy. In some embodiments, the compound is administered piOphylactically. In some embodiments, the method also includes selecting a subject at least in part

on the basis that the subject has been exposed to *Cryptosporidium* infection. In some embodiments, the subject has been diagnosed with a *Cryptosporidium* infection. In some embodiments, the *Cryptosporidium* infection of the subject includes infection by one or more of *C. parvum*, *C. hominis*, *C. canis*, *C. felis*, *C. meleagridis*, or *C. minis*. In certain embodiments, the subject is a vertebrate.

According to yet another aspect of the invention, use of a compound that modifies cell cholesterol metabolism in a subject in the manufacture of a medicament to treat a *Cryptosporidium* infection in a subject, wherein the compound is not a statin is provided.

According to another aspect of the invention, methods of treating an **apicomplexan** infection in a subject are provided. The methods including administering to a subject, an **effective** amount of a statin compound, a tetracycline compound, an anti-metabolite compound, a digoxin, docetaxol, idarubicin, daunorubicin, doxorubicin, homohaningtonine, oligomyein C, tegaserod, indomethacin, glipizide, triptolide, earvedilol, ketoconazole, mefloquine, Imodium, or aripiprazole compound, and an additional anti-apicomplexan therapeutic agent to treat the apicomplexan infection in the subject. In some embodiments, the anti-apicomplexan therapeutic agent is nitazoxanide (NTZ). In some embodiments, the apicomplexan infection is a *Cryptosporidia* infection.

According to another aspect of the invention, methods of identifying a test compound as a compound to treat an apicomplexan parasite infection in a subject or to reduce an apicomplexan parasite contamination of a substrate are provided. The methods including inoculating parasite oocysts onto a cell monolayer, priming the parasite oocysts, contacting the inoculated cells with a test compound, incubating the contacted inoculated cells, determining the level of a parasite in the cell monolayer, and comparing the level of the parasite in the cell monolayer contacted with the test compound to a control level of the parasite, wherein a decrease in the level of the parasite in the cells contacted with the test compound compared to the control level of the parasite identifies the test compound as a compound to treat an apicomplexan parasite infection in a subject or to reduce an apicomplexan parasite contamination of a substrate. In some embodiments, the oocyst is a *Cryptosporidium* oocyst. In some embodiments, the incubation is at least 24 hours, 36 hours, 48 hours, or 60 hours in duration. In certain embodiments, the method also includes fixing the contacted, inoculated, incubated cells. In some embodiments, determining the level of the parasite includes staining the cell nuclei and staining the parasites, visualizing the cells, and determining the number of parasites in the cell culture. In some embodiments, the control level of the parasite is the level of the parasite in an inoculated, incubated cell monolayer that is not contacted with the test

compound. In some embodiments, the cell monolayer is a human ileocecal adenocarcinoma (HCT-8) cell monolayer. In certain embodiments, the method also includes confirming efficacy of the candidate compound for treating a cell or subject having or at risk of having the apicomplexan parasite infection or for reducing contamination of a substrate by the apicomplexan parasite.

The present invention is not intended to be limited to a system or method that must satisfy one or more of any stated objects or features of the invention. It is also important to note that the present invention is not limited to the exemplary or primary embodiments described herein. Modifications and substitutions by one of ordinary skill in the art are considered to be within the scope of the present invention.

Brief Description of the Drawings

Fig. 1 provides graphs showing results of the sensitive and specific assay to detect *C. parvum* growth in intestinal epithelial cells. Fig. 1A shows results when primed oocysts were inoculated onto confluent HCT-8 cell monolayers at varying multiplicities of infection, incubated for 48 hours and then fixed, stained and imaged. Each data point is the mean of 16 technical replicates. The solid line represents a linear regression ($R^2 \approx 0.85$), demonstrating good correlation between the number of parasites detected and the MOI. Fig. 1B shows results when primed oocysts were inoculated onto confluent monolayers and then treated with varying concentrations of nitazoxanide at three hours following infection. The half maximal inhibitory concentration (IC₅₀) for nitazoxanide was determined to be 3.7 μ M (95% C.I. $\approx$ 3.5 - 3.8 μ M). $N=12$ for log [nitazoxanide] $\approx$ 1.9 and 24 for all other data points. Fig. 1C shows graph of results obtained when primed oocysts were inoculated onto confluent monolayers and then fixed at 3 ($n=33$), 12 ($n=21$), 24 ($n=22$), and 48 ($n=21$) hours, stained, imaged and analyzed. Figures 1A-C are representative of at least two biological replicates and error bars represent standard error of the mean.

Fig. 2 provides graphs demonstrating the assay is sufficiently robust for high throughput applications. Fig. 2A shows results obtained when primed oocysts were inoculated onto confluent monolayers ($n=286$) and 96 were left uninfected (gray triangles). Seventeen randomly located infected wells were treated with an inhibitory concentration of nitazoxanide (41 μ M; 689 open diamonds) while the remaining 269 wells were treated with DMSO vehicle only (filled circles). Dashed lines represent the signal band, the upper bound of which is equal to μ DMSO treated wells - 3σ DMSO treated wells and the lower bound is equal to μ uninfected wells - 3σ uninfected wells. Fig. 2B

provides results obtained when confluent monolayers were inoculated with primed oocysts ($n=154$ per plate; filled circles) or left uninfected ($n=152$ for plate 1 and 154 for plates 2 and 3; open triangles) and incubated for 48 hours at which point cells were processed as described in the Examples, and Z' scores (equation 1 Examples section) were calculated for each biological replicate. The signal band is shown for each plate. Vertical dotted lines separate the data from each of three experiments which were performed on separate days.

Fig. 3 provides plots of screening results. The results from screening the 727 compound MIF Clinical Collection libraries are shown for the first screening replicate as a scatter plot (Fig. 3A) and a **frequency** distribution (Fig. 3B). The solid horizontal lines in Fig. 3A represent the hit limit (80% inhibition) for each plate screened. The data are divided into their corresponding assay plates by the dashed vertical lines.

Fig. 4 provides a table showing hit confirmation information for a number of screened compounds. Confirmatory testing was performed on compounds that exhibited $\geq 8Q\%$ inhibition in at least one screening replicate. Compounds were recovered from source plates and used to determine dose-response curves. IC50 indicates the 50% inhibitory concentration.

Fig. 5 provides a table showing **confirmed** screening hits. **IC50** indicates 50% inhibitory concentration. **TC50** indicates 50% toxic concentration. **TI** indicates therapeutic index, calculated as TC50 divided by IC50.

Fig. 6 shows IC50 and toxicity results for ffoxuridine. Fig. 6A shows toxicity results. Fig. 6B shows refined IC50 results indicating number of parasites detected, and Fig. 6C shows rough IC50 results indicating number of parasites detected.

Fig. 7 shows **IC50** and toxicity results for digoxin. Fig. 7A shows toxicity results, Fig. 7B shows refined IC50 results indicating number of parasites detected, and Fig. 7C shows rough IC50 results indicating number of parasites detected.

Fig. 8 shows **IC50** and toxicity results for chloroxine. Fig. 8A shows **toxicity** results. Fig. 8B shows refined **IC50** results indicating number of parasites detected, and Fig. 8C shows rough **IC50** results indicating number of parasites detected.

5 Fig. 9 shows **IC50** and toxicity results for **docetaxel**. Fig. 9A shows toxicity results and Fig. 9B shows rough **IC50** results indicating number of parasites detected.

Fig. 10 shows **IC50** and toxicity results for **idarubicin**. Fig. 10A shows toxicity results and Fig. 10B shows rough **IC50** results indicating number of parasites detected.

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Fig. 11 shows **IC50** and toxicity results for **Daunorubicin**. Fig. 11A shows toxicity results, Fig. 11B shows refined **IC50** results indicating number of parasites detected, and Fig. 11C shows rough **IC50** results indicating number of parasites detected.

15 Fig. 12 shows **IC50** and toxicity results for Doxorubicin. Fig. 12A shows toxicity results, Fig. 12B shows refined **IC50** results indicating number of parasites detected, and Fig. 12C shows rough **IC50** results indicating number of parasites detected.

20 Fig. 13 shows **IC50** and toxicity results for homoharringtonine. Fig. 13A shows toxicity results and Fig. 13B shows rough **IC50** results indicating number of parasites detected.

Fig. 14 shows **IC50** and toxicity results for hexachlorophene. Fig. 14A shows toxicity results, Fig. 14B shows refined **IC50** results indicating number of parasites detected, and Fig. 14C shows rough **IC50** results indicating number of parasites detected.

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Fig. 15 shows **IC50** and toxicity results for **6-azauridine**. Fig. 15A shows **toxicity** results, Fig. 15B shows refined **IC50** results indicating number of parasites detected, and Fig. 15C shows rough **IC50** results indicating number of parasites detected.

30 Fig. 16 shows **IC50** and toxicity results for **mevastatin**. Fig. 16A shows toxicity results, Fig. 16B shows refined **IC50** results indicating number of parasites detected, and Fig. 16C shows rough **IC50** results indicating number of parasites detected. Refined **IC50** represents results from 5/31 infection.

Fig. 17 shows IC50 and toxicity results for oligomycin C. Fig. 17A shows toxicity results. Fig. 17B shows rough IC50 results indicating number of parasites detected.

5 Fig. 18 shows IC50 and toxicity results for Minocycline. Fig. 18A shows toxicity results. Fig. 18B shows refined IC50 results indicating number of parasites detected, and Fig. 18C shows rough IC50 results indicating number of parasites detected.

10 Fig. 19 shows **IC50** and toxicity results for **Carmofur**. Fig. 19A shows toxicity results. Fig. 19B shows refined IC50 results indicating number of parasites detected, and Fig. 19C shows rough IC50 results indicating number of parasites detected.

15 Fig. 20 shows **IC50** and toxicity results for Tegaserod. Fig. 20A shows toxicity results. Fig. 20B shows refined IC50 results indicating number of parasites detected, and Fig. 20C shows rough IC50 results indicating number of parasites detected.

20 Fig. 21 shows **IC50** and toxicity results for 5-fluorouracil. Fig. 21A shows toxicity results. Fig. 21B shows refined IC50 results indicating number of parasites detected, and Fig. 21C shows rough IC50 results indicating number of parasites detected.

Fig. 22 shows **IC50** and toxicity results for Indomethacin. Fig. 22A shows toxicity results. Fig. 22B shows refined IC50 results indicating number of parasites detected, and Fig. 22C shows rough IC50 results indicating number of parasites detected.

25 Fig. 23 shows **IC50** and toxicity results for doxycycline. Fig. 23A shows toxicity results. Fig. 23B shows refined IC50 results indicating number of parasites detected, and Fig. 23C shows rough IC50 results indicating number of parasites detected.

30 Fig. 24 shows **IC50** and toxicity results for glipizide. Fig. 24A shows toxicity results. Fig. 24B shows refined IC50 results indicating number of parasites detected, and Fig. 24C shows rough IC50 results indicating number of parasites detected.

Fig. 25 shows IC₅₀ and **toxicity** results for triptolide. Fig. 25A shows toxicity results. Fig. 25B shows rough IC₅₀ results indicating number of parasites detected.

Fig. 26 shows IC₅₀ results for iremulacin and provides rough IC₅₀ results indicating number of parasites detected.

Fig. 27 shows a summary table for statin structure-activity relationship (SAR.) compounds.

Fig. 28 shows **IC₅₀** and toxicity results for mevastatin. Fig. 28A shows toxicity results, Fig. **28B** shows refined IC₅₀ results indicating number of parasites detected, and Fig. 28C shows rough IC₅₀ results indicating number of parasites detected.

Fig. 29 shows **IC₅₀** and toxicity results for cerivastatin. Fig. 29A shows toxicity results, Fig. **29B** shows refined IC₅₀ results indicating number of parasites detected, and Fig. 29C shows rough IC₅₀ results indicating number of parasites detected.

Fig. 30 shows **IC₅₀** and toxicity results for **itavastatin**. Fig. 30A shows toxicity results, Fig. 30B shows refined IC₅₀ results indicating number of parasites detected, and Fig. 30C shows rough IC₅₀ results indicating number of parasites detected.

Fig. 31 shows **IC₅₀** and toxicity results for fluvastatin. Fig. 31A shows toxicity results, Fig. 31B shows refined IC₅₀ results indicating number of parasites detected, and Fig. 31C shows rough IC₅₀ results indicating number of parasites detected.

Fig. 32 shows **IC₅₀** and toxicity results for lovastatin. Fig. 32A shows toxicity results. Fig. **32B** shows refined IC₅₀ results indicating number of parasites detected, and Fig. 32C shows rough IC₅₀ results indicating number of parasites detected.

Fig. 33 shows **IC₅₀** and toxicity results for simvastatin. Fig. 33A shows toxicity **results**, Fig. **33B** shows refined IC₅₀ results indicating number of parasites detected, and Fig. 33C shows rough IC₅₀ results indicating number of parasites detected.

Fig. 34 shows a **summary** table for tetracycline structure-activity relationship (**SAR**) compounds.

Fig. 35 shows IC₅₀ results for roxithetracycline.

5 Fig. 36 shows IC₅₀ and toxicity results for **tetracycline**. Fig. 36A shows toxicity results, Fig. 36B shows refined IC₅₀ results indicating number of parasites detected, and Fig. 36C shows rough IC₅₀ results indicating number of parasites detected.

Fig. 37 shows **IC₅₀** and toxicity results for **demeclocycline**. Fig. 37A shows toxicity results, Fig.
10 37B shows refined IC₅₀ results indicating number of parasites detected, and Fig. 37C shows rough IC₅₀ results indicating number of parasites detected.

Fig. 38 shows **IC₅₀** and toxicity results for oxytetracycline. Fig. 38A shows toxicity results, Fig.
38B shows refined IC₅₀ results indicating number of parasites detected, and Fig. 38C shows rough
15 IC₅₀ results indicating number of parasites detected.

Fig. 39 shows **IC₅₀** and toxicity results for **minocycline**. Fig. 39A shows toxicity results, Fig. 39B shows refined IC₅₀ results indicating number of parasites detected, and Fig. 39C shows rough IC₅₀ results indicating number of parasites detected.

20 Fig. 40 shows **IC₅₀** and toxicity results for **doxycycline**. Fig. 40A shows toxicity results, Fig. 40B shows refined IC₅₀ results indicating number of parasites detected, and Fig. 40C shows rough IC₅₀ results indicating number of parasites detected.

25 Fig. 41 provides graphs illustrating results showing that exogenous isopentenyl-pyrophosphate (IPP) partly reverses *C. parvum* growth inhibition by **itavastatin**. Fig. 41A shows results obtained when infected monolayers were treated with various concentrations of itavastatin and then supplied growth medium with or without 50 μ M IPP. The IC₅₀ for itavastatin in wells that received IPP was >5-fold the IC₅₀ in wells with no IPP. Fig. 41B shows results obtained when infected monolayers
30 were treated with various concentrations of metazoxanide and then supplied growth medium with or without 50 μ M IPP. The IC₅₀ for metazoxanide was not affected by the addition of IPP.

Fig. 42 provides photomicrographic images of results analysis pipeline. images were exported from *NIS elements* as *.tif* files and imported to *ImageJ* where the batch process function was used to innumerate host cells and parasites. In the example shown, the image from Figure 1 was separated into individual channels (host cells (Fig. 42A); parasites (Fig. 42B). and processed using the appropriate *ImageJ* macro (see appendices). Fig. 42C (host cells) and Fig. 42D (parasites) are the count masks generated for each image, where each white object represents one counted object.

Fig. 43 is a graph showing results of a qPCR assay for *C. parvum* DNA abundance, establishing a method to compare the cidal activity of drugs or drug-drug combinations to that of NTZ. Cell monolayers in a 384-well plate were infected with *C. parvum*, followed at 24 hours by addition of NTZ at the 1C90 (7.2 μ M) or DMSO. *C. parvum* DNA abundance was followed by qPCR for an additional 48 hours. As a control, the kinetics of *C. parvum* DNA degradation in heat killed parasites added to non-infected monolayers was followed. Data are normalized to the 24 hour time point (mean and SB, n=3).

Detailed Description

In certain aspects of the invention, cell-based screening assays are provided that can be used to identify compounds for the treatment of apicomplexan infections and for the reduction or elimination of contamination by apicomplexan parasites. The invention also provides in some aspects, *in vitro* methods useful to characterize one or more compounds to determine whether or not they may be useful to treat an apicomplexan infection or to reduce or eliminate contamination by an apicomplexan parasite. The invention, in some aspects includes methods useful to overcome barriers to drug repurposing and to permit known drugs and compounds to be identified for use in a previously unknown manner to treat an apicomplexan infection and/or to reduce contamination by an apicomplexan parasite.

Some aspects of the invention include methods such as assays that may be used to identify compounds useful to treat an apicomplexan infection in a cell or subject, or to reduce or elimination apicomplexan contamination of a substrate. Methods of the invention may also be used to determine efficacy, cell toxicity, and other characteristics of compounds that may be used to treat an apicomplexan infection or to reduce or eliminate apicomplexan contamination of a substrate.

Compounds have now been identified that can be used in methods to treat apicomplexan infections in subjects and apicomplexan contamination of substrates. In some embodiments the

apicomplexan infection or contamination that is treated with a compound of the invention is a *Cryptosporidium* infection or contamination. As used herein, the term "anti-apicomplexan therapeutic agent" refers to a compound of the invention that can be used to treat an apicomplexan infection or to reduce contamination by an apicomplexan parasite. In some embodiments of the invention, an anti-apicomplexan therapeutic agent may be a previously identified therapeutic agent such as nitazoxanide (NTZ).

A number of **treatment** methods and compounds of the invention have been identified as useful to treat infection and/or contamination by *Cryptosporidium* species. Methods of the invention that relate to anti-cryptosporidial activity include treatment of cryptosporidiosis in both humans, cattle, and other animals. Thus, compounds and methods of the invention may be used to treat apicomplexan infections in subjects, and may also be administered to decontaminate apicomplexan contamination of a substrate. Some of the compounds that are newly identified as effective to treat apicomplexan infection and/or contamination are compounds that have received Food and Drug Administration (FDA)-approval for treatment in other diseases and conditions. It has now been identified that the compounds of the invention can be used, independent of any prior known use, to treat an apicomplexan infection in a subject.

Compounds that have now been identified as useful to treat apicomplexan infections and contamination, for example *Cryptosporidium* infections, include statin compounds, tetracycline compounds, anti-metabolite compounds, and additional compounds such as digoxin, docetaxol, idarubicin, daunorubicin, doxorubicin, homoharringtonine, oligomycin C, tegaserod, indomethacin, glipizide, or triptolide. Examples of statin compounds of the invention that may be used to treat apicomplexan infections such as *Cryptosporidium* infections include fluvastatin, cerivastatin, mevastatin, itavastatin, lovastatin, and simvastatin. Examples of tetracycline compounds of the invention that may be used to treat apicomplexan infections such as *Cryptosporidium* infections include doxycycline, **deraeflocycline**, tetracycline, oxytetracycline, and **minocycline**. Examples of anti-metabolite compounds of the invention that may be used to treat apicomplexan infections such as *Cryptosporidium* infections include floxuridine, 6-azauridine, carmofur, or 5-fluorouracil. Additional compounds now identified to treat apicomplexan infections such as *Cryptosporidium* infections include, digoxin, chloroxine, docetaxol, idarubicin, daunorubicin, doxorubicin, homoharringtonine, hexachlorophene, oligomycin C, tegaserod, indomethacin, glipizide, triptolide, and tremulacin.

As used herein an apicomplexan infection may be infection by any apicomplexan parasite. Examples of apicomplexan parasites include, but are not limited to: a Cryptosporidiosis parasite, a Babesia parasite, a Cyclosporiasis parasite, an Isosporiasis parasite, an Eimeria parasite, a Plasmodium parasite, or a Theileria parasite. Thus, in some embodiments of the invention, compounds of the invention may be administered to a subject to an apicomplexan infection such as, but not limited to a Cryptosporidiosis infection, a Babesia infection, Cyclosporiasis infection, an Isosporiasis infection, an Eimeria infection, a Plasmodium infection, or a Theileria infection. In some embodiments of the invention, the infection is not a Plasmodium infection. In some embodiments of the invention, the infection is not a Babesia infection.

The invention pertains, in part, to inhibiting parasite replication to treat an infection of a subject by a parasite. Compositions, compounds, and methods of the invention may be used for treating a subject having or at risk of having, a parasitic infection such as an infection by an apicomplexan parasite. Infection of a subject by an apicomplexan parasite may be characterized by the presence of an apicomplexan in a cell or tissue of the subject.

As used herein, a subject shall mean a human or vertebrate mammal including but not limited to a dog, cat, horse, cow, goat, and primate, e.g., monkey. Thus, the invention can be used to treat diseases or conditions in human and non-human subjects. For instance, methods and compositions of the invention can be used in veterinary applications as well as in human prevention and treatment regimens. In some embodiments of the invention, the subject is a human. In some embodiments of the invention, a subject does not have a condition that would otherwise be treated by the compound of the invention that is administered to the subject to treat the apicomplexan infection. In certain embodiments of the invention, a subject does not have a Plasmodium infection. In some embodiments of the invention, the subject does not have a Babesia infection. In some embodiments of the invention the apicomplexan infection or contamination is a cryptosporidiosis infection or contamination with Cryptosporidia, respectively.

Non-limiting examples of subjects to which the present methods and compounds of the invention can be applied are subjects who are diagnosed with, suspected of having, or at risk of having, a parasitic infection, such as an apicomplexan infection. Methods of the invention may be applied to a subject who, at the time of treatment, has been diagnosed as having a parasitic infection, such as an apicomplexan infection, or a subject who is considered to be at risk for having or developing a parasitic infection, for example, an apicomplexan infection. In some embodiments of the invention, a subject having or at risk of having an apicomplexan infection may also be at risk

for, or be diagnosed with another disease such as HIV, AIDS, or other disease characterized by immunodeficiency or immune system compromise.

In some aspects of the invention, a subject having a parasitic infection, for example, an apicomplexan infection, may be a subject who has sufficient symptoms of the infection to be considered suitable for treatment with a compound of the invention, or may be a subject who has been diagnosed with, and so has been confirmed to have the infection. **Examples** of symptoms that may indicate a parasitic infection are known by those of skill in the art, and may include, but are not limited to diarrhea, malnutrition, etc.

In some aspects of the invention, a subject is at risk of having or developing a parasitic infection, for example, an apicomplexan infection. A subject at risk of developing a parasitic infection such as an apicomplexan infection is one who has an increased probability of developing the infection, compared to a control risk of developing the infection. In some embodiments of the invention, a level of risk may be statistically significant compared to a control level of risk. A subject at risk may include, for instance, may be a subject in a geographic location known to put subjects at risk of infection by an apicomplexan parasite; a subject having a family and/or personal medical history of the infection; a subject exposed to substrates that are expected or known to be contaminated by an apicomplexan parasite; and/or a subject who has previously been treated for the parasitic infection, (e.g., an apicomplexan infection) and who may be considered by a medical professional to be at risk for recurrence or a chronic infection.

In some embodiments of the invention, a treatment of a subject is a prophylactic treatment and in certain embodiments, a subject is selected for treatment with a compound of the invention at least in part on the basis that the subject has been exposed to an apicomplexan infection. In some embodiments of the invention, the subject who is treated using a compound of the invention has been diagnosed with an apicomplexan infection.

Certain of the compounds of the invention that have now been found useful treat an apicomplexan infection (e.g., such as a Cryptosporidia infection) or contamination are compounds that have been previously used to treat diseases or conditions that are not Cryptosporidium infections or Cryptosporidium contamination. For example, anti-metabolite compounds floxuridine, 5-fluorouracil, 6-azauridine, and carmofur are all currently approved for treatment of various cancers. Several of the anti-metabolites for which *C. parvum* inhibitory activity was not detected in these studies are in use as antivirals (e.g., AZT, D4T, 3TC, penciclovir) or as disease modifying agents for inflammatory conditions such as rheumatoid arthritis (e.g., mercaptopurine and

azathioprine). Statins are widely prescribed for high cholesterol, and have shown mortality benefits when prescribed following acute myocardial infarction. Some tetracycline compounds are known to inhibit bacterial protein synthesis and widely used antibacterials. Doxycycline is also widely used for both prevention and treatment of malaria. Some tetracyclines are also used for treatment of
 5 acne. Doxycycline has been used for treatment and prevention of parasites that cause malaria (*Plasmodium species*), but has not been previously **used** to treat Cryptosporidia infections. Hexachlorophene is the active ingredient in surgical scrub detergent and Chloroxine is used in an **anti-microbial shampoo**. **Digoxin** has been used as a treatment for congestive heart failure and cardiac **arrhythmias** (to reduce heart rate in **atrial** fibrillation). The anthracyclines: doxorubicin,
 10 idarubicin, daunorubicin have been used to treat cancers and may be more toxic to a treated subject than other compounds identified in these studies. The microtubule inhibitor docetaxel has been used for treatment of cancers. Homoharringtonine is in development for treatment of cancers. Oligomycin C has been used as a topical antibacterial. Tegaserod is a partial agonist of the serotonin receptors 5-HT₄ and 5-HT₃. It was previously approved for treatment of irritable bowel
 15 syndrome and other gastrointestinal motility disorders. It was withdrawn from the market because of cardiac toxicity. Indomethacin is a non-steroidal anti-inflammatory agent that inhibits cyclooxygenase. It has been used as an anti-inflammatory and analgesic. Glipizide is a sulfa drug currently approved for use as an oral treatment for type II diabetes mellitus. Glipizide affects glucose by targeting an ATP-gated K⁺ channel. Triptolide is a traditional Chinese medicine used
 20 for treatment of cancer, inflammation, and autoimmune diseases. It induces intracellular calcium release via the polycystin 2 calcium channel. In some embodiments of the invention, the subject being treated using a compound of the invention is free of indications otherwise calling for treatment with the compound for a non-apicomplexan disease or condition.

In some embodiments of the invention a subject to be treated with a compound of the
 25 invention is a subject who is free of an indication that would otherwise call for treatment of that subject with the compound. In some embodiments the compound to be administered to the subject to treat the apicomplexan infection is a statin compound, a tetracycline compound, an anti-metabolite compound, or another compound such as a digoxin, doxorubicin, idarubicin, daunorubicin, doxorubicin, homoharringtonine, oligomycin C, tegaserod, indomethacin, glipizide, or triptolide
 30 compound. Thus, in some embodiments of the invention a subject to be treated with a compound of the invention is otherwise free of indications for treatment with a therapeutic agent comprising fioxuridine, digoxin, chloroxine, docetaxel, idarubicin, daunorubicin, doxorubicin,

homoharringtonine, hexachlorophene, 6-azauridine, oligomycin C, eamofur, tegaserod, 5-fluorouracil, indomethacin, glipizide, iriptolide, tremulacin; doxycycline, minocycline, demeclocycline; tetracycline, oxytetracycline, fluvastatin, cerivastatin, mevastatin, itavastatin, lovastatin, simvastatin, cerivastatin, ketoconazole, mefloquine, imodium, or aripiprazole.

5 In some embodiments of the invention, a subject administered a statin compound of the invention as a treatment is a subject who is not undergoing treatment with the statin for a condition that is not the **apicomplexan** infection. In some embodiments of the invention, a subject administered a tetracycline compound of the invention as a treatment is a subject who is not undergoing treatment with the tetracycline for a condition that is not the apicomplexan infection.

10 Similarly, in some embodiments of the invention, a subject administered an anti-metabolite compound of the invention as a treatment is a subject who is not undergoing treatment with the anti-metabolite for a condition that is not the apicomplexan infection. In some embodiments of the invention, a subject administered one of the other compounds of the invention such as a digoxin, docetaxol, idarubicin, daunorubicin, doxorubicin, homoharringtonine, oligomycin C, tegaserod, 15 indomethacin, glipizide, or triptolide compound as a treatment, is a subject who is not undergoing treatment with the compound for a condition that is not the apicomplexan infection.

Thus, some embodiments of the invention include methods of administering a compound to a cell, tissue, or subject in an amount effective to inhibit apicomplexan replication in the cell, tissue, or subject as a treatment for the parasitic infection. In some embodiments of the invention, methods 20 include inhibitory compounds of the invention to treat *Cryptosporidium* species. In some embodiments, methods to treat *Cryptosporidium* species include treatment of cryptosporidiosis in both humans and dairy cattle.

Inhibitory activity of compounds of the invention against *C. parvum* has now been shown and compounds of the invention may be used to treat *C. parvum* and additional *Cryptosporidium* 25 species. Infections and contamination by additional *Cryptosporidium* species that may be treated with methods and compounds of the invention, include, but are not limited to infection or contamination by *Cryptosporidium andersoni*, *Cryptosporidium bailey*, *Cryptosporidium hominis*, *Cryptosporidium cervine*, *Cryptosporidium canis*, *Cryptosporidium cuniculus*, *Cryptosporidium ducismarci*, *Cryptosporidium fayeri*, *Cryptosporidium felis*, *Cryptosporidium fragile*, 30 *Cryptosporidium galli*, *Cryptosporidium hominis*, *Cryptosporidium marcopodum*, *Cryptosporidium meleagridis*, *Cryptosporidium molnari*, *Cryptosporidium parvum*, *Cryptosporidium parvum*, *Cryptosporidium ryanae*, *Cryptosporidium saurophilum*, *Cryptosporidium serpentis*,

Cryptosporidium sin's, *Cryptosporidium ubiquilum*, *Cryptosporidium wrairi*, **and/or** *Cryptosporidium xiaoi*.

Cryptosporidium infection in young children is associated with malnutrition, and some embodiments of the invention include administration of one or more compounds of the invention to

5 treat malnutrition in a subject.

In addition to the use of compounds of the invention to treat apicomplexan infections in subjects, compounds of the invention may be **used** to treat (e.g., **decontaminate**) water or other substrate that may include one or more apicomplexan parasites, such as *Cryptosporidium*. The *Cryptosporidia* are well-described causes of waterborne outbreaks of infection. In addition,

10 *Cryptosporidium* oocysts are known to be resistant to many standard methods of disinfection, including bleach, and compounds of the invention may be used to decontaminate substrates that include a *Cryptosporidium* parasite. Examples, though not intended to be limiting, of substrates that may be treated with a compound of the invention to decontaminate are medical devices, first responder equipment, farm equipment, and recreational water such as swimming **pools** and wave

15 pools, etc. Chloroxine and hexachlorophene may be particularly useful in this regard, and additional compounds of the invention may also be used to decontaminate substrates such as food, drinking water, medicines, skin, etc.

In addition to the use of compounds of the invention to treat *Cryptosporidia* infection and contamination, such compounds may also be used to treat and/or decontaminate against other

20 organisms such as apicomplexan parasites, including *Plasmodium* species that cause malaria, *Toxoplasma gondii*, *Cyclospora cayetenensis*, *Isospora belli*, and the veterinary pathogens *Eimeria teneba*, *Theileria annulata*, and *Theileria parva*. In some cases, it may be useful to combine compounds of the invention or related compounds with other therapeutic agents/compounds that exhibit activity against these parasites. For example, compounds targeting non-mevalonate

25 dependent isoprenoid synthesis in the apicoplast of these parasites may act synergistically with therapeutic agents that interfere with mammalian cholesterol synthesis, such as the HMG-Co-A reductase inhibitor compounds of the invention and the co-administration of such compounds may be useful to treat a parasitic infection. In addition to use to treat apicomplexan infections and contamination, in some embodiments of the invention, compounds of the invention, for example

30 hexachlorophene and chloroxine, may be used to decontaminate equipment and/or to achieve water and food safety that results from fecal-oral pathogens, including *Clostridium difficile* (and

inaetivation of *C. difficile* spores), *Entamoeba histolytica*, *Giardia* species, *Cyclospora cayetenensis*, and *Isospora belli*.

An apicomplexan infection in a subject may be characterized by the presence of one or more genus of apicomplexan parasite in or on the subject. Apicomplexan contamination of a substrate may be characterized by the presence of one or more genus of apicomplexan parasite in or on the substrate.

There are varied life stages of apicomplexan parasites. In some embodiments of the invention, the stage of an apicomplexan parasite is a vegetative stage, for example, the parasite is a trophozoite, merozoite, meront, or stage considered to be vegetative by those skilled in the art. In certain embodiments, of the invention, etc) the stage of an apicomplexan parasite is an infectious stage, for example an oocyst stage. Some infections or contaminations by apicomplexan parasites that can be treated using methods and compounds of the invention may be characterized by the presence of apicomplexan parasites in a vegetative stage and apicomplexan parasites in an infectious stage.

A level or presence of a parasitic infection, such as an apicomplexan infection, or the level or presence of contamination of a substrate by a parasitic, for example an apicomplexan parasite, can be determined and compared to control values to assess efficacy of a treatment of the invention. For example, the level of a parasite found in cells, tissues, subjects that do not have a parasitic infection, (e.g., an apicomplexan infection), can be compared to the levels in a treated cell, tissue, or subject to determine efficacy of a compound and treatment of the invention. Similarly, control values such as a level of parasites on a substrate, or the infectivity of a contaminated substrate can be compared with levels and infectivity following treatment with a compound of the invention as a measure of the effectiveness of the compound and/or treatment.

A control may be a predetermined value, which can take a variety of forms. It can be a single cut-off value, such as a median or mean. It can be established based upon comparative groups, such as in groups not having a parasitic infection or parasitic contamination and groups having a parasitic infection or parasitic contamination. Another example of comparative groups may be groups having one or more symptoms of, or a diagnosis of, a parasitic infection, for example an apicomplexan infection, and groups without having one or more symptoms of or a diagnosis of the parasitic infection, (e.g., an apicomplexan infection). Another comparative group may be a group with a personal medical history of a parasitic infection, (e.g., an apicomplexan infection) and a group without such a personal medical history. A predetermined value can be

arranged, for example, where a tested population is divided equally (or unequally) into groups, such as a low-risk group, a medium-risk group and a high-risk group or into quadrants or quintiles, the lowest quadrant or quintile being individuals with the lowest risk (for example of a parasitic infection, such as an apicomplexan infection) and the lowest level of parasite, or parasite replication and the highest quadrant or quintile being individuals with the highest risk (for example of a parasitic infection, such as an apicomplexan infection) and highest levels of parasite or parasite replication.

The predetermined value, of course, will depend upon the particular population selected. For example, an apparently healthy population will have a different "normal" range than will a population that is known to have a parasitic infection, (e.g., an apicomplexan infection) or presence. Accordingly, the predetermined value selected may take into account the category in which an individual or cell falls. Appropriate ranges and categories can be selected with no more than routine experimentation by those of ordinary skill in the art. As used herein, "abnormal" means significantly different as compared to a normal control. By abnormally high levels of a parasite (which may indicate a parasitic infection and/or a parasitic contamination) it is meant high relative to a selected control, and may include an increase in a parasite level of at least 0.1%, 0.5%, 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, or more, in a subject, cell, tissue, or substrate as compared to the level in a normal control. It will be understood that a control may have zero apicomplexan parasites and that any level higher than such a control may indicate the presence of an apicomplexan infection or contamination as compared to that control.

Treatment with a compound of the invention, may result in a reduction in the level or replication of a parasite compared to an abnormal control (e.g., a level that indicates infection or contamination) and include a decrease of at least 0.5%, 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% of the level or replication of a parasite in a subject, cell, tissue, or surface as compared to the level in the abnormal control. Typically, a normal control will be based on apparently healthy normal individuals in an appropriate age bracket or apparently healthy cells; and an abnormal control will be based on individuals who have a parasitic infection and/or substrates known to be contaminated with the parasite, for example, with a *Cryptosporidium* parasite.

In some aspects of the invention, levels of a parasite and/or parasite replication may be determined for a subject may serve as control values for later determinations of the parasite in that same subject, thus permitting assessment of changes from a "baseline" parasitic infection in a subject. Thus, an initial level of parasite and/or parasite replication may be determined in a subject

on/in a substrate and methods and compounds of the invention may be used to decrease the level of parasite and/or parasite replication in the subject or in/on the substrate, with the initial level serving as a control level for that subject or substrate, respectively. Using methods and compounds of the invention, the level of parasite and/or parasite replication in the subject or on the substrate may be
5 decreased by at least 0.5%, 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 100%, compared to the initial level by administering a **treatment** for the parasite infection to the subject or in/on the substrate, respectively.

It will be understood that controls according to the invention may be, in addition to predetermined values, samples of materials tested in parallel with the experimental materials.

10 Examples include samples from control populations or control samples generated through manufacture to be tested in parallel with the experimental samples.

Treatment

The invention in some aspects relates to methods for reducing a parasitic infection (for
15 example an apicomplexan infection) in a subject or in/on a substrate. As used herein the term "reducing" means lowering the amount of a parasite in a subject or in/on a substrate. In some embodiments of the invention, parasitic infection is reduced by reducing the replication of the parasite in the subject, or in/on a substrate. Certain embodiments of the invention methods may include directly decreasing the number of parasites in or on a subject or substrate to a level that is
20 effective to treat the parasitic infection or contamination.

As used herein, the terms "treat", "treated", or "treating" when used with respect to a parasitic infection of a subject may refer to a prophylactic treatment that decreases the likelihood of a subject developing the parasitic infection, and also may refer to a treatment after the subject has developed the parasitic infection in order to eliminate or reduce the level of the parasitic infection,
25 prevent the parasitic infection from becoming more advanced (e.g., more severe), and/or slow the progression of the parasitic infection compared to in the absence of the therapy.

As used herein, the terms "treat", "treated", or "treating" when used with respect to a parasitic infection in or on a substrate may refer to reducing the amount of an infectious parasite in or on the substrate. Treating a substrate with a compound of the invention may reduce the amount
30 of parasite in or on the substrate and may also reduce the likelihood of infection of a subject who contacts the treated substrate. For example, if treatment of a substrate with a compound of the invention reduces the amount of infective parasite on a surface, or in water, etc., the likelihood of

infection of a subject who contacts the treated substrate, may be reduced by up to 0.5%, 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more as compared to the likelihood of infection of the untreated substrate by the subject. Thus, the treatment of the substrate (e.g., the surface or water, etc.) reduces the likelihood of infection of a subject who contacts the treated substrate.

Compounds of the invention may be administered to a cell, tissue, or subject in the form of a **medicament**. A medicament of the invention may be manufactured for the treatment of an apicomplexan infection, for example for the treatment of a **cryptosporidiosis** infection. As used herein the term "therapeutic agent" refers to a compound that is administered to a cell, subject, or substrate to reduce an infection or reduce contamination. Thus, compounds of the invention that are useful to treat an apicomplexan infection or contamination may be referred to as therapeutic agents. Additional compounds that are anti-apicomplexan therapeutic agents are known in the art and include, but are not limited to, NTZ.

Compounds of the invention may be administered singly or in combination with one or more additional compounds. In some embodiments, a compound of the invention may act in a synergistic manner with one or more other therapeutic agents or treatments and increase the effectiveness of the one or more therapeutic agents or activities. Thus, for example, administration of a compound that reduces apicomplexan replication may be administered with another compound that treats the infection, for example, an anti-apicomplexan therapeutic agent. A compound of the invention may act synergistically to increase the effectiveness of one or more agents or treatments that can be administered to treat an apicomplexan infection or contamination. In some embodiments of the invention, an anti-apicomplexan therapeutic agent may be a previously known anti-apicomplexan therapeutic agent such as nitazoxanide (**NTZ**). Compounds of the invention may be administered in combination with other anti-apicomplexan therapeutic agents such as other compounds of the invention or NTZ, etc. In certain embodiments of the invention, a compound administered with NTZ or other anti-apicomplexan therapeutic agent is carvedilol, ketoconazole, mefloquine, imodium, or aniprazole.

Compounds of the invention described herein can be used alone or in conjugates or compositions with other molecules such as targeting agents and/or labeling agents in treatment methods of the invention. Targeting agents useful according to the methods of the invention are those that direct a compound of the invention to a specific cell type or tissue type for treatment. A targeting compound of choice will depend upon the nature of the stage of the parasitic infection or

contamination. In some instances it may be desirable to target the agent to skeletal muscle, cardiac muscle, kidney, liver, brain, etc.; to aid a compound of the invention in crossing the blood-brain-barrier, etc., depending on whether the infection is new, long-term, chronic, etc. Those of ordinary skill in the art will be aware of and able to select and use suitable targeting agents for use in methods of the invention. In some embodiments of the invention, a targeting agent is an agent that targets an epithelial tissue. In certain embodiments of the invention, a targeting agent may be an agent that permits conversion of a compound by linkage of the compound to a peptide to alter solubility of the compound. Methods of altering solubility of therapeutic compounds by peptide linkage are known in the art, see for example Tsume, Y., et al., (2008) Molecular Pharmaceutics; Vol. 5, No. 5 717-727.

Labeling agents may be used in methods of the invention for *in vitro* and *in vivo* assays, to determine the location of compounds of the invention after administration, and may be used to assess the location of treatment compounds that have been administered to a subject or substrate. Procedures for attaching labels to compounds of the invention, and for utilizing labeling agents such as enzymatic labels, dyes, radiolabels, fluorescent labels, etc. **are well known** in the art.

Treatment methods of the invention that include administration of a compound of the invention to a subject can be used at any stages of a parasitic infection in a subject including, early-stage, mid-stage, and late-stage of the parasitic infection including all times before and after any of these stages. Methods of the invention may also be used for subjects who have previously been treated with one or more other medicaments that were not successful, were minimally successful, and/or are no longer successful at slowing or stopping progression of the parasitic infection in the subject.

Treatment methods of the invention that include administration of a compound of the invention to a substrate can be used at any stages of a parasitic infection and can also be used in advance of potential contact with a parasite, for example, as a preventive treatment. Methods of the invention may also be used for substrates that have been previously treated with one or more other compounds to treat a parasitic infection that were not successful, were minimally successful, and/or are no longer successful at removing the parasites, and/or slowing or stopping progression of the parasitic contamination on the substrate.

Effective amounts for treatments

Compounds of the invention are administered to a subject or with a substrate, in an effective amount for treating the parasitic infection. An "effective amount for treating a parasitic infection" is an amount necessary or sufficient to realize a desired biologic effect. For example, an effective amount of a compound of the invention could be that amount necessary to (i) slow or halt progression of the parasitic infection; or (ii) reverse one or more symptoms of the parasitic infection. According to some aspects of the invention, an effective amount is that amount of a compound of the invention alone or in combination with another medicament or treatment, which when combined or **co-administered** or administered alone, results in a therapeutic response in the parasitic infection, either in the prevention or the treatment of the parasitic infection. The biological effect may be the amelioration and or absolute elimination of symptoms resulting from the parasitic infection. In another embodiment, the biological effect is the complete abrogation of the parasitic infection or the parasites, as evidenced for example, by a diagnostic test that indicates the subject or substrate is free of the parasites and/or infection. Diagnostic tests for apicomplexan infections are well known in the art and may include analysis of blood, stool, tissue samples, cell samples, etc. obtained from a subject. A non-limiting example of a diagnostic test may include microscopy, staining, detection of antibodies, an acid-staining test, etc. The analysis may in some embodiments include assessment of one or more samples obtained from a subject, for the presence, absence, level, or changes of a level of an apicomplexan parasite. A sample from a subject, for diagnostic testing may be a blood sample, a stool sample, a tissue sample, etc. In some diagnostic tests a sample from a subject may be cultured and then tested for the presence, absence, and/or level of a apicomplexan parasite, or changes in the level over time, e.g., by comparison of subsequent samples with an initial sample. In some embodiments of the invention a diagnostic test is used to test for and diagnose a *Cryptosporidia* parasitic infection.

Typically an effective amount of a compound of the invention to treat a parasitic infection will be determined in clinical trials (for treating subjects), trials (for treating substrates), establishing an effective dose for a test population versus a control population in a blind study, etc. In some embodiments, an effective amount will be that which results in a desired response, e.g., an amount that diminishes an infection or likelihood of infection in a subject. An effective amount of a compound of the invention to treat substrate may be the amount that when contacted with the substrate reduces the amount of parasite on or in the substrate. In some embodiments of the invention, an effective amount of a compound of the invention may be the amount that when contacted with the substrate reduces the likelihood that a subject contacting the treated substrate

will result in the parasitic infection in the subject, as compared to the likelihood of an infection if the subject contacted the substrate that was not treated with the compound of the invention.

Similarly, an effective amount to treat a parasitic infection in a subject may be the amount that when administered decreases the level of parasite and/or parasite replication in the subject to an amount **that** that is below the amount that would occur in the subject or tissue without the administration of the compound of the invention. In the case of treating a parasitic infection **the** desired response may be reducing or eliminating one or more symptoms of the infection in a subject. The reduction or elimination may be temporary or may be permanent. The status of the parasitic infection can be monitored using methods **of determining** the **amount** of parasite **and/or** the replication status of the parasite, etc. In some aspects of the invention, a desired response to treatment of the parasitic infection can be delaying the onset or even preventing the onset of the parasitic infection.

An effective amount of a compound of the invention to treat a parasitic infection (which may also be also referred to herein as a pharmaceutical compound) may also be determined by assessing physiological effects of administration on a cell or subject, such as a decrease of a parasitic infection in a subject or on a surface following administration. Assays suitable to determine efficacy of a compound of the invention will be known to those skilled in the art and can be employed for measuring the level of the response to a treatment and an amount of a pharmaceutical compound administered to a subject, or an amount of a compound contacted with a substrate, can be modified based, at least in part, on such measurements. Diagnostic tests useful to assess the effects of administration of a compound of the invention on an apicomplexan infection in the subject are known in the art.

The amount of a treatment may be varied for example in a treatment of a subject, by increasing or decreasing the amount of a therapeutic composition, by changing the therapeutic composition administered, by changing the route of administration, by changing the dosage timing and so on. The effective amount will vary with the particular parasitic infection being treated, the age and physical condition of the subject being treated; the severity of the parasitic infection, the duration of the treatment, the nature of the concurrent therapy (if any), the specific route of administration, and additional factors within the knowledge and expertise of the health care professional and/or practitioner.

The effective amount of a compound of the invention in the treatment of a parasitic infection, treatment of a parasitic contamination, or in the reduction of the risk of developing a

parasitic infection may vary depending upon the specific compound used, the mode of delivery of the compound, and whether it is used alone or in combination. The effective amount for any particular application can also vary depending on such factors as the specific parasitic infection or contamination being treated, the particular compound being administered, the size of the subject or substrate, or the severity of the parasitic infection. A skilled artisan can empirically determine the effective amount of a particular compound of the invention without necessitating undue experimentation. Combined with the teachings provided **herein**, by choosing among the various active compounds and weighing factors such as potency, relative bioavailability, substrate size and make up, subject **body** weight, severity of adverse side-effects and preferred mode of administration, an effective prophylactic or treatment regimen can be planned that does not cause substantial toxicity and yet is entirely effective to treat the particular subject or substrate.

When treating a subject, a pharmaceutical compound dosage may be adjusted by an individual health care provider or veterinarian, particularly in the event of any complication. A therapeutically effective amount typically varies from 0.01 mg/kg to about 1000 mg/kg, from about 0.1 mg/kg to about 200 mg/kg, or from about 0.2 mg/kg to about 20 mg/kg, in one or more dose administrations daily, for one or more days. The absolute amount will depend upon a variety of factors including a concurrent treatment, the number of doses and the individual subject parameters including age, physical condition, size and weight. These are factors well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation. In some embodiments, a maximum dose can be used, that is, the highest safe dose according to sound medical judgment.

When treating a substrate, the concentration of a compound of the invention may be applied to the substrate as a component in a composition. The concentration of a compound of the invention in a composition that is in contact to the substrate and/or the final concentration of the compound of the invention in or on the substrate, can be readily determined. In some embodiments of the invention, a compound of the invention may be at a concentration of at least 0.2%, 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10% or more weight per **volume** in a composition that may be applied to a substrate. In certain embodiments of the invention, a compound of the invention may be at a concentration of at approximately 3% weight per volume in a composition that may be applied to a substrate. In certain embodiments, it may be desirable to have a final concentration in or on a substrate in a range of from 0.1% to 33% weight to volume of a compound of the invention, including all amounts within the range. In some embodiments a final concentration in or on a

substrate may be at least 30%, 40%, 50%, or more weight to volume of a compound of the invention.

Multiple doses of compounds of the invention are also contemplated. In some instances, a compound of the invention can be administered at least daily, every other day, weekly, every other
5 week, monthly, etc. to a subject or substrate. Doses may be administered once per day or more than once per day, for example, 2, 3, 4, 5, or more times in one 24 hour period.

Pharmaceutical compounds of the invention to treat subjects, and compounds of the invention to treat substrates, may be administered alone, in combination with other compounds of the invention, and/or in combination with other drug therapies, or other treatment regimens that are
10 administered to subjects or substrates. Pharmaceutical compositions for use in subjects and compositions for use on or in substrates in the foregoing methods may be sterile and contain an effective amount of a compound of the invention to produce the desired response in a unit of weight or volume suitable for administration to a subject or substrate.

The doses of a composition of the invention to treat a parasitic infection, (for example, an
15 apicomplexan infection) can be chosen in accordance with different parameters, in particular in accordance with the mode of administration used and the state of the subject or substrate. Other factors include the desired period of treatment. In the event that a response in a subject or substrate is insufficient at the initial doses applied, higher doses (or effectively higher doses by a different, more localized delivery method or route) may be employed to the extent that subject and substrate
20 tolerance permits.

Administration methods to Subjects

A variety of routes are available to administer a compound of the invention to a subject. The particular delivery mode selected will depend, of course, upon the particular condition being
25 treated and the dosage required for therapeutic efficacy. In some embodiments of the invention, a compound of the invention is administered orally, sublingually, intravenously, intramuscularly, subcutaneously, intrathecally, transdermally, intraperitoneally, transmucosally, dermally, or intranasally. In some embodiments, the administration is a prophylactic administration.

Methods of this invention, generally speaking, may be practiced using any mode of
30 administration that is medically acceptable, meaning any mode that produces effective levels of protection without causing clinically unacceptable adverse effects. In some embodiments of the invention, a compound of the invention may be administered via an oral, enteral, mucosal.

percutaneous, and/or parenteral route. The term "parenteral" includes subcutaneous, intravenous, intramuscular, intraperitoneal, and intrasternal injection, or infusion techniques. Other routes include but are not limited to nasal (e.g., via a gasfro-nasai tube), dermal, vaginal, rectal, and sublingual. In some embodiments of the invention, a delivery route may include contacting a compound of the invention with the skin or dermis of a subject. Delivery routes of the invention may include intrathecal, intraventricular, or intracranial. In some embodiments of the invention, a compound of the invention may be placed within a slow release matrix and administered by placement of the matrix in the subject.

Compounds of the invention may be administered in formulations, which may be administered in pharmaceutically acceptable solutions, which may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers, adjuvants, and optionally other therapeutic ingredients. According to methods of the invention, the compound may be administered in a pharmaceutical composition. In general, a pharmaceutical composition comprises the compound of the invention and a pharmaceutically-acceptable carrier. Pharmaceutically-acceptable carriers are well-known to those of ordinary skill in the art. As used herein, a pharmaceutically acceptable carrier means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients, e.g., the ability of the compound of the invention to treat the parasitic infection.

Pharmaceutically acceptable carriers include diluents, fillers, salts, buffers, stabilizers, solubilizers and other materials that are well-known in the art. Exemplary pharmaceutically acceptable carriers are described in US. Pat. No. 5,211,657 and others are known by those skilled in the art. Such preparations may routinely contain salt, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents. When used in medicine, the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically-acceptable salts thereof and are not excluded from the scope of the invention. Such pharmacologically and pharmaceutically-acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, citric, formic, malonic, succinic, and the like. Also, pharmaceutically-acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts.

In certain embodiments of the invention, a pharmaceutical composition may also include one or more agents that reduce solubility of the compound in the subject's intestine, thereby preventing absorption and increasing availability of the compound with the intestine of the subject.

Compounds of the invention may be administered directly to a tissue. In some
5 embodiments, the tissue to which the compound is administered is a tissue in which the parasitic infection is likely to arise. Direct tissue administration may be achieved by direct injection. Compounds may be **administered** once, or **alternatively** they may be **administered** in a plurality of administrations. If administered multiple times, the compounds may be administered via different routes. For example, the first (or the first few) administrations may be made directly into the
10 affected tissue while later administrations may be systemic.

The compounds, when it is desirable to deliver them systemically, may be **formulated** for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with or without an added preservative. The compositions may take such forms as suspensions,
15 solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic **esters** such as ethyl oleate. Aqueous
20 carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-
25 oxidants, chelating agents, and inert gases and the like. Lower doses will result from other forms of administration, such as intravenous administration. In the event that a response in a subject is insufficient at the initial doses applied, higher doses (or effectively higher doses by a different, more localized delivery route) may be employed to the extent that patient tolerance permits. Multiple
30 doses per day may be used as needed to achieve appropriate systemic or local levels of compounds.

In yet other embodiments, a delivery vehicle is a biocompatible microparticle or implant that is suitable for implantation into the mammalian recipient. Exemplary bioerodible implants that are useful in accordance with this method are described in PCT Publication No. WO 95/24929

(incorporated by reference herein), which describes a biocompatible, biodegradable polymeric matrix for containing a biological macromolecule. Such delivery means are well known in the art and can be used to achieve sustained release of a compound of the invention in a subject; and may be selected not to degrade, but rather, to release by diffusion over an extended period of time.

5 Both non-biodegradable and biodegradable polymeric matrices can be used to deliver the compounds of the invention to the subject. In some embodiments, a matrix may be biodegradable. Matrix polymers may be natural or synthetic polymers. A polymer can be selected based on the period of time over which release is desired, generally in the order of a few hours to a year or longer. Typically, release over a period ranging from between a few hours and three to twelve
10 months can be used. The polymer optionally is in the form of a hydrogel that can absorb up to about 90% of its weight in water and further, optionally is cross-linked with multivalent ions or other polymers.

In general, compounds of the invention may be delivered using the bioerodible implant by way of diffusion, or by degradation of the polymeric matrix. Exemplary synthetic polymers for
15 such use are well known in the art. Biodegradable polymers and non-biodegradable polymers can be used for delivery of compounds of the invention using art-known methods. Bioadhesive polymers such as bioerodible hydrogels (see H. S. Sawhney, C. P. Pathak and J. A. Hubell in *Macromolecules*, 1993, 26, 581-587, the teachings of which are incorporated herein) may also be used to deliver compounds of the invention for treatment. Additional suitable delivery systems can
20 include time-release, delayed release or sustained release delivery systems. Such systems can avoid repeated administrations of the compound, increasing convenience to the subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. (See for example: U.S. Pat. Nos. 5,075,109; 4,452,775; 4,675,189; 5,736,152; 3,854,480; 5,133,974; and 5,407,686 (the teaching of each of which is incorporated herein by reference). In addition,
25 pump-based hardware delivery systems can be used, some of which are adapted for implantation.

Single or multiple dose administration, short-term administration, and other treatment methods described herein may be used for prophylactic treatment of a subject at risk of having a parasitic infection. In addition, use of a long-term sustained release implant may be suitable for
30 prophylactic treatment of subjects and for subjects at risk of developing a recurrent or chronic parasitic infection. Long-term release, as used herein, means that the implant is constructed and arranged to delivery therapeutic levels of the active ingredient for at least 30 days, 60 days, 90 days

or longer. Long-term sustained release implants are well-known to those of ordinary skill in the art and include some of the release systems described above.

Therapeutic formulations of compounds of the invention may be prepared for storage by mixing the compound having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers [Remington's Pharmaceutical Sciences 21st edition, (2006)], in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl **paraben**; catechol; resorcinol; cyclohexanol; **3-pentanol**; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins, hydrophilic polymers such as polyvinylpyrrolidone; amino acids **such as** glycine, glutamine, **asparagine**, histidine, arginine, **or** lysine; **monosaccharides**, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g., Zn-protein complexes); and/or non-ionic surfactants such as TWEEN[®], PLURONICS[®] or polyethylene glycol (PEG).

Decontamination Treatments for Substrates

As described elsewhere herein, methods of the invention may include contacting a substrate with a compound of the invention to decrease or eliminate the contamination by the apicomplexan parasite. Thus, in some embodiments, methods of the invention include decontaminating a substrate that has apicomplexan contamination or is at risk of having apicomplexan contamination. In some embodiments, the methods include contacting the substrate with an effective amount of at least one compound of the invention, such as a statin compound of the invention, a tetracycline compound of the invention, an anti-metabolite compound of the invention, or another compound of the invention such as a digoxin, docetaxol, idarubicin, daunorubicin, doxorubicin, homoharringtonine, oligomycin C, tegaserod, indomethacin, glipizide, or triptolide compound. The substrate is contacted with an effective amount of the compound of the invention to reduce the apicomplexan contamination of or the risk of apicomplexan contamination of the substrate. As described herein, the contamination may be due to the presence of one or more genus of apicomplexan parasite such as Cryptosporidia,

Babesia, Plasmodium, **Cyclosporin Isosporia, Eimeria**, or Theileria parasite. In some embodiments of the invention, the substrate may also be contaminated with Clostridium, Entamoeba, Giardia, which in addition to the reduction of the apicomplexan infection, may also be reduced by contact with the compound of the invention.

5 Reduction in the contamination of a substrate may include a reduction in the amount or activity of the apicomplexan parasite **on** the substrate. Such **a** reduction may result in the substrate being less "infectious" when contacted by a subject. In some embodiments **of the** invention the efficacy of **a** treatment **to** decontaminate a substrate may be **determined** by measuring and assessing the amount of apicomplexan parasite in or on the substrate **following** contact with a compound of
10 **the** invention. In certain embodiments of the invention the efficacy of a treatment to decontaminate a substrate may be determined **by** measuring and assessing the infectivity of the substrate when contacted by a subject after treatment **of the** substrate compared to infectivity of an untreated substrate (e.g., a control, untreated substrate).

 The term "substrate" as used in reference to compounds and treatments of the invention,
15 includes any type of material that may be contaminated by an apicomplexan parasite of the invention. In some embodiments of the invention, a substrate is an aqueous substrate, which includes a liquid that in some embodiments of the invention is water. In some embodiments of the invention, a substrate includes one or more of a metal, wood, plastic, glass, cork, fiber, a polymer, or a fabric, etc. A substrate that is contaminated may be at least a portion of a tool, work surface, a
20 medical device, body of water, clothing, skin, or an edible substance (e.g., a beverage or food). In certain embodiments of the invention, the substrate may be the skin of a subject. Contacting the skin with a compound of the invention may be useful to reduce infection from parasitic presence on the skin. Lotions, ointments, wipes, gels, body-washing liquid, hand-washing liquid, etc., which may be non-sterile or sterile, may be useful to apply one or more compounds of the invention to the
25 skin of a subject to reduce parasitic contamination on that substrate.

 A contaminated substrate that can be decontaminated using a method and/or compound of the invention may have one or more apicomplexan parasites in or on the substrate. A parasite that contaminates a substrate may be a vegetative (e.g., sporozoites, merozoites, meronts, etc) apicomplexan parasite or an infectious apicomplexan parasite (e.g., oocysts). In certain
30 embodiments of the invention a contaminated substrate includes contamination by both vegetative and infectious apicomplexan parasites.

A compound of the invention may be contacted with a substrate using any suitable means. In some embodiments of treatment methods of the invention the contaminated substrate is contacted with an aqueous composition comprising the compound of the invention. The aqueous composition may also include a detergent, colorant, scent, soap, carriers or other components that are suitable for delivery of a compound of the invention to a potentially contaminated substrate. A compound of the **invention** can be delivered to a substrate via any suitable process; including, but not limited to, spraying, or wiping, coating, etc. the substrate with a solution that contains the compound of the invention. Additional means of contacting a substrate with a compound of the invention may include dipping, immersion, etc. of the substrate into a solution that contains the compound. In addition, some substrates such as food, aqueous solutions, water, etc. may have a compound of the invention added to the substrate directly, by pouring, mixing etc. of a solution that contains the compound into the substrate. In some embodiments of the invention, a compound of the invention is part of a composition that is contacted with a substrate to treat an apicomplexan infection. In some embodiments of the invention such a composition may be sterile and in certain embodiments of the invention the composition may be non-sterile.

Kits

Also within the scope of the invention are kits that comprise one or more compositions of the invention and instructions for use. A kit of the invention may be referred to herein as an "article of manufacture" and the terms may be used interchangeably herein. Kits of the invention may include one or more of a compound of the invention that may be used to treat a parasitic infection or treat a parasitic contamination. Kits containing compounds of the invention can be prepared for use in treatment methods for subjects and kits containing compounds of the invention can be prepared for use in treatment methods for substrates. Components of kits of the invention may be packaged either in aqueous medium or in lyophilized form. A kit of the invention may comprise a carrier being compartmentalized to receive in close confinement therein one or more container means or series of container means such as test tubes, vials, flasks, bottles, syringes, or the like. A first container means or series of container means may contain one or more compounds of the invention. A second container means or series of container means may contain a targeting label or Sink-label intermediate capable delivering a compound to a cell or tissue, etc.

A kit of the invention may also include instructions. Instructions typically will be in written form and will provide guidance for carrying-out the assay or treatment embodied by the kit and for making a determination based upon that treatment.

In some embodiments, a kit of the invention may include two or more anti-apicomplexan therapeutic agents, one or more of which may be a compound of the invention. In certain
 5 embodiments, a kit of the invention may **include** an **anti-apicomplexan** therapeutic agent such as NTZ and also one or more compounds of the invention. In some embodiments a compound of the invention included **in** a kit with NTZ may be a statin compound, a **tetracycline** compound, an anti-metabolite compound, another compound such as digoxin, docetaxol, idarubicin, daunorubicin,
 10 doxorubicin, homoharringtonine, olivomycin C, tegaserod, indomethacin, glipizide, nifedipine, carvedilol, ketoconazole, mefloquine, imodium, or antiprazole.

Methods to Identify Candidate Compounds

Certain aspects of the invention include methods of identifying and/or screening additional
 15 candidate compounds to identify compounds that may be used to treat a parasitic infection or contamination (e.g., an apicomplexan infection or contamination), in a subject or substrate, respectively.

Assay methods of the invention, may include primed apicomplexan oocysts inoculated onto confluent cell monolayers and incubated for a period of time, such as up to 3, 6, 12, 24, 48, 60, or
 20 72 hours. Incubation of the cell monolayer may be at a temperature suitable for survival of the monolayer and development of the oocysts into parasites. In certain embodiments of the invention, an incubation temperature may be 37°C. In some embodiments of the invention the apicomplexan oocysts are *Cryptosporidium* oocysts. Cultivation methods for some *Cryptosporidia* are known in the art, for example, **Arrowood** MJ (2002) *Clin Microbiol Rev* **15**: 390-400, the teaching of which is
 25 incorporated herein by reference. Priming of the oocysts can be done using art-known methods, for example, as set forth in Gut J. and R.G. Nelson, (2009) *J Eukaryotic Biol.* **46(5):56S-57S**, the teaching of which is incorporated herein by reference. Priming of oocysts comprises **excystation** of the oocysts, which includes escape from the cyst by a parasite. Thus, some embodiments of methods the invention include inducing excystation of oocysts and inoculating them onto a
 30 monolayer of cells. In some embodiments of the invention, the cell monolayers are **HCT-8** monolayers and after the incubation, the cells are fixed and parasites are stained to visualize the parasites. Various means of labeling the parasites to permit determination of parasite levels (e.g.,

parasite number) in an assay may be utilized in embodiments of methods of the invention. Methods of staining parasites may include the use of detectable labels. In certain embodiments of the invention, a staining method useful to visualize the parasites comprises staining with labeled *Vicia villosa* lectin (VVL) and streptavidin. In certain embodiments of the invention, the VVL is biotinylated VVL and the streptavidin is labeled with a detectable label such as, but not limited to Alexa Fluor 568 and the nuclei may be counterstained using an art-known label and procedure. A non-limiting example of a nuclei stain that may be used in embodiments of the invention is Hoechst staining. Following the staining procedures, imaging and image analysis may be **performed** using art-known imaging methods including for example, microscopy, video imaging, etc. In some embodiments of the invention, the imaging is automated imaging. It will be understood that in some embodiments of the invention, other labels, labeling methods, and detection methods may also be used.

In some embodiments, the invention includes methods of assessing efficacy of a compound for the treatment of a parasitic infection or contamination by a parasite. The invention, in some aspects may include mixing an identified compound with cells or tissues or in a subject and testing to see the effect on an apicomplexan parasitic infection. Examples of testing means that may be used include but are not limited to, determining whether the compound modifies cholesterol metabolism in the cells, tissue, or subject; determining whether the compound reduces cell replication using an assay as set forth in Example 1, etc. Methods can be used that permit assessment of apicomplexan infection and/or contamination before and after contact with the compound, which may also be referred to herein as a candidate agent. A decrease in the amount of replication and/or the amount of parasite in comparison to a suitable control is indicative of a compound agent capable of treating an apicomplexan infection or contamination.

An assay mixture useful to assess a treatment candidate for a parasitic infection, such as an apicomplexan infection comprises a candidate compound. The candidate compound may be an antibody, small organic compound, small molecule, polypeptide, nucleic acid, etc., and accordingly can be selected from combinatorial antibody libraries, combinatorial protein libraries, small organic molecule libraries, or any other suitable source. Typically, a plurality of reaction mixtures is run in parallel with different candidate compound concentrations to obtain a different response to the various concentrations. Typically, one of these concentrations serves as a negative control, i.e., at zero concentration of the candidate compound or at a concentration of compound below the limits of assay detection.

Candidate compounds may be obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides, synthetic organic combinatorial libraries, phage display libraries of random or non-random polypeptides, combinatorial libraries of proteins or antibodies, and the like. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are available or readily produced. Additionally, natural and synthetically produced libraries and compounds can be readily be modified through conventional chemical, physical, and biochemical means. Further, known compounds, which may be FDA approved compounds to treat other diseases or conditions, may be subjected to directed or random chemical modifications such as acylation, alkylation, esterification, amidification, etc. to produce structural analogs of the compounds.

Non-limiting examples of assays and methods to test a candidate compound, identify a compound that may treat a parasitic infection or contamination, and to assess efficacy of compounds such as assays of parasite number and/or parasite replication are set forth in the Examples, including, but not limited to, in Example 1.

The following examples are provided to illustrate specific instances of the practice of the present invention and are not intended to limit the scope of the invention. As will be apparent to one of ordinary skill in the art, the present invention will find application in a variety of compositions and methods.

Examples

Example I

Methods:

In the studies, primed oocysts were inoculated onto confluent HCT-8 monolayer and incubated for 48 hours. The cells were fixed and parasites stained with biotinylated *Vicia villosa* lectin (VVL) and streptavidin Alexa Fluor 568. The nuclei counterstained with Hoechst. Automated imaging and image analysis were performed. Note that the designations: IC (inhibitory concentration) and EC (effective concentration) are used interchangeably herein.

Cell Culture and Infection:

Human ileocecal adenocarcinoma cells (HCT-8) were obtained from ATCC and maintained in T-75 tissue culture flasks with RPMI-1640 medium with HEPES, sodium pyruvate (Imm), and L-Glutamine (ATCC; Cat No: 30-2001) supplemented with 10% horse serum (ATCC; Cat No: 30-2040), and 120U/mL penicillin and 120µg/mL streptomycin. Cells were then harvested and plated into 384 well, tissue culture treated black, clear bottom microwell plates (**ED** Falcon; Cat No: 353962) at a density of 8850 cells/well. Cells were allowed to grow to confluency, at which point each well was inoculated with 5.5×10^3 primed *C. parvum* oocysts (Bunch Grass Farms, Deary, ID) suspended in inoculation medium (RPMI-1640 as above without horse serum). Oocysts were primed for excystation following a previously described protocol [Gut, J. & R.G. Nelson, (1999) J Eukaryotic. Biol, 46(5): 56S-57S]. Briefly, oocysts were centrifuged for 5 minutes at 9,000g and treated for 10 minutes with 10 mM HCS at 37° C. The oocysts were then centrifuged again, the supernatant discarded, and treated with a 2 mM solution of sodium taurocholate (Sigma-Aldrich, St Louis, MO) in PBS with Ca^{2+} and Mg^{2+} . The suspension was incubated for 10 minutes at 16° C and then diluted in inoculation medium and added to each well. Infected cells were incubated at 37° C for three hours, at which point an equal volume of growth medium containing 20% horse serum (total serum concentration of 10%) and each experimental compound (see below) was added. The infected cells were then incubated for 48 hours.

Experimental Compounds:

High-throughput Screening (HTS):

The NIH Clinical Collection (NCC) libraries (Evotec, San Francisco, **CA**) were received as 10 mM compounds in DMSO. The compounds were further diluted by a factor of 1:14 in 100% DMSO and arrayed in duplicate into the center 308 wells of V-bottom polypropylene 384 well source plates (Whatman; Cat no: 7701-5101) which were stored at -80°C until use. A 384 solid pin blot multi-blot replicator (V&P Scientific, San Diego; Cat no: **VP386**) was used to transfer approximately 120 nL of compound from the source plate to the assay plate for a final concentration of approximately 10 µM of each compound. Controls included EC₉₀ concentrations of nitazoxanide in various locations to ensure consistent loading across the pin transfer tool, as well as DMSO only wells (vehicle). For the testing, the cells were prepared and infected, and a determined amount of a compound for testing was added to the infected cell preparation and incubated up to 48 hours. For NTZ, IC₅₀ dose (50% inhibitory concentration) was determined to be 3.5 micromolar, which is equal to 1.1 micrograms/milliliter concentration. The IC₉₀ dose for NTZ (90% inhibitory), was

determined to be 7.2 micromolar, which is equal to 2.2 micrograms/milliliter. The EC₅₀ dose for NTZ is a sub-inhibitory concentration of NTZ. The EC₅₀ dose used was the concentration that inhibited 10% of the parasites was 2.2 micromolar NTZ, which was equal to a concentration of NTZ of 0.676 micrograms/milliliter in the test well.

5

Secondary Assays:

Compounds were selectively picked from the NCC plates ("**Confirmatory**" IC₅₀ curves) or refined ("refined" IC₅₀ curves), diluted in 100% **DMSO** and further diluted in growth medium with 20% **serum** (total **[DMSO]** never exceeded **0.5%**) and **immediately** added to infected **monolayers**.

10 **Cenvastatin** (SML0005), **simvastatin** (S6196), fluvastatin (SML0038), mevastatin (M2537), lovastatin (M2I47), and pravastatin (P4498) were purchased from Sigma Aldrich, St. Louis, MO) and itavastatin (SRP0239Qi) was purchased from Sequoia Research Products (Pangbourne, UK). Compound purity was assessed using liquid chromatography/mass spectrometry (LC/MS). LC/MS mass spectra were obtained on an Agilent 6120 single **quadrupole** mass spectrometer with Agilent
15 1200 HPLC system with a Zorbax Eclipse XDB-C8 reversed-phase column (4.6 x 50 mm, 3.5 μ m). Samples were eluted using a linear gradient from 95:5 to 5:95 water/0.1% formic acid-acetonitrile/0.1% formic acid at a flow of 1 mL/min, over a 6 min period.

Immunofluorescence Assay:

20 Following 48 hour incubation, the monolayers were washed three times with PBS containing **20mM** D-Galactose with an **EL406** automated plate washer (Biotek, Winooski, **Vermont**) fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature, and then permeabilized with **0.25%** triton-X in PBS for 10 minutes at 37°C. The monolayers were then washed three times in PBS with 0.1% Tween-20, and blocked with 4% bovine serum albumin (BSA) for two hours. *Vicia*
25 *vittata* lectin (VVL, Vector Labs, Burlingame, CA, Cat no: L-1230) and streptavidin-conjugated Alexa fluor 568 Invitrogen Life Technologies, Grand Island, NY; Cat no: S11226) were diluted in 1% BSA/PBS with 0.1% Tween-20 (optimal concentrations were determined empirically for each application), added to the wells containing fixed cells and 4% BSA, and allowed to incubate for one hour. 15 μ L of 0.29 μ M Hoechst 33258 (Anaspec, Fremont **CA**; Cat no: 83219) in water was
30 added, and the plates were incubated for an additional 15 minutes.

The monolayers were then washed 5 times with PBS with 0.1% tween and imaged utilizing a Nikon Eclipse 17 **epifluorescent** microscope with motorized stage. Images were captured with an

EXi Blue Fluorescent Microscopy Camera (QImaging, Surrey, British Columbia). *NIS-Elements Advanced Research* software (Nikon USA, Melville, NY) was used to program the instrument to automatically focus on the center of each of the middle 308 wells of the plate, and then acquire a three-hy-three 20x-field image around the focal point (corresponding to approximately 13% of the surface area of the well). The images were then exported as .tif files and imported into ImageJ where the batch process function was used to execute a macro to segment and enumerate the hoechst-stained nuclei (Appendix A) and streptavidin-labeled parasites (Appendix B).

Data Handling and Statistical Analysis:

The ImageJ output was imported into excel for data organization and cleaning. Additional statistical analysis and construction of charts and graphs was conducted in *GraphPad Prism* version 5.0 (GraphPad Software, San Diego, CA).

High-throughput Screening (HTS):

The robustness of the HTS assay was determined by calculating Z' scores as previously described [Zhang, J.H., (1999) *J. Biomolecular Screening*, 4(2). p. 67-73]. (equation 1).

$$(1) \quad Z' = 1 - \frac{3 \cdot \sigma \text{ infected wells} + 3 \cdot \sigma \text{ uninfected wells}}{|\mu \text{ infected wells} - \mu \text{ uninfected wells}|}$$

The percent inhibition was calculated for each experimental compound (equation 2), and compounds that exhibited $\geq 80\%$ in one of two biological replicates were picked for confirmation.

$$(2) \quad \% \text{ inhibition} = \frac{\# \text{ parasites DMSO treated wells} - \# \text{ parasites experimental compound treated wells}}{\# \text{ parasites DMSO treated wells}}$$

Confirmatory EC₅₀ Curves (also referred to herein as IC₅₀ curves):

The selectively picked compounds were sent to the NERCE/BEID for evaluation of purity and confirmation of identity by liquid chromatography/mass spectrometry (LC/MS). All compounds were then retested at 0.12, 0.37, 1.1, 3.3, and 10 μM ($n=14$ for each concentration) for the generation of IC₅₀ curves. In experiments utilizing isopentenyl pyrophosphate (IPP), IPP was obtained as the dry triammonium salt (Isoprenoids.com; Tampa, FL). Stocks were reconstituted in

inoculation medium at 8.12 mM, and then further diluted into assay plates to the indicated concentration.

All curves were generated using the *lag/inhibitor? vs. response- variable slope* equation in *Graphpad Prism* (equation 3) with the bottom constraint set equal to 0

5

$$(3) \quad Y \sim \text{Bottom} + \frac{\{ \text{Top} - \text{Bottom} \}}{(1 + 10^{((\text{LogEC}_{50} - X) * \text{HillSlope}))}}$$

Refined EC₅₀ Curves:

Compounds were purchased from Sigma Aldrich (St Louis, MO) or Sequoia Research Products (United Kingdom), reconstituted in .100% DMSO and analyzed by LC/MS. EC₅₀ curves were then generated by diluting the compounds to 0.03x, 0.1x, 0.33x, 0.8x, 0.9x, 1.0x, 1.1x, 3x, 9x (where possible), and 30x (where possible) the EC₅₀ determined in the confirmatory EC₅₀ experiments (n= 1.1 for each concentration). Three wells were left uninfected but treated with the corresponding concentration of compound to assess for background staining. The average signal of all of these wells was calculated (average background) and subtracted from the signal in each experimental well to yield a background corrected parasite count for each well. Additionally, 22 wells per plate were infected and treated with DMSO to assess infection quality. EC₅₀ curves were then generated using equation 3.

Cell Toxicity Assays:

HCT-8 cells were grown to confluence in 384 well plates as described above. Growth medium was then replaced with growth medium spiked with 5 μM, 10 μM, 20 μM, 50 μM, or 100 μM (n=> replicates per concentration) of experimental compound, and plates were incubated for 48 hours. Following incubation, the outer 76 wells of the plate were treated with trypsin (blank wells), and medium was replaced in the inner 308 wells with 20 μL of fresh growth medium. The *CeliTiter AQ_{neo}* (Promega, Madison, WI; cat no: TB169) was then used to assess cell viability, according to manufacturer's instructions. Briefly, 5 μL of MTS solution was added to each well and plates were incubated for 70 minutes, at which point absorbance was read at 490 nm with a *PowerWave* microtiter plate reader (Biotek, Wmooski, VT). The average absorbance of all blank wells was subtracted from the absorbance of experimental wells, and the IC₅₀ doses were calculated in *Graphpad Prism* (equation 3) with the bottom constraint set equal to 0.

30

Bioinformatic Analysis:

Evidence for the presence or absence of *Cryptosporidium* metabolic pathways was collected using BLAST; all alignments were based on amino acid sequence using default parameters [Altschul SF, et al., (1997) Gapped BLAST Nucleic Acids Res 25: 3389-3402.66]. A number of representative proteins were chosen from each pathway using KEGG [Ogata H, et al., (1999) Nucleic Acids Res 27: 29-34] and ApiCyc annotation [Aurrecochea C, et al, (2010) Nucleic Acids Res 38: D415-419]. *Plasmodium falciparum* sequences were used for methylerythritol phosphate pathway analysis, and *Homo sapiens* sequences for all other pathways. The bioinformatics analysis extended the results to *C. hominis*.

Appendix A: ImageJ macro for nucleus separation and enumeration

```
run("8-bit");
run("Subtract Background...", "rolling=50");
run("Unsharp Mask...", "radius=2 mask=0.70");
//run("Threshold...");
setAutoThreshold("Huang dark");
run("Convert to Mask");
run("Watershed");
run("Set Measurements...", "area mean min centroid center perimeter integrated median kurtosis
area_fraction stack limit display redirect=None decimal=2"),
run("Analyze Particles...", "size=159.5125-Infinity circularity=0.00-1.00 show=Nothing display
clear summarize add").
```

Appendix B: ImageJ macro for parasite separation and enumeration

```
run("Subtract Background. ...", "rolling=3");
setAutoThreshold("Huang dark");
//run("Threshold...");
setThreshold(1000, 16383);
run("Convert to Mask");
run("FillHoles");
run("Watershed");
```

run("Analyze **Particles...**", "size≈16.48282906-1 85.4936891 circularity≈0.00-1.00 show-Nothing display clear summarize add").

Example 2

The assay methods **described** in Example 1 were **validated** using **nitazoxanide** **contact** with cultured cells infected with *Cryptosporidium*. Results were determined for infection rates, percent of *Cryptosporidium* inhibition **by nitazoxanide** treatment. Results of several **validity** tests are shown in Figs. 1-3 and confirmed the validity of the assay methods.

Example 3

Two preliminary screens were performed using methods as set forth in Example 1.
(A.) A straight screen was performed of the NIH Clinical Collection of 727 compounds with history of use in human trials and known safety profiles; many of the compounds have FDA approval, were screened at ≈10 μM. **Screening** methods were as set forth in Example 1. Overall results are shown in Fig. 2, which provides data from one of two screening replicates, and shows that eleven compound hits demonstrated over 90% *Cryptosporidium* inhibition.

Results of the straight screen of 727 compounds resulted in 11 hits with ≥ 90% inhibition of the cultured cells. The hit rate was approximately 1.5% of the compounds tested. IC₅₀ and host cell toxicity curves for the screening hits showed >80% inhibition in the primary screen. Figs. 4 and 5 show tables of results and follow-up results of screening hits.

Screening hits were defined as >8Q% inhibition in either of two screening replicates. Hit compounds were picked from the compound library and used to generate approximate IC₅₀ data. Confirmed hits were then purchased from a different vendor and used to generate refined IC₅₀ data. TC₅₀ is the 50% toxic concentration based on the cellular dehydrogenase activity assay, (see above, **CedTiterAqueous**, Promega, Madison, Wf).

Follow-up screening was performed on hits identified in the straight screening. Results for tested compounds are shown in Figs. 6-26.

Additional Information and Results from Examples 1-3

I. Drug classes and currently approved uses

a. Summary of Screening and Results: Seven hundred twenty seven (727) compounds in the NIH Clinical Collections 1 and 2 were screened as duplicate, as described in the methods section above.

Of the screened compounds, 24 compounds were identified as potential inhibitors based on occurrence of $>\sim 80\%$ inhibition in at least one of the screening replicates. The remaining 703 compounds were negative (i.e., showed no inhibition) by these criteria. The 24 screening hits and, in the cases of statins and tetracyclines, related compounds were pulled from the screening libraries and retested to **confirm** inhibition. Twenty of the twenty four (20/24) **of the** re-tested compounds inhibited *C. parvum* growth and IC50 curves (termed rough IC50) were determined. Fifteen (15) of these confirmed inhibitors were re-purchased for follow-up testing. **IC50** curves and host cell toxicity data were then generated for each of the re-purchased compounds (termed refined IC50 and **TC50** respectively). In the cases of statins and tetracyclines, testing of additional related compounds enabled generation of structure activity relationship (SAR) data.

Identities of screening hits, summary of currently approved uses, and anti-fxirasic action:

Screening hits fell into several broad categories: 1) anti-metabolites; 2) tetracyclines; 3) statins (HMG-CoA-reductase inhibitors); 4) anti-microbial detergents; and 5) miscellaneous. The hits included compounds that showed inhibitory activity against *Cryptosporidium* in assays performed as set forth in Example 1.

(1) Anti-metabolites:

Anti-metabolites that were confirmed screening hits included: Floxuridine, **6-azauridine**, carmofur, and 5-fluorouracil.

Anti-metabolites that were tested (in the library) but for which no inhibitory activity was detected at the doses tested included: 5-fluorocytosine, **(2,3)-dideoxycytidine**, **AZT**, 5-Azacytidine, D4T, 3TC, 3'-deoxyadenosine, DDI, penciclovir, azathioprine, mercaptopurine, and fludarabine.

All of the anti metabolites that inhibited *C. parvum* growth were pyrimidine analogues, and none of the 6 purine analogues tested showed inhibition. These data suggest that mechanisms of pyrimidine acquisition and phosphorylation may be targetable for treatment of cryptosporidiosis.

(2) Statins:

Mevastatin was identified as a confirmed screening hit. Because these drugs have an outstanding safety profile, Structure-activity relationship (SAR) studies were conducted using a number of statins present in the screening library and re-purchased statins. Fig. 27 shows results of statins tested. Figs. 28-33 show IC50 and toxicity results for tested statins.

SAR studies using repurchased statins identified itavastatin and cerivastatin as the most potent statin inhibitors of *C. parvum* growth. IC₅₀s for the statins tested ranged from 0.27 micromolar to non-inhibitory, supporting the specificity of the findings.

The statins inhibit mammalian cholesterol synthesis by inhibiting the enzyme HMG-CoA-reductase, which is the rate limiting step in cholesterol biosynthesis. Potency of the statins tested against *C. parvum* correlated with published potency against human HMG-CoA-reductase, suggested that the anti-parasitic effect is via inhibition of the host enzyme. Apicomplexan parasites generally synthesize isoprenoid precursors using a non-mevalonate dependent mechanism and enzymes encoded within a unique, DNA containing organelle called the apicoplast. It has been shown that this is the primary function of the apicoplast in malaria parasites, and that, if malaria parasites lose their apicoplast, then isopentyl pyrophosphate (which is an intermediate product cholesterol synthesis (downstream of the role of HMG-CoA reductase) rescues their ability to grow. On the other hand, *Cryptosporidium* species are apicomplexan parasites that do not have an apicoplast. Results support a conclusion that the *Cryptosporidium* species may be completely-dependent on the host for synthesis of isoprenoid precursors, and inhibition of this process with HMG-CoA-reductase inhibitors inhibits *Cryptosporidium* growth for this reason. Similarly, the results suggest that a drug that interferes with host cholesterol synthesis would be useful to inhibit *Cryptosporidium* growth.

(3) Tetracyclines:

Tetracyclines that were screening hits included: doxycycline and minocycline.

SAR studies done after repurchasing these and other tetracyclines present in the screening libraries demonstrated that Demeclocycline, tetracycline, and oxytetracycline also have inhibitory activity (with varying potency). Rolitetracycline did not demonstrate inhibitory activity under the assay conditions and was not purchased for follow-up. Fig. 34 shows results of tetracyclines tested. Figs. 35-40 show IC₅₀ and toxicity results for tested tetracyclines.

(4) Anti-microbial detergents:

Both hexachlorophene and chloroxine were found to have potent activity against *C. parvum* with selective toxicity for the parasite rather than the host cells. These agents may be used for decontamination of environmental surfaces, substrates, etc.

(5) Miscellaneous screening hits:

The following compounds were determined to have anti-cryptosporidal activity included:

Digoxin.

Anthracyclines: doxorubicin, idarubicin, and daunorubicin.

5 Docetaxol - a microtubule inhibitor. Results of the studies described herein support targeting tubulin for treatment of *Cryptosporidium*.

Homoharringtonine.

Oligomycin C.

10 Tegaserod - a partial agonist of the serotonin receptors 5-HT₄ and 5-HT₃. It was previously approved for treatment of irritable **bowel** syndrome and other gastrointestinal **motility** disorders. It was **withdrawn** from the market because of cardiac toxicity, but a number of other compounds targeting these serotonin receptors are in development, and the data from the experiments outlined above suggest that these or tegaserod for use in treatments for *Cryptosporidium*.

15 Indomethacin - a non-steroidal anti-inflammatory agent that inhibits cyclooxygenase.

Glipizide - a sulfa drug that affects glucose by targeting an ATP-gated K⁺ channel.

Triptolide - a traditional Chinese medicine that induces intracellular calcium release via the polycystin 2 calcium channel.

20 Previously, advances in the field of drug development for cryptosporidiosis have been very limited. The lack of methods to continuously and reliably culture *Cryptosporidium* species in the laboratory [Karanis P & Aidarybi HM (2011) Int J Parasitol 41: 1231-1242] and the parasite's genetic intractability limited the utility of target-based approaches (which require genetic manipulation to validate presumed drug targets) for discovery of anti-cryptosporidials. Cell-based
25 high-throughput screening (HTS) has now been used to facilitate development of therapeutics for cryptosporidiosis, making it a more effective approach to drug repurposing than target based screening. As described herein, a cell-based HTS to identify novel repurposing candidates for the treatment of cryptosporidiosis has been developed and used to identify candidate compounds to treat **Cryptosporidia**. Assays have been used to screen the NIH Clinical Collections (NCC), two
30 libraries of FDA approved drugs and drug-like molecules that have been used in human clinical trials. The assay has now been developed and validated and used to identify lead repurposing candidates for the treatment of cryptosporidiosis. In addition, assays described herein have been

used to obtain *in vitro* data which has been used in conjunction with bioinformatics data to support a model for a potential mechanism of action for β -hydroxy- β -methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors against *C. parvum*.

Screening studies yielded **21 confirmed** inhibitors of *C. parvum* growth, with a hit confirmation rate of 87.5%. Through additional follow up including structure activity relationship studies, the *HMG-CoA reductase* inhibitor itavastatin was identified as a promising **repurposing** candidate for treatment **of cryptosporidiosis**. Itavastatin was potent against *C. parvum* (**IC₅₀**= 0.62 μ M (95% **CX** = 0.45 - **0.86**)) and exhibited no host cell toxicity at concentrations up to 100 μ M. Analysis of the *Cryptosporidium* genome databases indicated that the Cryptosporidia lack all known enzymes required for synthesis of isoprenoid precursors, and itavastatin-induced growth inhibition of *C. parvum* was specifically attenuated by the addition of exogenous isopentenyl pyrophosphate. These data suggested that ***Cryptosporidium*** species are reliant on host cell synthesis of isoprenoid precursors, which is blocked by statin drugs. The studies included the first cell-based high throughput screen in *Cryptosporidium*, which identified itavastatin (among others) as a promising drug repurposing lead. The studies also validated the host mevalonate dependent pathway for isoprenoid synthesis as a target for drugs to treat cryptosporidiosis.

A sensitive and specific screening assay for C. parvum growth:

A robust, sensitive, and specific cell-based assay in a 384 well **microliter** plate **format** was developed. Briefly, excystation of *C. parvum* oocysts was induced and they were inoculated onto a confluent monolayer of ileocecal adenocarcinoma (HCT-8) cells, and then incubated them for 48 hours. After staining of host cell nuclei and parasites, large (3x3 tiled) low magnification images were acquired using automated epifluorescence microscopy, and then parasites and host cell nuclei were enumerated with *Imaged utilizing* macros developed **in-house** (Appendix A and B). This workflow provided a straightforward means to detect anti-cryptosporidial activity and potential host cell toxicity of experimental compounds.

The number of *C. parvum* oocysts inoculated onto a confluent monolayer (multiplicity of infection; MOI) correlated strongly with the number of parasites detected following a 48 hour incubation period ($R^2 = 0.85$) (Figure 1A). The half maximal inhibitory concentration (IC₅₀) of nitazoxanide (3.7 μ M; 95% C.I. = 3.5 - 3.8) (Figure 1B) determined by this assay was in agreement with previously published findings utilizing quantitative RT-PCR [Cai X, et al., (2005) Antimicrob Agents Chemother 49: 4437-4442. 23] and microscopy [Gargasa G, et al. (2000) J Antimicrob

Chemother 46: 57-60. 24] to detect *C. parvum* growth *in vitro*. Moreover, *C. parvum* growth kinetics observed in the assay matched those previously described for *in vitro* culture systems [Arrowood MJ (2002) Clin Microbiol. Rev 15: 390-400].

Despite low infection efficiency (the number of intracellular parasites detected at 3 hours is approximately one log less than the original inoculum), exponential parasitic growth was observed *in* infected wells that were fixed and stained at 3, 12, 24, and 48 hours following infection, ($R^2 = 0.80$) with a calculated doubling time between 15 and 17 hours (Figure 1C). These data illustrate the sensitivity and specificity of this **miniaturized**, semi-automated assay to measure *C. parvum* growth *in vitro*.

Assay was sufficiently robust for high throughput screening applications:

Assays were run to determine whether the assay could be used to specifically identify infected wells treated with a known *C. parvum* inhibitor from a larger group of infected, untreated wells in order to evaluate the utility of the assay in a higher throughput context. The assay readily distinguished infected wells that were randomly selected and treated with an inhibitory concentration of nitazoxanide (41 μ M) ($n = 17$) 3 hours following infection from those that were treated with DMSO vehicle only ($n = 269$) (Fig. 2A). The screening window coefficient known as the Z-score was used to assess the robustness of the assay as a HTS. A Z-score ≥ 0.5 is considered ideal for high throughput applications; however successful screens have been reported that employed assays with Z-scores as low as 0.2 [Zhang JH (1999) J Biomol Screen 4: 67-73]. The Z'-score, a permutation of the Z-score in which only control data are considered, assesses the quality of the assay itself [Zhang JH (1999) J Biomol Screen 4: 67-73, Zhang J-H, et al., (2000) J Comb Chem 2: 258-265]. The Z'-score was calculated for the assay for three biological replicates (performed on different days) and it was determined to be between 0.27 - 0.41 (Fig. 2B). When combined with the fact that the current workflow facilitates testing of >1700 compounds per week in duplicate, these data support a finding that the assay is well suited to conduct high throughput screening.

Screening of a compound library:

After validating the utility of the assay as a HTS, a screen of the NCI Clinical Collections libraries was conducted. The libraries are comprised of 727 FDA approved drugs or drug-like compounds with a history of use in human clinical trials. Each compound was screened at approximately 10 μ M in two separate biological replicates (performed on separate days) with good

agreement between screening replicates. Fig. 3 shows screening results as a scatter plot (Fig. 3A) and as a frequency distribution of percent inhibition by each compound (Fig. 3B) for screening replicate number one. Compound names, simplified molecular input line entry specification (SMILES) strings, PubChem identification numbers, supplier information, compound purity (when available, provided by supplier), and percent inhibition for both screening replicates were obtained for each of the **compounds** in the library. **Compounds** that exhibited $\geq 80\%$ inhibition in **at least** one replicate were selected for follow-up testing. Twenty four compounds met this definition, and these were recovered from the original library source plates, assessed **for** purity with LC/MS, and IC50 curves were generated **by** testing each drug **in** the growth assay at 5 different concentrations (0.1, 0.37, 1.1, 3.3, and 10 μM ; $n=14$ wells for each dose). Nineteen compounds had **confirmatory** IC50 values $< 10 \mu\text{M}$, while 2 compounds were inhibitory at all doses tested, and 3 compounds did not show any inhibition (see Fig. 4 for hit result information table). Overall, the assay exhibited a 3.3% hit rate with a true positive rate of 87.5% when a hit limit of 80% inhibition was applied.

Secondary assays:

Hit prioritization and Confirmation:

An ideal drug candidate to repurpose for the treatment of cryptosporidiosis would be safe enough for administration in a pediatric population, exhibit potent and selective toxicity against the Cryptosporidia, be formulated for oral administration, be available at low cost, and have FDA approval. This product profile was used as part of a framework to inform further prioritization of candidate compounds based on the preliminary data, as well as information compiled from the *Drugbank* database [Knox C, et al., (2011) *Nucleic Acids Res* 39: D1035-D1041; Gaulton A, et al., (2012)], *ChEMBL database* [Nucleic Acids Res 40: D1100-D1107], additional literature sources, and clinical experience. Additionally, careful review of the preliminary screening data revealed that potent inhibitors of *C. parvum* with well characterized mechanisms of action had been identified, and that the **compounds** may prove useful as chemical probes to help elucidate *Cryptosporidium* biology. Based on these criteria, 16 compounds were identified for additional follow up, which were repurchased, determined to be $\geq 85\%$ pure by LC/MS (with the exception of tetracycline (purity $< 50\%$), and hexachlorophene, oligomycin C, and floxuridine, which could not be tested by LC/MS), and used to generate IC50 curves. These curves were generated using variable concentrations of each compound based on the preliminary IC50 values generated with the compounds recovered from the original source plates in order to saturate the curve around the

suspected IC₅₀. Candidiaie compounds that did not demonstrate host cell toxicity as evidenced by a significant decrease in nuclear counts were further assessed for potential toxicity using a commercially available assay of cellular dehydrogenase activity (The *CellTiter AQueous*, Promega, WI). These findings are summarized in table in Fig. 5.

5

Follow up assays:

The screen identified representative chemical scaffolds from three chemical families that were considered to have therapeutic potential against cryptosporidiosis.

(A) The screen identified fioxuridine, a pyrimidine analog nucleoside, was the most potent candidate compound (IC₅₀ = 0.0063 μ M (95% C.I. = 0.0020 - 0.020)), while eaxmofur and 5-fluorouracil (analogs of fioxuridine), as well as 6-azauridine (another pyrimidine analog nucleoside) exhibited potency in the single micromolar range, suggesting that pyrimidine analogs may be a rich source of anti-eryptosporidial compounds.

(2) The screen also identified two tetracycline analogs (doxycycline and minocycline) a family of drugs that has demonstrated activity against other medically important apicomplexan parasites [Andersen SL, et al., (1998) *Clin Infect Dis* 26: 146-150, Chang HR, et al. (1990) *Antimicrobial agents and chemotherapy* 34: 775-780; Dahl EL & Rosenthal PJ (2007) *Antimicrob Agents Chemother* 51: 3485-3490; Dahl EL, et al., (2006) *Antimicrob Agents Chemother* 50: 3124-3131; Pang LW, et al., (1987) *Lancet* 1: 1161-1164; and Pradmes B, et al, (2001) *Antimicrob Agents Chemother* 45: 1746-1750].

(3) The results revealed that mevastatin, the prototypical p-hydroxy-p-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor (statin), has single micromolar efficacy *in vitro* against *C. parvum*. The statins fit well into the product profile, and numerous clinically utilized chemical analogs with excellent safety profiles already exist. Consequently, although the pyrimidine analogs and tetracyclines appear to have potential as therapeutics, follow-up studies were focused around the chemical scaffold represented by mevastatin.

HMG-CoA reductase inhibitors:

The statins are a widely used family of competitive *HMG-CoA reductase* inhibitors, the rate-limiting enzyme in the mevalonate-dependent pathway of isoprenoid biosynthesis. While mevastatin was never developed as a therapeutic, it provided the chemical scaffold for additional structure activity relationship (SAR) studies that gave rise to numerous clinically useful statins

[Endo A (1992) J Lipid Res 33: 1569-1582]. Confirmatory testing with mevastatin demonstrated **inhibition** of *C. parvum* growth *in vitro* with an **IC₅₀** $\approx$ 5.1 μ M (**95% C.I.** $\approx$ 3.7 - 7.0), a **TC₅₀** >100 μ M, and a **TI** > 20. Additionally, a number of **additional** statins in the NCC library came close to, but failed to meet the hit limit of 80% inhibition. These data, combined with mevastatin's fruitful history as a chemical scaffold and the outstanding safety profile of many of the clinically utilized statins in the general population [Tonelli M, et al., (2011) CMAJ 183:E1189-E1202], prompted follow up SAR studies to be conducted utilizing additional statin drugs. Analog compounds were purchased, **determined** to have >85% purity by LC/MS, and used to determine the **IC₅₀** against *C. parvum* and the **TC₅₀** on confluent HCT-S monolayers. Cerivastatin (**IC₅₀** = 0.27 μ M (95% C.I. = 0.21 - 0.36), **TC₅₀** > 100, **TI** > 250) was the most potent statin derivative tested. Itavastatin (also known as pravastatin) (**IC₅₀** = 0.62 μ M (95% **C.I.** = 0.45 - 0.86), **TC₅₀** > 100, **TI** > 167) was selected for examination in additional follow up studies.

Itavastatin reduced isopentenyl pyrophosphate pools:

HMG-CoA reductase is the rate limiting step in the mevalonic acid (MVA) pathway that produces isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). These 5 carbon molecules provide the building blocks for isoprenoid synthesis by a family of enzymes known as prenyltransferases [Liang **PH**, et al., (2002) Eur J Biochem 269: 3339-3354]. It was reasoned that the addition of exogenous IPP to the growth medium might relieve the inhibition of *C. parvum* growth by itavastatin (a specific inhibitor of the MVA pathway). Consistent with this, the addition of 50 μ M IPP to infected **monolayers** treated with itavastatin increased the **IC₅₀** greater than 5-fold (**IC₅₀** itavastatin + IPP $\approx$ 2.3 μ M (95% C.I. $\approx$ 0.7 - 7.5) vs. **IC₅₀** itavastatin only $\approx$ 0.44 μ M (95% **C.I.** = 0.31 - 0.62), (see Fig. 41A). Moreover, the ability of IPP to rescue *C. parvum* growth was specific to itavastatin, as the **IC₅₀** did not change in infected monolayers treated with nitazoxanide and up to 200 μ M IPP (**IC₅₀** NTZ + IPP = 3.2 μ M (95% **C.I.** = 2.6 - 3.9) vs. **IC₅₀** NTZ only = 2.9 μ M (95% C.I. = 2.4 - 3.5), (see Fig. 41B). These data suggested that itavastatin inhibited *Cryptosporidium* growth, at least in part, through inhibition of human *HMG-CoA reductase*, rather than off-target effects of the drug.

Six of seven p-hydroxy-p-methylglutaryl-coenzyme **A (HMG-CoA)** reductase inhibitors tested had 50% **inhibitory** concentrations (**IC₅₀s**) under 10 μ M against *Cryptosporidium parvum*. The most potent FDA approved HMG-CoA reductase inhibitor tested was itavastatin (**IC₅₀** $\approx$ 0.62 μ M). Furthermore, addition of exogenous isopentenyl pyrophosphate (**IPP**) (a downstream product

of HMG-CoA reductase in mammalian **cells**) reduced the effects of itavastatin on *C. parvum* growth (addition of IPP resulted in a > 5-fold increase in the IC_{50} , but did not affect the IC_{50} of the non-statin *C. parvum* inhibitor nitazoxanide). The ability of exogenous IPP to reduce the effect of statin drugs on *Cryptosporidium* growth indicates that the growth effect is via specific inhibition of host HMG-CoA reductase, rather than an off-target of the statin drugs, and that IPP is an essential metabolite for *Cryptosporidium* parasites. This is consistent with the bioinformatics analysis of the *C. parvum* and *C. hominis* genomes, which revealed strong evidence for the existence of multiple biochemical pathways in the Cryptosporidia that utilize isoprenoids derived from IPP and its isomer dimethylallyl pyrophosphate (DMAPP). However, like other apicomplexan parasites, there is no evidence to suggest that either species is capable of IPP or DMAPP synthesis. Thus, the Cryptosporidia appear to be completely dependent on the host for synthesis of these precursors. Collectively, the data validate the statins as promising drug **repurposing** leads for treatment of cryptosporidiosis in humans and animals, and validate the host mevalonic acid pathway of isoprenoid synthesis as a drug target for treatment of *Cryptosporidium* parasites.

The experimental results support the statins as promising leads, and also validate the host mevalonic acid pathway (which is inhibited by the statins) as a target for treatment of cryptosporidiosis. The results suggest that a drug that interferes with host synthesis of the isoprenoid precursors isopentenyl pyrophosphate and dimethylallyl pyrophosphate, or parasite acquisition of these precursors may be used to inhibit *Cryptosporidium* growth.

Example 4

A synergy screen was also performed. The testing included contacting the cultured cells with compounds from the NIH Clinical Collection as described in Example 1, with the addition of also contacting the cells with nitazoxanide at EC10 dose, in combination with each of the NIH clinical **collection** compounds. The EC 10 dose for NTZ is a **sub-inhibitory** concentration of NTZ. The EC 10 dose used was the concentration that inhibited 10% of the parasites (2.2 micromolar NTZ), which was equal to a concentration of NTZ of 0.676 **micrograms/ml** **1H**lter in the test **well**.

All 727 of the library compounds were tested alone and in combination with NTZ. Results in Table 1 show the percent inhibition of *C. parvum* growth when each compound was administered to the cells alone (plain screen) or was administered in the presence of a sub-inhibitory concentration of NTZ (2.2 μ M). Test compounds were used at 10 μ M. The results indicated a synergistic effect of the combination of the compounds with the NTZ administration.

Table 1. Examples of identified compounds with synergistic activity against *C. parvum* when used in combination with nitazoxanide

Drug	Plain screen % inhibition (mean $\pm$ SD, n=2)	NTZ synergy screen % inhibition (mean $\pm$ SD, n=2)
carvedilol	11 $\pm$ 9%	89 $\pm$ 5%
ketoconazole	-16 $\pm$ 36%	88 $\pm$ 6%
mefloquine	19 $\pm$ 15%	89 $\pm$ 8%
Imodium	18 $\pm$ 3%	86 $\pm$ 6%
aripiprazole	-3 $\pm$ 27%	88 $\pm$ 4%

5

For these experiments, and for additional assays described in the Examples, the IC₅₀ dose for NTZ (50% inhibitory concentration) was determined to be 3.5 micromolar, which is equal to 1.1 micrograms/milliliter. The IC₉₀ dose for NTZ (90% inhibitory), was determined to be 7.2 micromolar, which is equal to 2.2 micrograms/milliliter.

10

Example 5

Assays were performed as follows: primed oocysts were inoculated onto confluent Human ileocecal adenocarcinoma (HCT-8) cell monolayers and incubated for approximately 48 hours. After the incubation, the cells were fixed and parasites were stained with biotinylated *Vicia villosa* lectin (VVL) and streptavidin Alexa Fluor 568 to permit visualization of the parasites. The nuclei were then counterstained using standard Hoechst **staining** methods. Following the staining procedures, imaging, including automated imaging, was performed and the images analyzed.

15

Images of the stained parasites and nuclei were analyzed as described in Example 1 using the Appendix A macro for nucleus separation and enumeration, which provided a cell count. Analysis as described in Example 1 using the Appendix B macro for parasite separation and enumeration was also performed, which provided a parasite count. (see Fig. 42).

20

The assay was validated using procedures set forth in Example 2, with results of some validation assays shown in Figure 1.

25 Example 6

Testing was performed to examine the basis for the failure of nitazoxanide (NTZ) administration as a treatment for *Cryptosporidium* in immunocompromised subjects, which was possibly the result of the use of NTZ as a static drug for *Cryptosporidium* species. A goal of the study was to determine whether it would be possible to improve the efficacy of NTZ using synergistic drug combinations -- for example, by administering one or more drugs in addition to NTZ to increase the treatment efficacy. Results are shown in Fig. 43.

Elimination of parasite DNA unambiguously indicates a reduction in the abundance of viable parasites. Therefore, **experiments** took advantage of the fact that the DNA of *C. parvum* was rapidly degraded after parasites were killed (>90% in 12 hours) (see Fig. 43 (heat-killed)), and used qPCR to monitor *C. parvum* DNA abundance following drug exposure to determine if nitazoxanide treatment failure might be due to static rather than cidal anti-parasitic effect. **HCT-8** cells **grown** in 384-well plates were infected, DMSO or an **IC90** dose of NTZ was added after 24 hours, and qPCR was used to monitor the relative abundance of *C. parvum* and HCT-8 DNA for an additional 48 hours (Fig. 43). The rate of DNA degradation for heat-killed parasites added to uninfected monolayers was also assayed. As expected, *C. parvum* DNA abundance increased in the untreated wells (DMSO control), and the DNA of killed parasites was rapidly degraded. However, NTZ treatment with the IC90 dose did not reduce the quantity of *C. parvum* DNA during 48 hours of incubation. The basis for NTZ failure in this assay may have been because NTZ at this concentration and duration was static or only slowly cidal for *C. parvum*, and/or that some parasites in the population have pre-existing NTZ resistance (i.e., some are killed, but others continue to replicate). Also, the time resolution of this assay was limited by the kinetics of DNA degradation, which may vary with the mechanism of cell death. Unlike NTZ, however, any drug or drug combination that results in elimination of *C. parvum* in this cell-based assay might be capable of eliminating *Cryptosporidium* *in vivo* in the absence of effective host immunity.

Although several embodiments of the present invention have been described and illustrated herein, those of ordinary skill in the art **will** readily envision a variety of other means **and/or** structures for performing the functions and/or obtaining the results and/or one or more of the advantages described herein, and each of such variations and/or modifications is deemed to be within the scope of the present invention. More generally, those skilled in the art will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be exemplars and that the actual parameters, dimensions, materials, and/or configurations will

depend upon the specific application or applications for which the teachings of the present invention is/are used. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of
5 example only and that, within the scope of the appended claims and equivalents thereto; the invention may be practiced otherwise than as specifically described and claimed. The present invention is directed to each individual feature, system, article, material, and/or method described herein. In addition, any combination **of two** or more such features, systems, articles, materials, and/or methods, if such features, systems, articles, materials, **and/or** methods are not mutually
10 inconsistent, is included within the scope of the present invention.

All definitions, as defined and used herein, should be understood **to** control over dictionary definitions, definitions in documents incorporated **by** reference, and/or ordinary meanings of the defined terms.

The indefinite articles "a" and "an," as used herein in the specification and in the claims,
15 unless clearly indicated to the contrary, should be understood to mean "at least one."

The phrase "and/or," as used herein in the specification and in the claims, should be understood to mean "either or both" of the elements so conjoined, i.e., elements that are conjunctively present in some cases and disjunctively present in other cases. Other elements may optionally be present other than the elements specifically identified by the "and/or" clause, whether
20 related or unrelated to those elements specifically identified, unless clearly indicated to the contrary.

All references, patents and patent applications and publications that are cited or referred to in this application are incorporated in their entirety herein by reference.

What is claimed is:

Claims

1. A method of treating an apicomplexan infection in a subject, the method comprising:
administering to a subject having or at risk of having an apicomplexan infection an effective amount
5 of at least one of a statin compound, a tetracycline compound, an anti-metabolite compound, a
digoxin, docetaxol, idarubicin, daunorubicin, doxorubicin, homoharringtonine, olivomycin C,
tegaserod, indomethacin, glipizide, or iriptolide compound to treat the apicomplexan infection.
2. The method of claim 1, wherein the apicomplexan infection is a Cryptosporidiosis infection.
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3. The method of claim 1, wherein the apicomplexan infection is a Babesia infection, a
Plasmodium infection, a Cyclosporiasis infection, an Isosporiasis infection, an Eimeria infection, or
a Theiiera infection.
- 15 4. The method of claim 1, wherein the statin compound is fluvastatin, cerivastatin, mevastatin,
itavastatin, lovastatin, or simvastatin.
5. The method of claim 1, wherein the tetracycline compound is doxyeyeiine, demeciocycline,
tetracycline, oxytetracycline, or minocycline.
20
6. The method of claim 1, wherein the anti-metabolite compound is fioxuridine, 6-a2airridine,
carmofur, or 5-fluorouraeil.
7. The method of claim 1, wherein the subject is free of indications otherwise calling for
25 treatment with the statin compound, the tetracycline compound, the anti-metabolite compound, or
the digoxin, docetaxol, idarubicin, daunorubicin, doxorubicin, homoharringtonine, olivomycin C,
tegaserod, indomethacin, glipizide, or iriptolide compound, administered to treat the apicomplexan
infection.
- 30 8. The method of claim 1, wherein the subject is otherwise free of indications for treatment
with a therapeutic agent comprising fioxuridine, digoxin, chioroxine, docetaxel, idarubicin,
daunorubicin, doxorubicin, homoharringtonine, hexachlorophene, 6-azauridine, oligomycin C,

carmofur, tegaserod, 5-fluorouracil, indomethacin, glipizide, triptolide, tremulacin; doxycycline, minocycline, demeclocycline; tetracycline, **oxytetracycline**, fluvastatin, **cerivastatin**, mevastatin, itavastatin, lovastatin, or simvastatin.

5 9. The method of claim 1, wherein the compound is a statin and the subject is not undergoing treatment with the statin for a condition that is not the apicomplexan infection.

10. The method of claim 1, wherein the compound is a tetracycline and the subject is not undergoing treatment with the tetracycline for a condition that is not the apicomplexan infection.

10

11. The method of claim 1, wherein the compound is an anti-metabolite compound and the subject is not undergoing treatment with the anti-metabolite compound for a condition that is not the apicomplexan infection.

15 12. The method of claim 1, wherein the compound is digoxin, docetaxol, idarubicin, daunorubicin, doxorubicin, homohanningtonine, oligomycin C, tegaserod, indomethacin, glipizide, or triptolide and the subject is not undergoing treatment with the compound for a condition that is not the apicomplexan infection.

20 13. The method of any one of the preceding claims wherein the apicomplexan infection comprises the presence of one or more apicomplexan parasites in or on the subject.

14. The method of claim 13, wherein the apicomplexan parasites comprise vegetative (e.g., sporozoites, merozoites, meronts, etc) and/or infectious apicomplexan parasites (e.g., oocysts).

25

15. The method of claim 1, further comprising administering one or more additional anti-apicomplexan therapeutic agents to the subject.

16. The method of claim 15, wherein the additional anti-apicomplexan therapeutic agent is

30 NTZ.

17. The method of any one of claims 1-16, wherein the compound is administered in a pharmaceutical composition.

18. The method of claim 17, wherein the pharmaceutical composition further comprises a pharmaceutically acceptable carrier.

19. The method of claim 17 or 18, wherein the pharmaceutical composition further comprises a targeting agent.

20. The method of claim 19, wherein the targeting agent is an agent that targets an epithelial tissue.

21. The method of claim 17 or 18, wherein the pharmaceutical composition further comprises an agent to reduce solubility of the compound in the subject's intestine.

22. The method of any one of claims 1-21, wherein the compound is administered orally, sublingually, intravenously, intramuscularly, subcutaneously, intrathecal, transdermal, intraperitoneal, transmucosal, or intranasally.

23. The method of any one of claims 1-22, wherein the compound is administered prophylactically.

24. The method of any one of claims 1-23, further comprising selecting a subject at least in part on the basis that the subject has been exposed to an apicomplexan infection.

25. The method of any one of claims 1-24, wherein the subject has been diagnosed with an apicomplexan infection.

26. The method of claim 2, wherein the *Cryptosporidium* infection of the subject comprises infection by one or more of *C. parvum*, *C. hominis*, *C. canis*, *C. felis*, *C. meleagridis*, or *C. parvum*.

27. The method of any one of claims 1-26, wherein the subject is a vertebrate, and optionally is a mammal.

28. An article of manufacture comprising: one or more containers housing a statin compound, a tetracycline compound, an anti-metabolite compound, a digoxin, docetaxol, idarubicin, daunorubicin, doxorubicin, homohanningionine, olivomycin C, tegaserod, indomethacin, glipizide, triptolide, capecitabine, ketoconazole, mefloquine, Imodium, or axipiprazole compound and instructions for administering the compound to a subject having or at risk of having an apicomplexan infection.

29. The article of manufacture of claim 28, wherein the apicomplexan infection is a Cryptosporidiosis infection.

30. The article of manufacture of claim 28, wherein the apicomplexan infection is a Babesia infection, a Plasmodium infection, a Cyclosporiasis infection, an Isosporiasis infection, an Eimeria infection, or a Theiliana infection.

31. The article of manufacture of claim 28, wherein the statin compound is fluvastatin, cerivastatin, mevastatin, pravastatin, lovastatin, or simvastatin.

32. The article of manufacture of claim 28, wherein the anti-metabolite compound is floxuridine, 6-azauridine, capecitabine, or 5-fluorouracil.

33. The article of manufacture of claim 28, wherein the tetracycline compound is doxycycline, demeclocycline, tetracycline, oxytetracycline, or minocycline.

34. The article of manufacture of any one of claims 28-44, further comprising a container comprising an additional anti-apicomplexan therapeutic agent.

35. The article of manufacture of claim 34, wherein the additional anti-apicomplexan therapeutic agent is nitazoxanide (NTZ).

36. The article of manufacture of any one of claims 29~35, wherein the *Cryptosporidium* infection is an infection by one or more of *C. parvum*, *C. hominis*, *C. canis*, *C. felis*, *C. meleagridis*, or *C. maris*.

37. The article of manufacture of any one of the claims 28~36, wherein the apicomplexan infection comprises the presence of one or more apicomplexan parasites in or on a subject.

38. The article of manufacture of claim 37, wherein the apicomplexan parasites are vegetative apicomplexan parasites (e.g., **sporozoites**, merozoites, **meronts**, etc) or infectious apicomplexan parasites (e.g., oocysts).

39. A **pharmaceutical** composition for **treatment** of an apicomplexan infection, the composition comprising one or more of a statin compound, a tetracycline compound, an anti-metabolite compound, a digoxin, doxycycline, idamycin, daunorubicin, doxorubicin, homoharringtonine, thioguanosine, tegaserod, trimethoprim, glipizide, triptolide, carvedilol, ketoconazole, mefloquine, Imodium, or aripiprazole compound.

40. The pharmaceutical composition of claim 39, wherein the apicomplexan infection is a *Cryptosporidiosis* infection.

41. The pharmaceutical composition of claim 39, wherein the apicomplexan infection is a *Babesia* infection, a *Plasmodium* infection, a *Cyptosporiasis* infection, an *Isosporiasis* infection, an *Eimeria* infection, or a *Theilera* infection.

42. The pharmaceutical composition of claim 39, wherein the statin compound is fluvastatin, cerivastatin, mevastatin, itavastatin, lovastatin, or simvastatin.

43. The pharmaceutical composition of claim 39, wherein the anti-metabolite compound is floxuridine, 6-azauridine, carmofur, or 5-fluorouracil.

44. The pharmaceutical composition of claim 39, wherein the tetracycline compound is doxycycline, demeclocycline, tetracycline, **oxytetracycline**, or **minocycline**.

45. The pharmaceutical composition **of** claim 39, further comprising one or more additional anti-apicomplexan therapeutic agents.

46. The pharmaceutical composition of claim 45, wherein an additional anti-apicomplexan therapeutic agent is nitazoxanide (NTZ).

47. The pharmaceutical composition **of** any one **of** claims 39-46, further comprising a targeting agent.

48. **The** pharmaceutical **composition** of claim 47, wherein the targeting agent is an agent that targets an epithelial tissue.

49. The **pharmaceutical** composition of any one of claims 39-46, wherein the pharmaceutical composition further comprises an agent that reduces solubility of the compound in the subject's intestine.

50. The pharmaceutical composition of any one of claims 39-49, wherein the composition is formulated oral, sublingual, intravenous, intramuscular, subcutaneous, cutaneous, dermal, intrathecal, transdermal, intraperitoneal, transmucosal, or intranasal delivery.

51. The pharmaceutical composition of any one of claims 39-50, wherein the composition is formulated for prophylactic delivery.

52. The pharmaceutical composition of any one of claims 40, wherein the Cryptosporidium infection **comprises** infection by one or more of *C. parvum*, *C. hominis*, *C. canis*, *C. felis*, *C. meleagridis*, or *C. tritrichosporii*.

53. A method of decontaminating a substrate that comprises or is at risk of comprising apicomplexan contamination, the method comprising: contacting the substrate with an effective amount of at least one of a statin compound, a tetracycline compound, an anti-metabolite compound, a digoxin, doxorubicin, idarubicin, daunorubicin, doxorubicin, homoharringtonine,

oligomycin C, tegaserod, indomethacin, glipizide, triptolide, carvedilol, ketoconazole, mefloquine, Imodium, or aripiprazole compound to reduce the apicomplexan contamination of or the risk of apicomplexan contamination of the substrate.

5 54. The method of claim 53, wherein the apicomplexan contamination is a Cryptosporidiosis contamination.

55. The method of claim 53, wherein the apicomplexan contamination is a Babesia contamination, a Plasmodium contamination, a Cyclosporiasis contamination, an isosporiasis
10 contamination, an Eimeria contamination, or a Theileria contamination.

56. The method of claim 54, wherein the substrate is also **contaminated** with Clostridium, Entamoeba, Giardia, and wherein the method reduces the apicomplexan contamination and the Clostridium contamination, Entamoeba contamination, or Giardia contamination.

15 57. The method of claim 53, wherein reducing the apicomplexan contamination of the substrate comprises reducing the level of apicomplexan parasite on the substrate.

58. The method of claim 53, wherein the statin compound is fluvastatin, cerivastatin,
20 mevastatin, itavastatin, lovastatin, or simvastatin.

59. The method of claim 53, wherein the tetracycline compound is doxycycline, demeclocycline, tetracycline, oxytetracycline, or minocycline.

25 60. The method of claim 53, wherein the anti-metabolite compound is floxuridine, 6-azauridine, carmofur, or 5-fluorouracil.

61. The method of claim 53, wherein the compound is chloroxine, hexachlorophene, or a combination thereof.

30 62. The method of claim 53, wherein the substrate comprises a liquid.

63. The method of claim 62, wherein the liquid comprises water.

64. The method of claim 53, wherein the substrate comprises one or more of a metal, wood, plastic, glass, cork, fiber, a polymer, or a fabric.

5

65. The method of claim 53, wherein the substrate comprises a food substance.

66. The method of claim 53, wherein the substrate is at least a portion of a tool, work surface, a medical device, body of water, clothing, skin, or an edible substance (e.g., a beverage or food).

10

67. The method of any one of claims 53-66, wherein the contaminated substrate comprises one or more apicomplexan parasites.

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68. The method of claim 67, wherein the apicomplexan parasite includes one or more of a vegetative (e.g., sporozoites, merozoites, meronts, etc) apicomplexan parasite or an infectious apicomplexan parasite (e.g., oocysts).

69. The method of any one of claims 53-68, wherein the substrate is contacted by an aqueous composition comprising the compound.

20

70. The method of claim 54, wherein *Cryptosporidium* is *C. parvum*, *C. hominis*, *C. canis*, *C. felis*, *C. meleagridis*, or *C. muris*.

71. A composition comprising a statin compound, a tetracycline compound, an anti-metabolite compound, a digoxin, docetaxol, idarubicin, daunorubicin, doxorubicin, homoharringtonine, oligomycin C, tegaserod, indomethacin, glipizide, triptolide, carvedilol, ketoconazole, mefloquine, imodium, or aripiprazole compound, and an additional anti-apicomplexan therapeutic agent.

25

72. The composition of claim 71, wherein the apicomplexan infection is a *Cryptosporidiosis* infection.

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73. The composition of claim 71, wherein the apicomplexan infection is a Babesia infection, a Plasmodium infection, a Cyclosporiasis infection, an Isosporiasis infection, an Eimeria infection, or a Theileria infection.

74. The composition of claim 71, wherein the composition comprises carvedilol, ketoconazole, mefloquine, Imodium, or aripiprazole.

75. The composition of claim 71, wherein the statin compound is fluvastatin, cerivastatin, mevastatin, itavastatin, lovastatin, or simvastatin.

76. The composition of claim 71, wherein the anti-metabolite compound is floxuridine, 6-azauridine, carmofur, or 5-fluorouracil.

77. The composition of claim 71, wherein the tetracycline compound is doxycycline, demeclocycline, tetracycline, oxytetracycline, or minocycline.

78. The composition of claim 71, further comprising a detergent.

79. The composition of any one of claims 71-78, further comprising a pharmaceutically acceptable carrier.

80. The composition of claim 72, wherein the Cryptosporidium infection comprises infection by one or more of *C. parvum*, *C. hominis*, *C. canis*, *C. felis*, *C. meleagridis*, or *C. muris*.

81. The composition any one of claims 71-80, wherein the apicomplexan infection comprises infection by one or more apicomplexan parasites.

82. The composition of claim 81, wherein the apicomplexan parasites comprise a vegetative apicomplexan parasite (e.g., sporozoite, merozoite, meront, etc) and/or an infectious apicomplexan parasite (e.g., oocyst).

83. The composition of any one of claims 71-82, wherein the additional anti-apicomplexan therapeutic agent is nitazoxanide (NTZ).

84. The composition of claim 83, wherein the composition comprises earvedilol, ketoconazole,
5 mefloquine, Imodium, or aripiprazole and NTZ.

85. A method of decontaminating a substrate that comprises or is at risk of comprising Clostridium contamination, the method comprising; contacting the substrate with an effective amount of at least one of a chlofoxine compound or a hexachlorophene compound to reduce the
10 Clostridium contamination of or the risk of Clostridium contamination of the substrate.

86. The method of claim 85, wherein reducing the Clostridium contamination of the substrate comprises reducing the level of Clostridium parasite on the substrate.

87. The method of claim 85, wherein the substrate comprises a liquid.
15

88. The method of claim 87, wherein the liquid comprises water.

89. The method of claim 85, wherein the substrate comprises one or more of a metal, wood,
20 plastic, glass, cork, fiber, a polymer, or a fabric.

90. The method of claim 85, wherein the substrate is at least a portion of a tool, work surface, a medical device, body of water, clothing, skin, or an edible substance (e.g., a beverage or food).

91. The method of claim 85, wherein the Clostridium contamination comprises contamination by Clostridium spores.
25

92. The method of any one of claims 85-91, wherein the substrate is contacted by an aqueous composition comprising the compound.
30

93. A method of treating a Cryptosporidium infection in a subject, the method comprising: administering to a subject having or at risk of having a Cryptosporidium infection an effective

amount of at least one of a compound that modifies cell cholesterol metabolism in the subject to treat the *Cryptosporidium* infection, wherein the compound is not a statin.

94. The method of claim 93, wherein the subject is not undergoing treatment with a statin compound.

95. The method of claim 93, wherein the *Cryptosporidium* infection comprises the presence of one or more *Cryptosporidium* parasites in or on the subject.

96. The method of any one of claims 93-95, wherein the *Cryptosporidium* parasites comprise vegetative (e.g., sporozoites, merozoites, meronts, etc) **and/or** infectious *Cryptosporidium* parasites (e.g., oocysts).

97. The method of claim 93, further comprising administering one or more additional anti-apicomplexan therapeutic agents to the subject.

98. The method of claim 93, wherein the compound is administered in a pharmaceutical composition.

99. The method of claim 98, wherein the pharmaceutical composition further comprises a pharmaceutically acceptable carrier.

100. The method of claim 98, wherein the pharmaceutical composition further comprises a targeting agent.

101. The method of claim 100, wherein the targeting agent is an agent that targets an epithelial tissue.

102. The method of claim 98, wherein the pharmaceutical composition further comprises an agent to reduce solubility of the compound in the subject's intestine.

103. The method of any one of claims **93-102**, wherein the compound is administered orally, sublingually, intravenously, intramuscularly, subcutaneously, intrathecally, transdermally, intraperitoneally, transmucosally, or intranasally.

104. The method of any one of claims **93-103**, wherein the compound is administered prophylactically.

105. The method of any one of claims **93-104**, further comprising selecting a subject at least in part on the basis that the subject has been exposed to *Cryptosporidium* infection.

106. The method of any one of claims **93-105**, wherein the subject has been diagnosed with a *cryptosporidium* infection.

107. The method of any one of claims **93-106**, wherein the *Cryptosporidium* infection of the subject comprises infection by one or more of *C. parvum*, *C. hominis*, *C. cants*, *C. felis*, *C. meleagridis*, or *C. muris*.

108. The method of any one of claims **93-107**, wherein the subject is a vertebrate.

109. Use of a compound that modifies cell cholesterol metabolism in a subject in the manufacture of a medicament to treat a *Cryptosporidium* infection in a subject, wherein the compound is not a statin.

110. A method of treating an apicomplexan infection in a subject, the method comprising administering to a subject an effective amount of a statin compound, a tetracycline compound, an anti-metabolite compound, a **digoxin**, docetaxol, idarubicin, **daunorubicin**, doxorubicin, homoharringtonine, olgomycin C, tegaserod, indomethacin, glipizide, triptolide, carvedilol, ketoconazole, mefloquine, imodium, or aripiprazole compound, and an additional anti-apicomplexan therapeutic agent, to treat the apicomplexan infection in the subject.

111. The method of claim **110**, wherein the anti-apicomplexan therapeutic agent is nitazoxanide (NTZ).

1 12. A method of identifying a test compound as a compound to treat an apicomplexan parasite infection in a subject or to reduce an apicomplexan parasite contamination of a substrate, the method comprising,

5 inoculating parasite oocysts onto a cell monolayer;
priming the parasite oocysts;
contacting the inoculated cells with a test compound;
incubating the contacted inoculated cells,
fixing the incubated cells;

10 determining the level of a parasite in the cell monolayer, and
comparing the level of the parasite in the cell monolayer contacted with the test compound to a **control** level of the parasite, wherein a decrease in the level **of the** parasite in the cells contacted with the test compound compared to the control level of the parasite identifies the test compound as a compound to treat an apicomplexan parasite infection in a subject or to reduce an apicomplexan
15 parasite contamination of a substrate.

113. The method of claim 112, wherein the oocyst is a *Cryptosporidium* oocyst.

114. The method of claim 112, wherein the incubation is at least 24 hours, 36 hours, 48 hours, 60
20 hours in duration.

115. The method of claim 112, wherein determining the level of the parasite comprises staining the cell nuclei and staining the parasites, visualizing the cells, and determining the number of parasites in the cell culture.

25 116. The method of claim 112, wherein the control level of the parasite is the level of the parasite in an inoculated, incubated cell monolayer that is not contacted with the test compound.

117. The method of claim 112, wherein the cell monolayer is a human ileocecal adenocarcinoma
30 (HCT-8) cell monolayer.

1 18. The method of claim 112, further comprising confirming efficacy of the candidate compound for treating a cell or subject having or at risk of having the apicomplexan parasite infection or for reducing contamination of a substrate by the apicomplexan parasite.

Figure 1

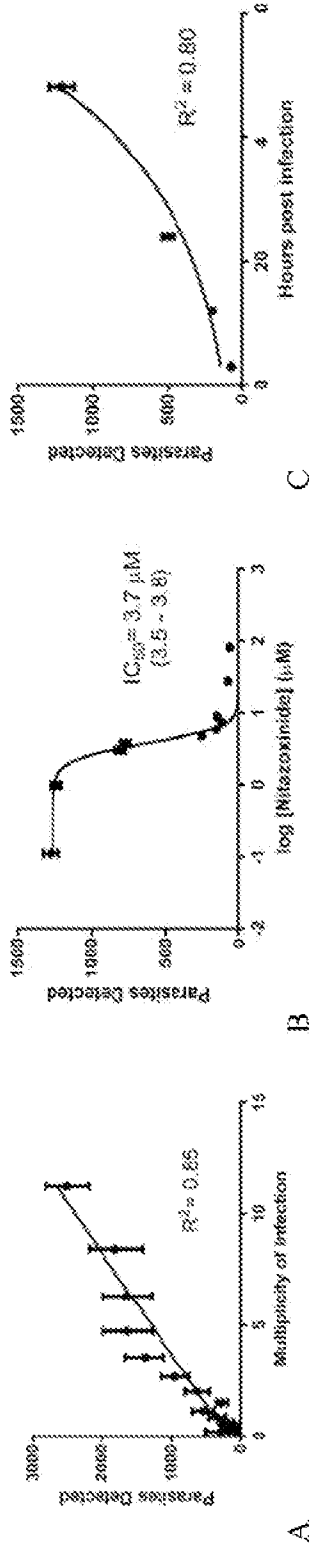


Figure 2

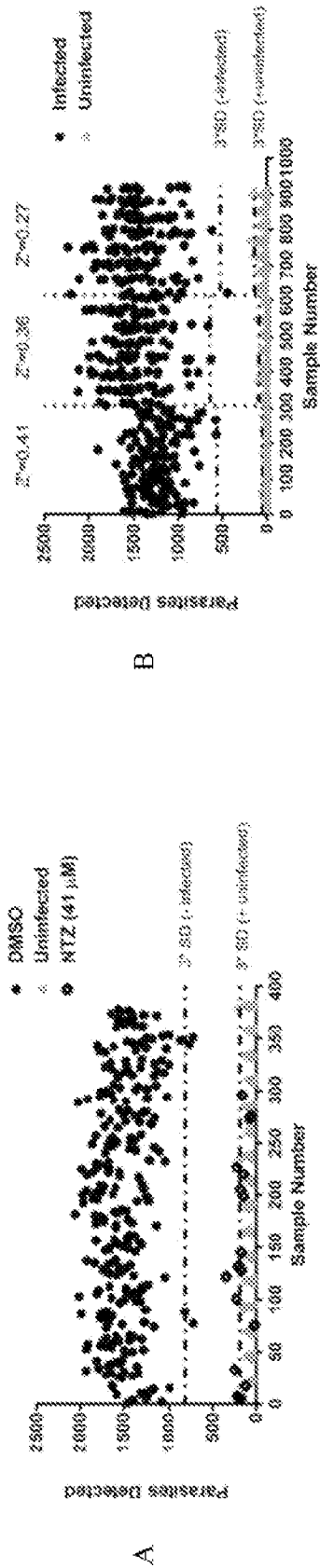


Figure 3

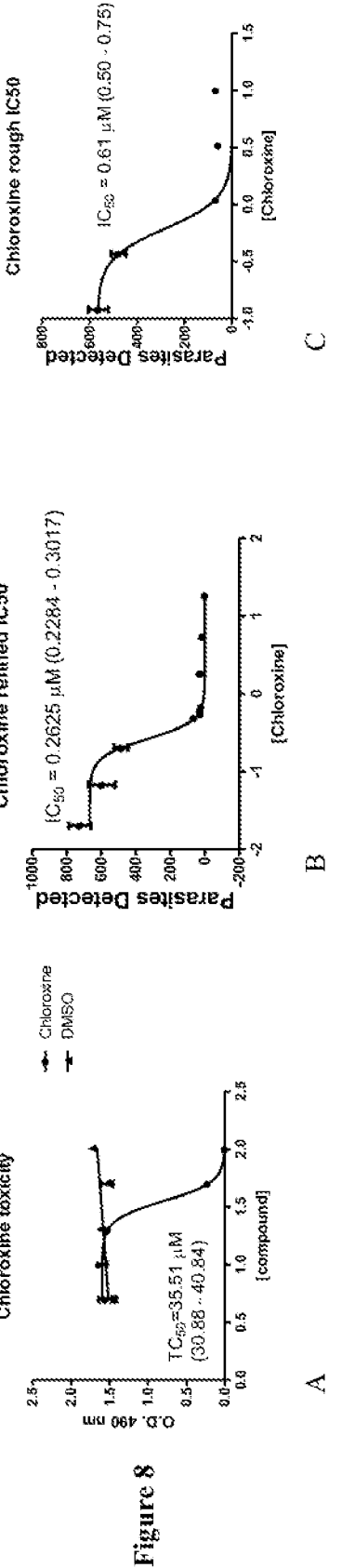
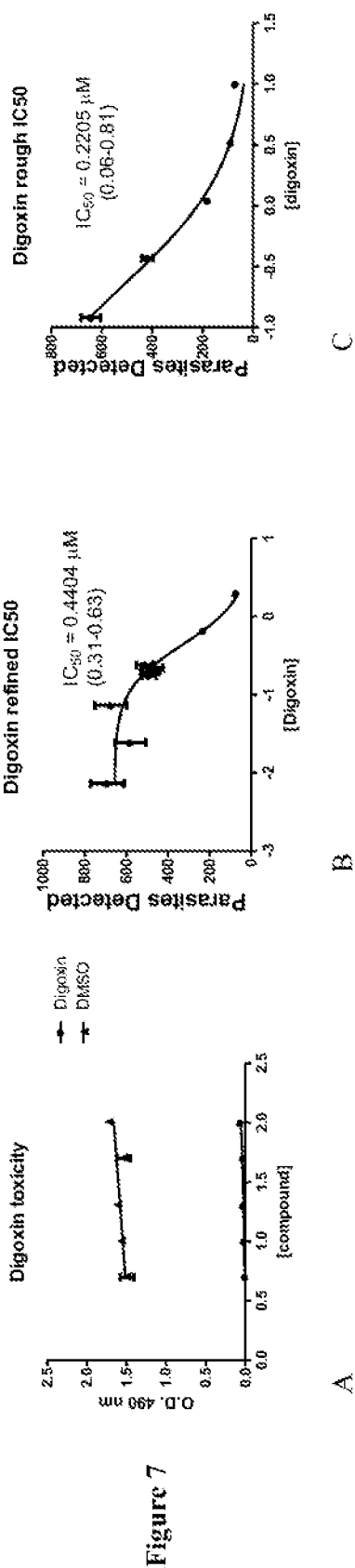
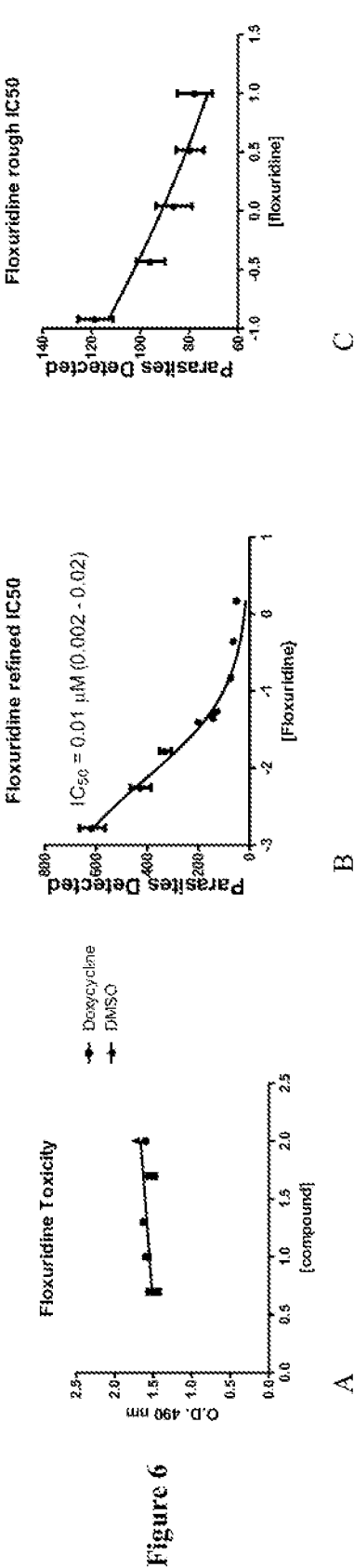


Figure 4

Compound Name	PubChem Identifier	Classification	% Inhibition Replicate 1	% Inhibition Replicate 2	Average % Inhibition	Compound Purity	IC ₅₀ in μ M ¹ (95% C.I.)
Idarubicin Hydrochloride	636362	Anthracycline	96%	90%	93%	>85%	0.77 (0.57-1.0)
Daunorubicin Hydrochloride	3065106	Anthracycline	89%	86%	87%	>85%	1.2 (0.75-1.9)
Doxorubicin Hydrochloride	443939	Anthracycline	84%	81%	82%	>85%	2.2 (1.5-3.0)
Chloroxine	2722	Antibacterial-topical	94%	93%	93%	>85%	0.61 (0.50-0.75)
Hexachlorophene	3586	Antibacterial-topical	95%	89%	92%	ND	1.3 (1.2-1.7)
Clofazimine	2794	Anti-mycobacterial	89%	83%	86%	>85%	no inhibition
Digoxin	30322	Channel inhibitor	86%	83%	85%	<50%	0.22 (0.060-0.81)
Gilgizide	3476	Channel inhibitor	91%	71%	81%	>85%	7.2 (5.6-9.4)
Oligomycin C	93857	Channel inhibitor	86%	77%	82%	ND	7.8 (5.8-10.6)
Indomethacin	3715	Cyclooxygenase inhibitor	100%	80%	90%	>85%	5.4 (4.5-5.9)
Homoharringtonine	285033	Experimental	70%	66%	78%	>85%	1.1 (cannot calculate)
Tremulacin	442544	Experimental	94%	87%	90%	<20%	inhibitory at all doses tested
Nobiletin	72344	Experimental	83%	21%	52%	>85%	no inhibition
Mevastatin	64715	HMG-CoA reductase inhibitor	61%	51%	66%	>85%	3.2 (2.6-4.1)
Docetaxel	148124	Microtubule agent	91%	83%	87%	>85%	0.31 (0.079-1.2)
Vincristine Bisulfate	23681803	Microtubule agent	60%	66%	68%	<80%	no inhibition
Triptolide	107965	NF κ B inhibitor	89%	83%	86%	<20%	9 (cannot calculate)
Camptofur	2677	Pyrimidine analog	67%	76%	81%	ND	4.1 (3.2-5.2)
5-Fluorouracil	3385	Pyrimidine analog	83%	82%	82%	>85%	4.1 (3.1-5.5)
6-Azauridine	6801	Pyrimidine analog	67%	83%	85%	>85%	0.9 (0.6-1.2)
Fluoridine	3363	Pyrimidine analog	93%	93%	93%	ND	inhibitory at all doses tested
Minocycline Hydrochloride	54685925	Tetracycline analog	91%	87%	89%	>85%	4.1 (3.4-4.8)
Doxycycline	54671203	Tetracycline analog	80%	90%	90%	>85%	6.0 (4.5-8.0)
Tegaserod Maleate	6918366	5HT ₄ receptor agonist	90%	70%	80%	>85%	5.6 (4.5-6.9)

Figure 5

Compound Name	PubChem Identifier	Classification	Supplier (catalog number)	Purity	IC ₅₀ in μM ¹ (95% C.I.)	TC ₅₀ ² (μM)	Therapeutic Index ³ (TI)
Chloroxine	2722	Topical antibacterial	Sigma (D54650)	>85%	0.28 (0.22 - 0.29)	35	135
Hexachlorophene	3596	Topical antibacterial	Sigma (H4625)	ND	1.4 (1.1 - 1.7)	90	64
Digoxin	30322	Channel inhibitor	Sigma (D6003)	>85%	0.44 (0.3 - 0.60)	< 5	<11
Glipizide	3478	Channel inhibitor	Sigma (G117)	>85%	5.8 (5.2 - 6.4)	>100	>17
Oligomycin C	93857	Channel inhibitor	Sequoia (SRP01150a)	ND	5.7 (4.5 - 10.1)	>100	15
Indomethacin	3715	Cyclooxygenase inhibitor	Sigma (I8280)	>85%	5.4 (4.9 - 5.9)	>100	>19
Homoharringtonine	285033	Experimental	Sequoia (SRP02125h)	>85%	0.86 (0.50 - 1.5)	ND	ND
Mevastatin	54715	HMG-CoA reductase inhibitor	Sigma (A1882)	>85%	5.1 (3.7 - 7.0)	>100	>20
Docetaxel	148124	Microtubule agent	Sigma (1885)	>85%	0.13 (0.079 - 0.20)	>100	>769
Floxuridine	3363	Pyrimidine analog	Sigma (F0503)	ND	0.0063 (0.0020 - 0.020)	>100	>15873
6-Azaundine	5901	Pyrimidine analog	Sigma (A1882)	>85%	3.2 (2.9 - 3.6)	>100	>31
Camofur	2577	Pyrimidine analog	Sigma (C1494)	>85%	4.4 (3.5 - 5.6)	>100	>23
5-Fluorouracil	3385	Pyrimidine analog	Sigma (F6627)	>85%	4.9 (3.7 - 6.3)	>100	>20
Doxycycline	54671203	Tetracycline analog	Sigma (D9891)	>85%	6.3 (5.7 - 6.9)	50	8
Minocycline Hydrochloride	54685825	Tetracycline analog	Sigma (M9511)	>85%	6.5 (5.0 - 8.5)	>100	>15
Tegaserod Maleate	6918369	5HT ₄ receptor agonist	Sequoia (SRP011871)	>85%	4.8 (3.9 - 5.8)	24	5



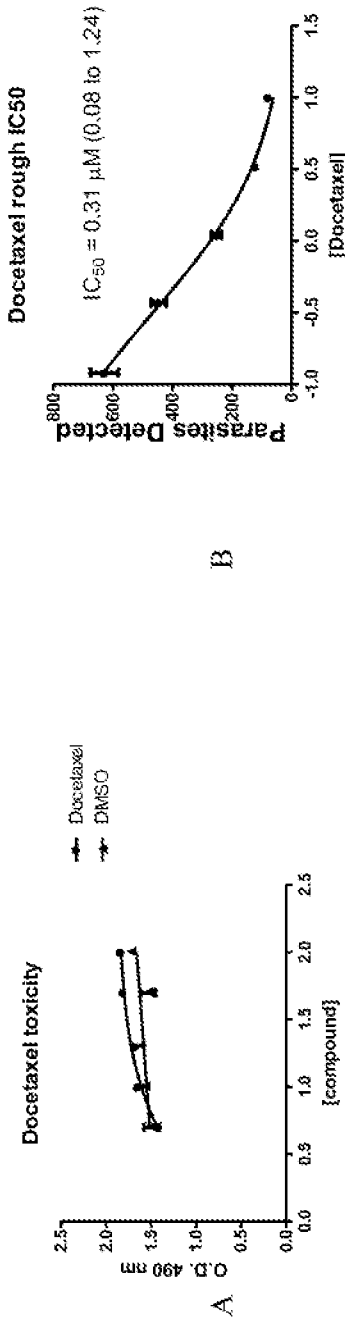


Figure 9

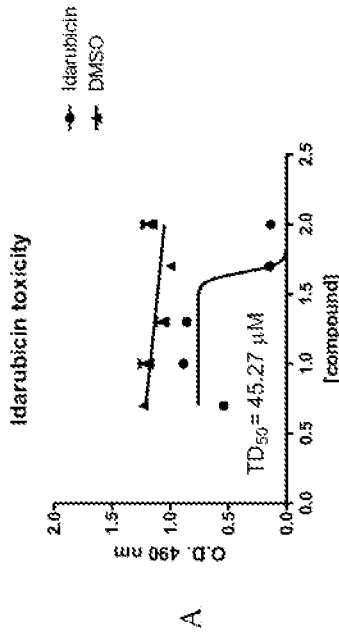


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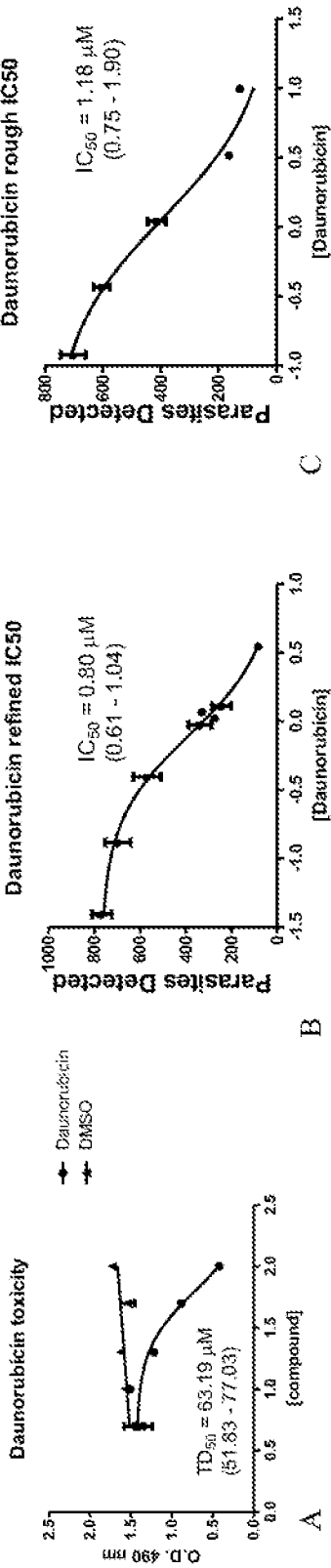


Figure 11

Figure 12

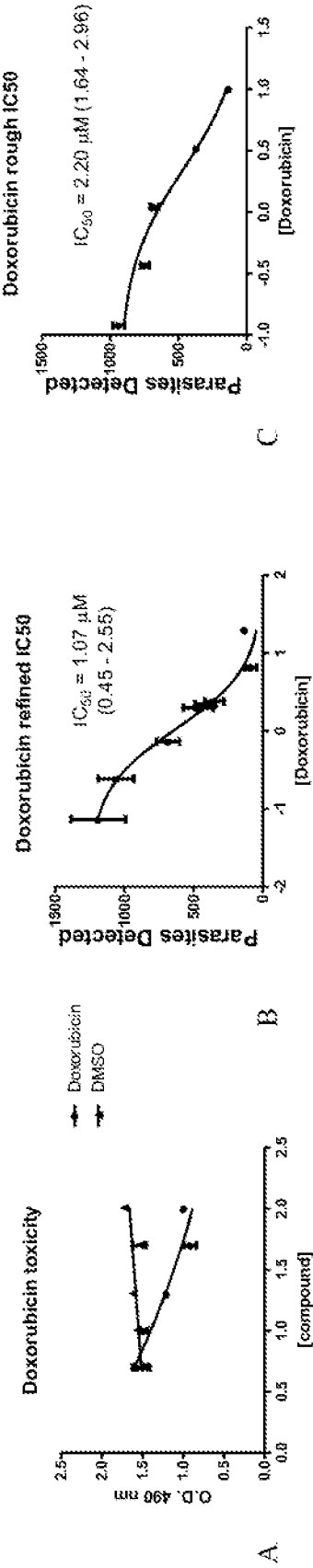


Figure 13

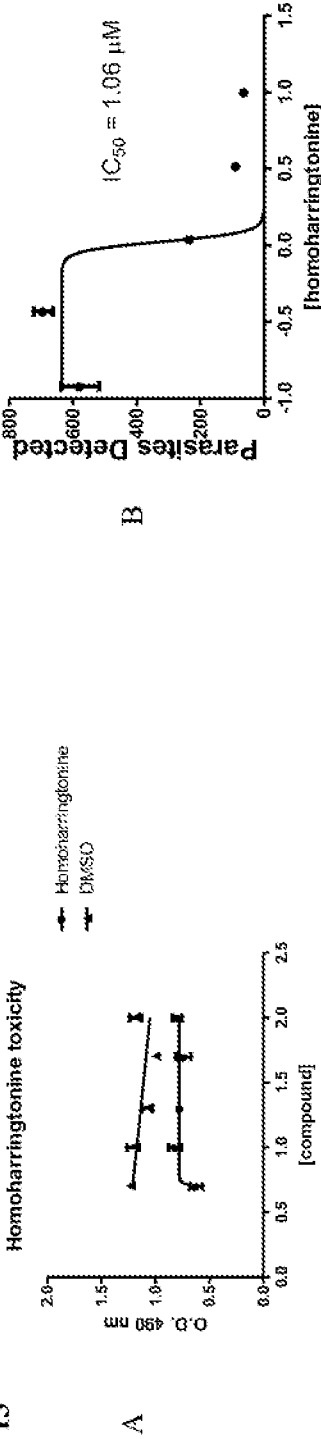
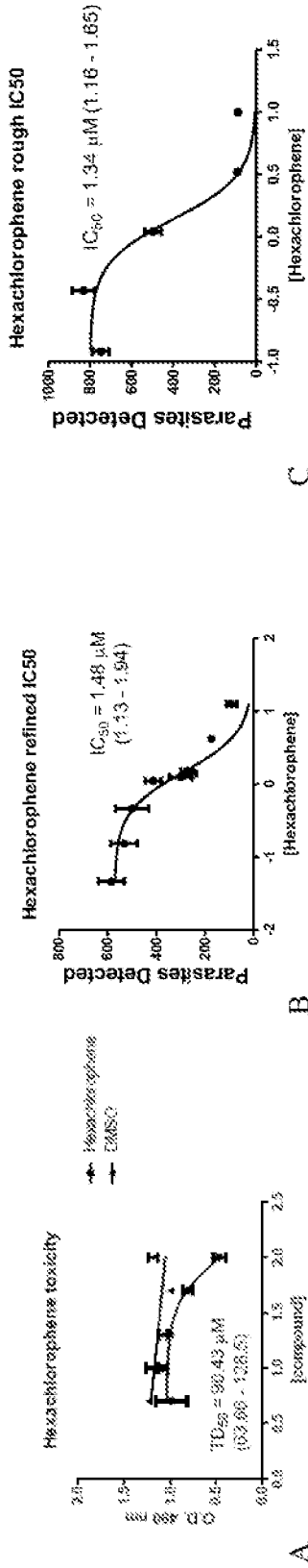


Figure 14



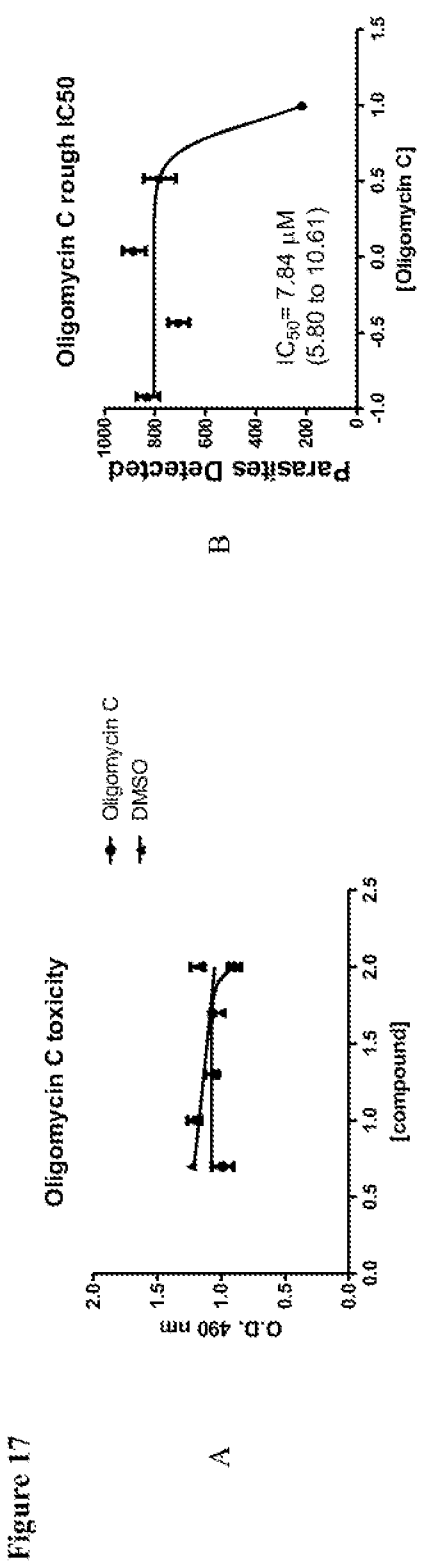
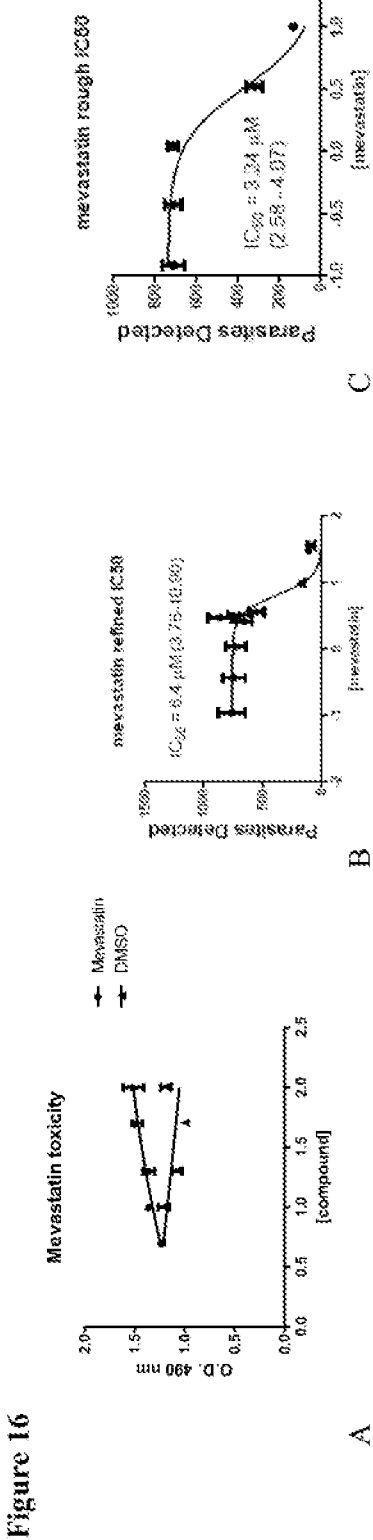
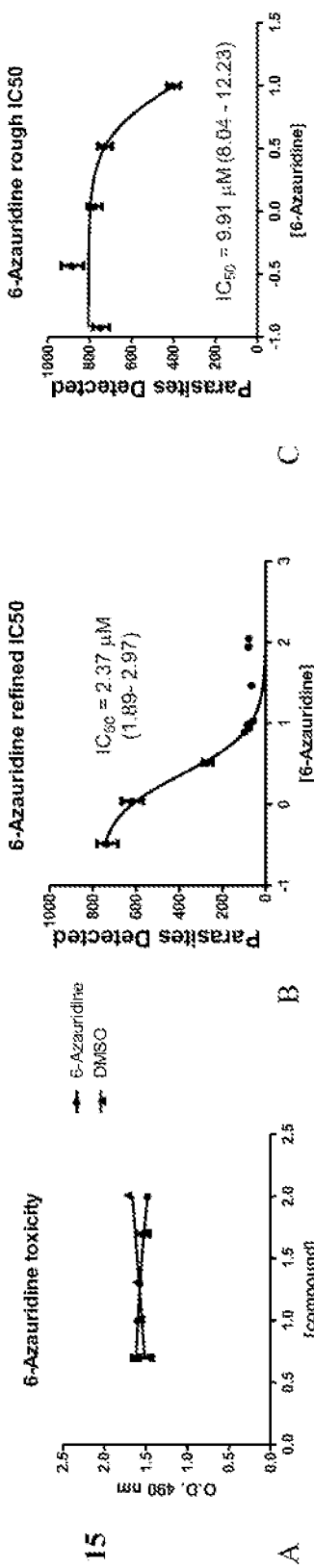


Figure 18

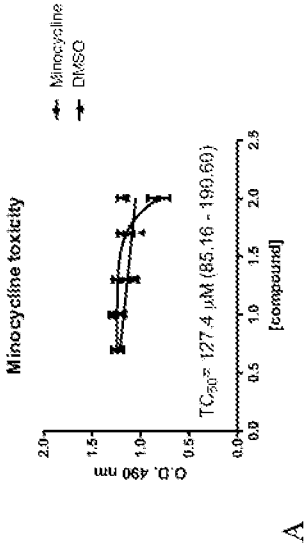


Figure 19

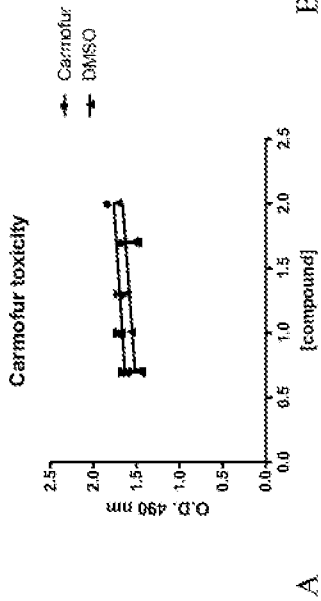
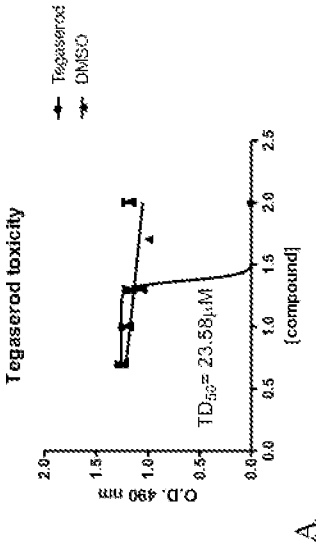
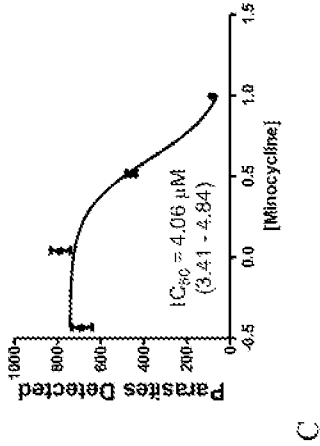


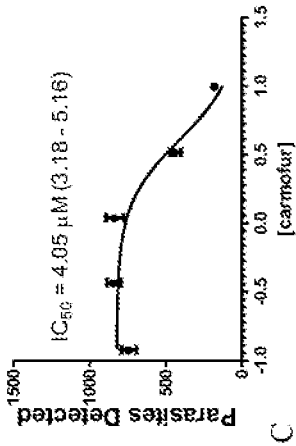
Figure 20



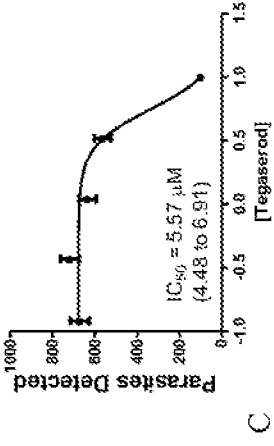
Minocycline rough IC50



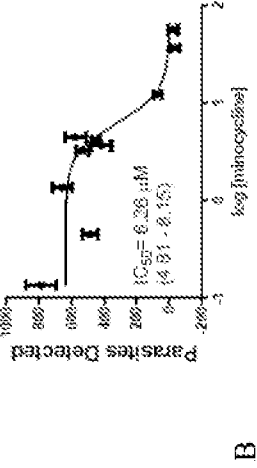
Carmofur rough IC50



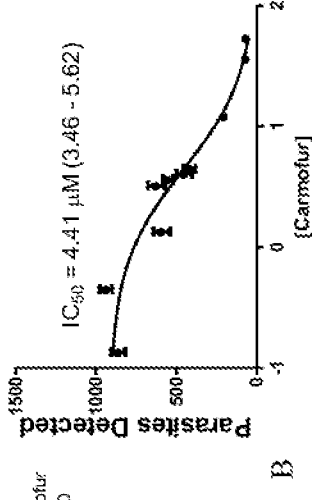
Tegaserod rough IC50



Minocycline refined IC50



Carmofur refined IC50



Tegaserod refined IC50

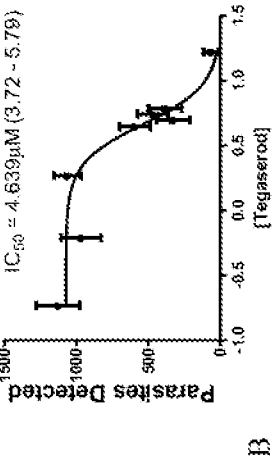


Figure 21

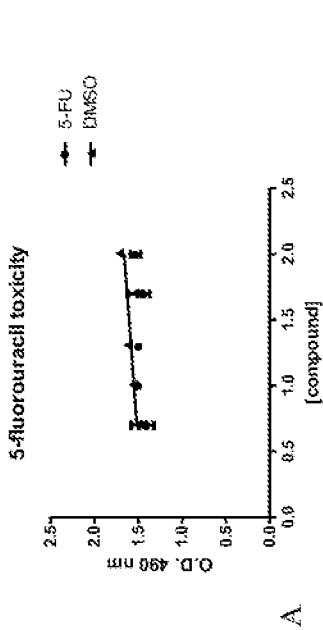


Figure 22

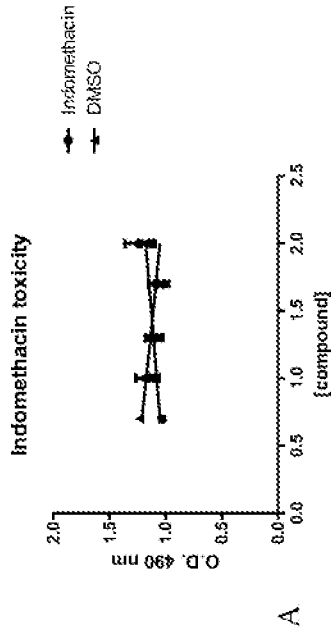


Figure 23

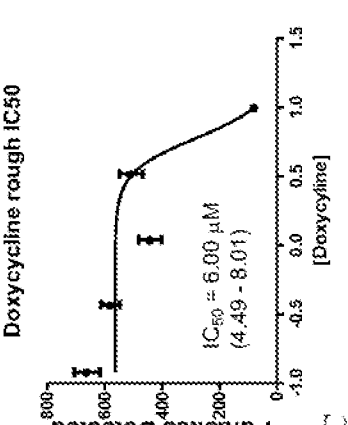
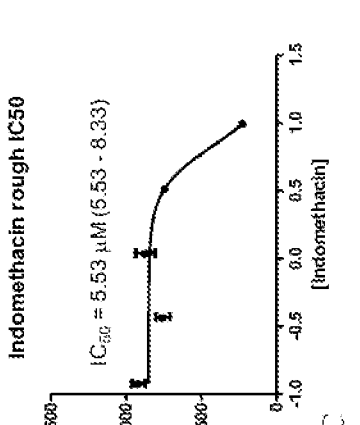
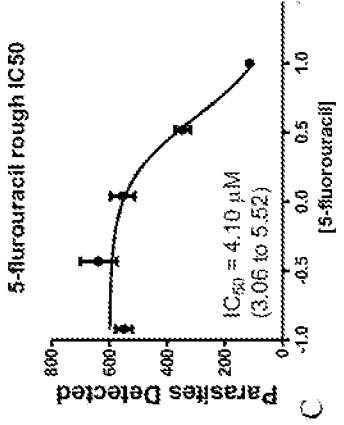
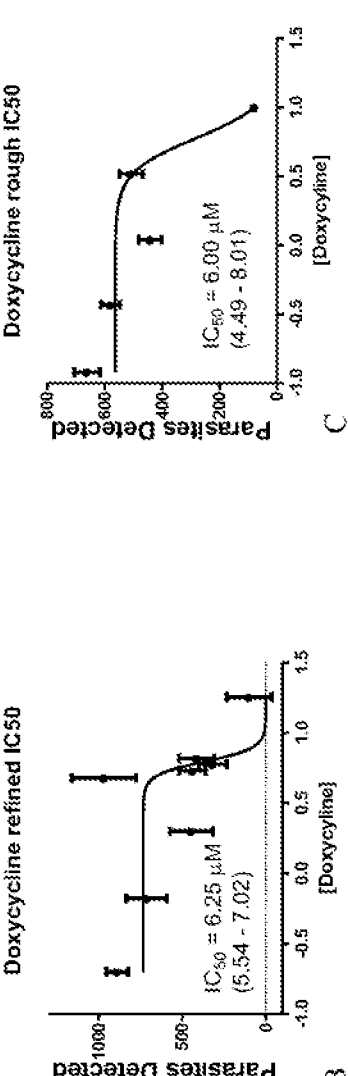
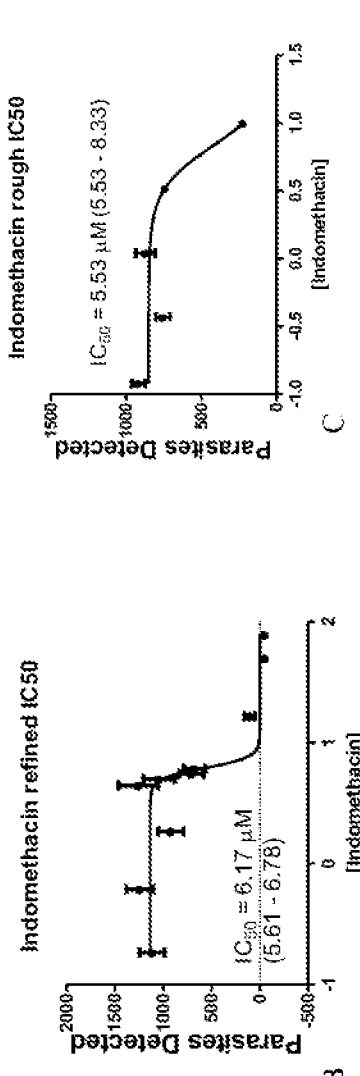
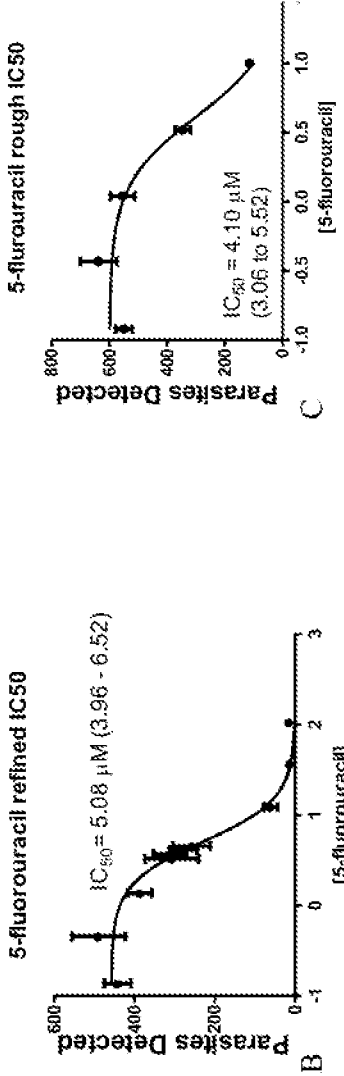
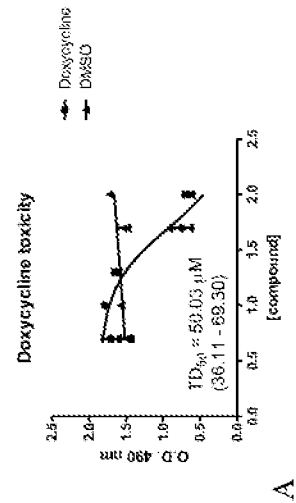


Figure 24

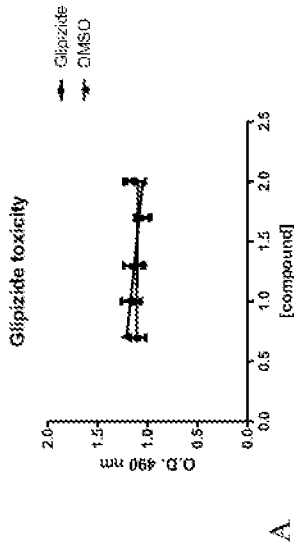


Figure 25

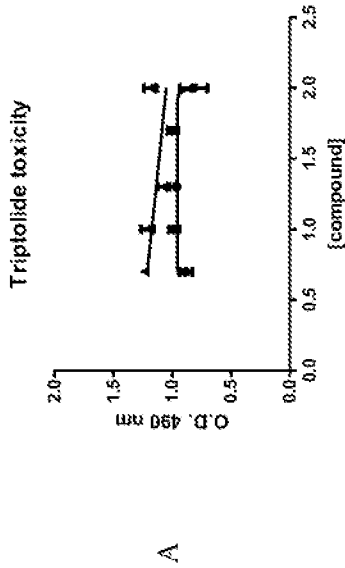


Figure 26

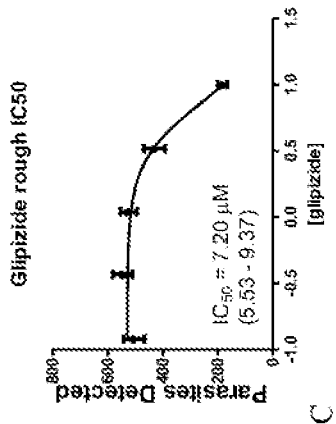
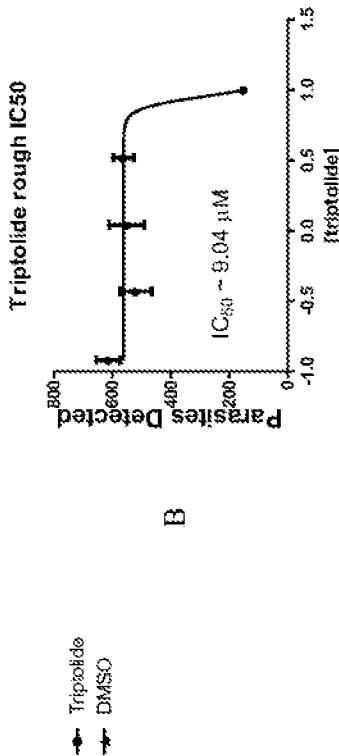
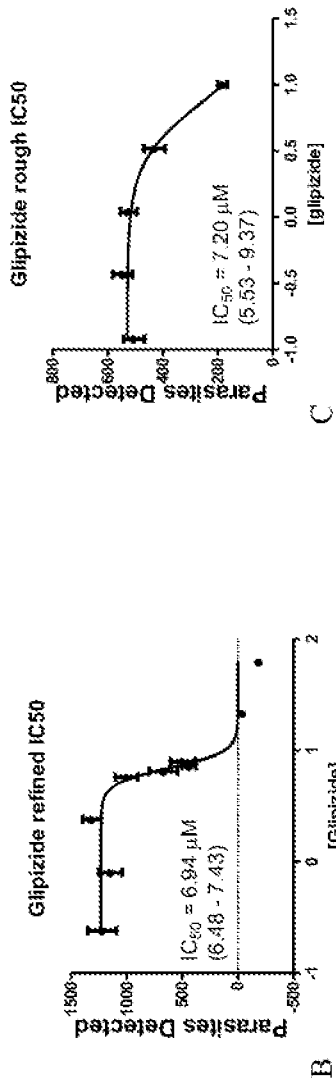
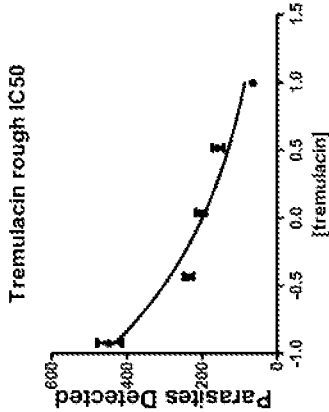


Figure 27

Compound name	% inhibition replicate 1	% inhibition replicate 2	Average % inhibition	Approximate IC50 (µM)	Approximate IC50 95% C.I. (µM)	Refined IC50 (µM)	Refined IC50 95% C.I. (µM)	TC50- metabolic assay (µM; 95% C.I.)
Mevastatin	81%	51%	66%	3.24	2.58 - 4.07	6.40	3.76 - 10.90	>100
Pravastatin	28%	20%	24%	non-inhibitory	n/a	non-inhibitory	n/a	>100
Cilastatin	23%	35%	6%	non-inhibitory	n/a	Not done	Not done	Not done
Cerivastatin	79%	73%	76%	cannot calculate	n/a	27	0.21-0.36	>100
Itavastatin	73%	73%	73%	0.14	0.003 - 5.22	.62	0.45-0.85	>100
Fluvastatin	78%	69%	74%	7.14	5.20 - 9.81	4.56	3.80 - 5.46	>100
Lovastatin	61%	21%	41%	10.69	8.45 - 13.52	5.46	3.99 - 7.49	>100
Simvastatin	41%	64%	52%	7.33	5.84 - 9.20	2.25	1.75-2.88	~91

Figure 28

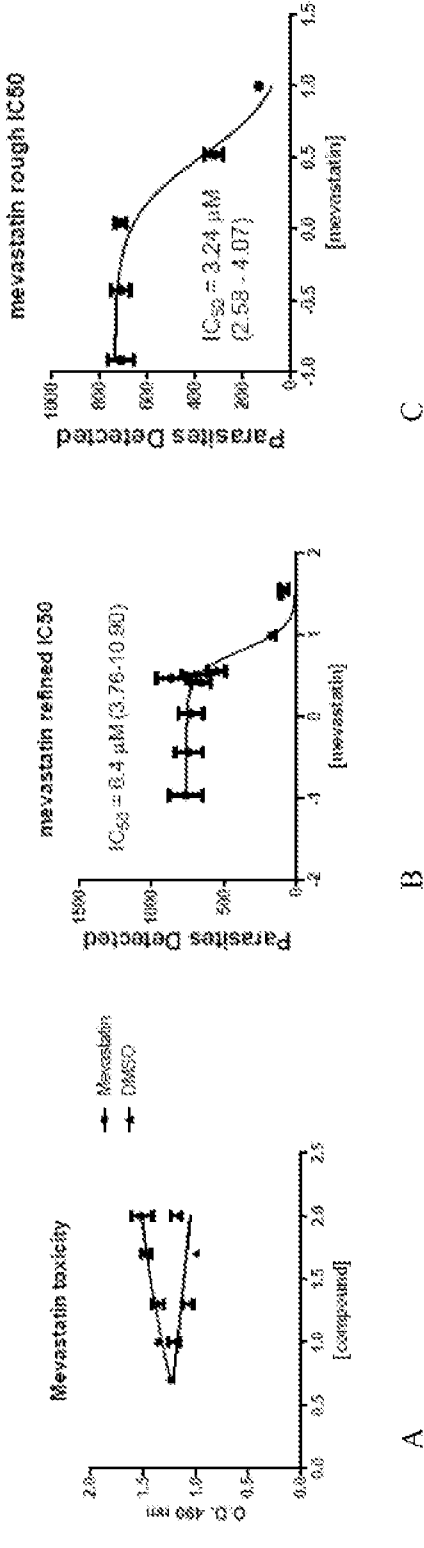


Figure 29

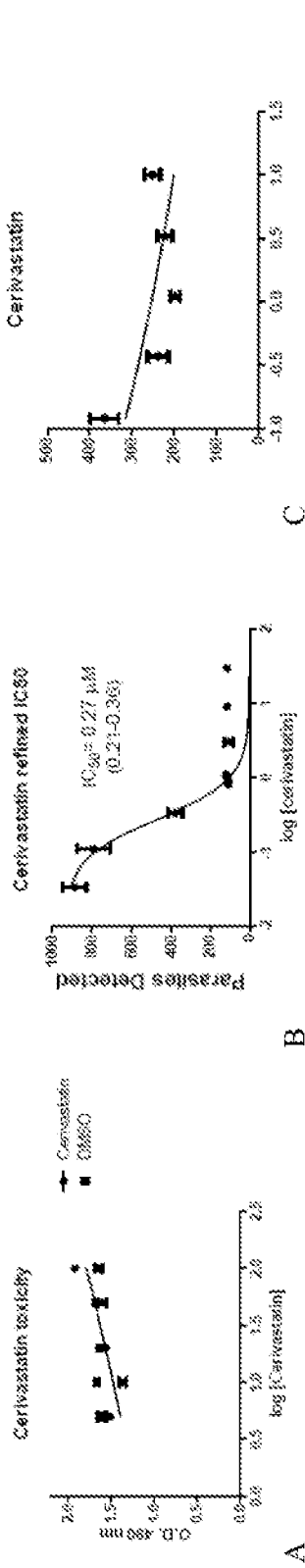


Figure 30

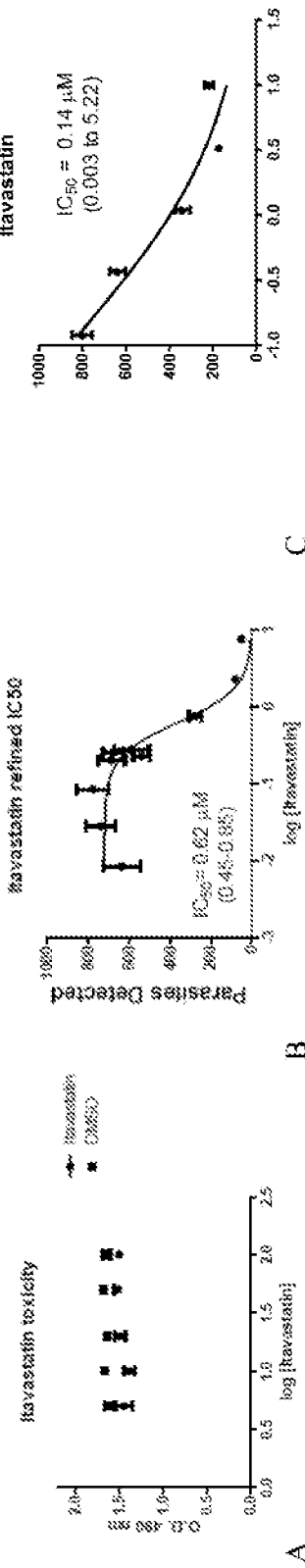


Figure 31

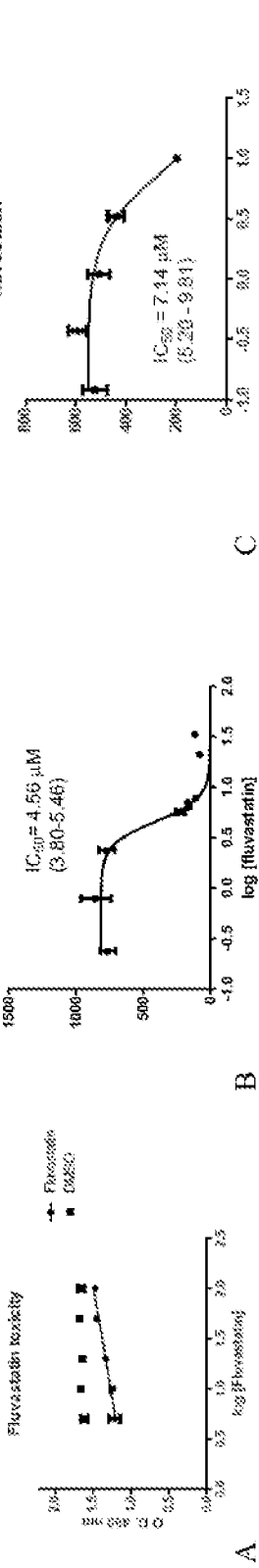


Figure 32

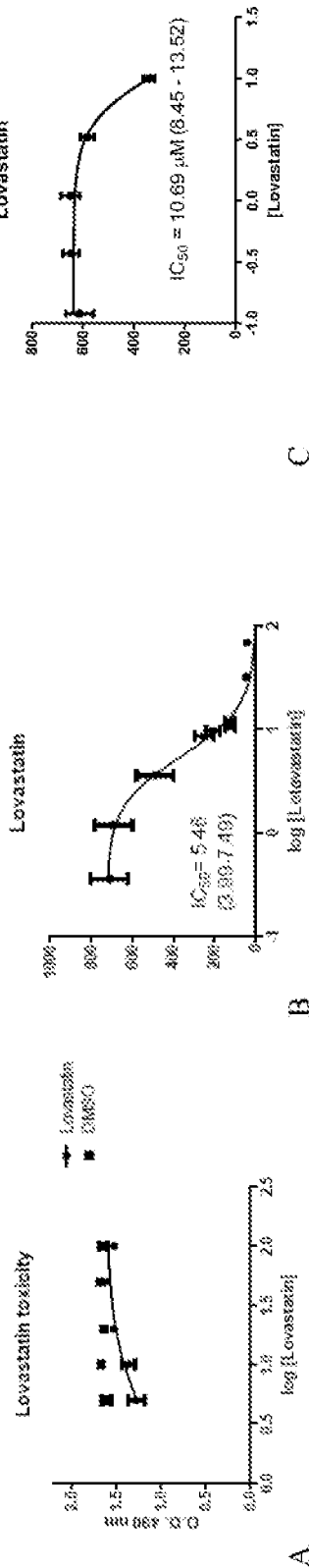


Figure 33

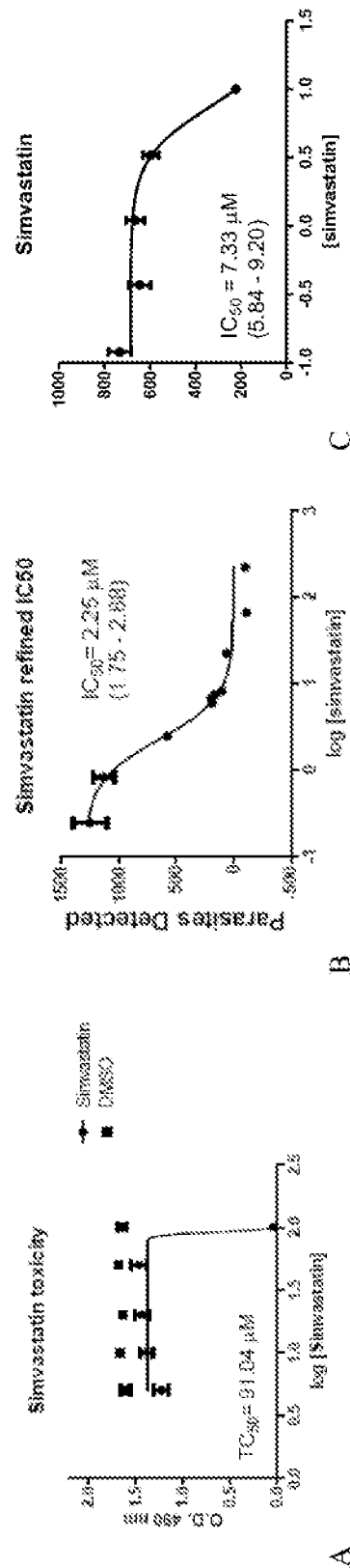


Figure 34

Compound name	% inhibition replicate 1	% inhibition replicate 2	Average % inhibition	Approximate IC ₅₀ (μm)	Approximate IC ₅₀ 95% C.I. (μm)	Refined IC ₅₀ (μM)	Refined IC ₅₀ 95% C.I. (μM)	TC ₅₀ - metabolic assay (μM; 95% C.I.)
Rolitetraacycline	-23%	-21%	-22%	cannot calculate		Not done		Not done
Tetraacycline	-8%	-42%	-25%	cannot calculate		12.35	6.54 - 23.31	>100
Demeclocycline	41%	50%	45%	cannot calculate		4.28	2.69 - 6.81	69.5 (57.8-83.5)
Oxytetraacycline	-15%	53%	19%	cannot calculate		9.09	7.16 - 11.54	>100
Minocycline Hydrochloride	91%	87%	89%	4.06	3.41 -- 4.84	6.26	4.81 -- 8.15	~127.4
Doxycycline	90%	90%	90%	6.00	4.49 -- 8.01	6.25	5.54 -- 7.02	50.03 (36.11-69.30)

Figure 35

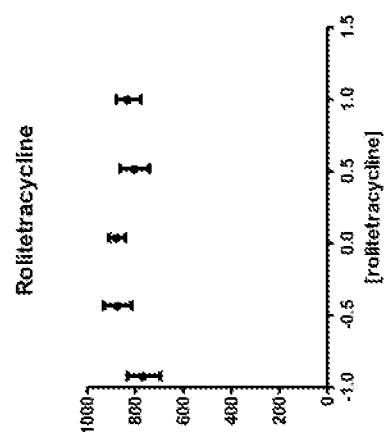


Figure 36

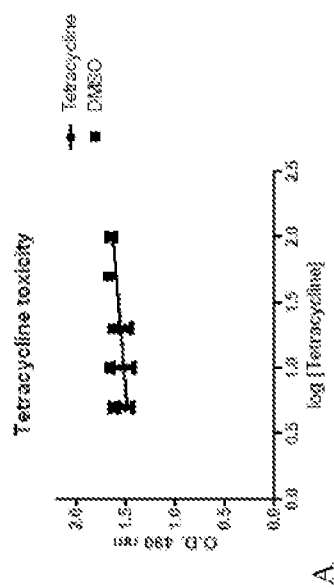


Figure 37

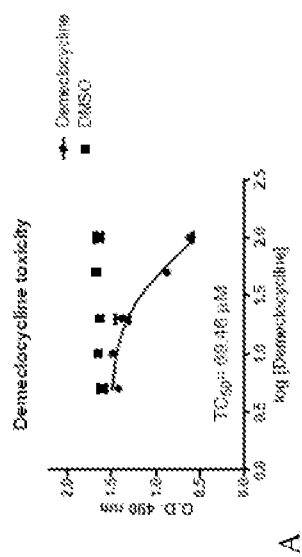


Figure 38

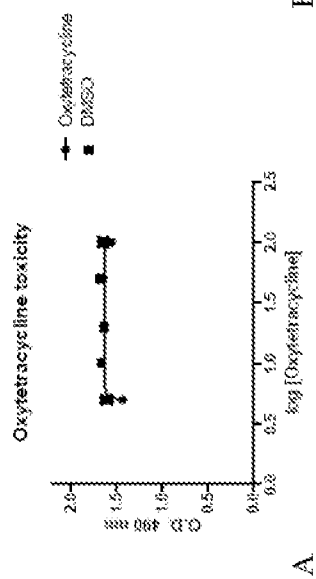


Figure 39

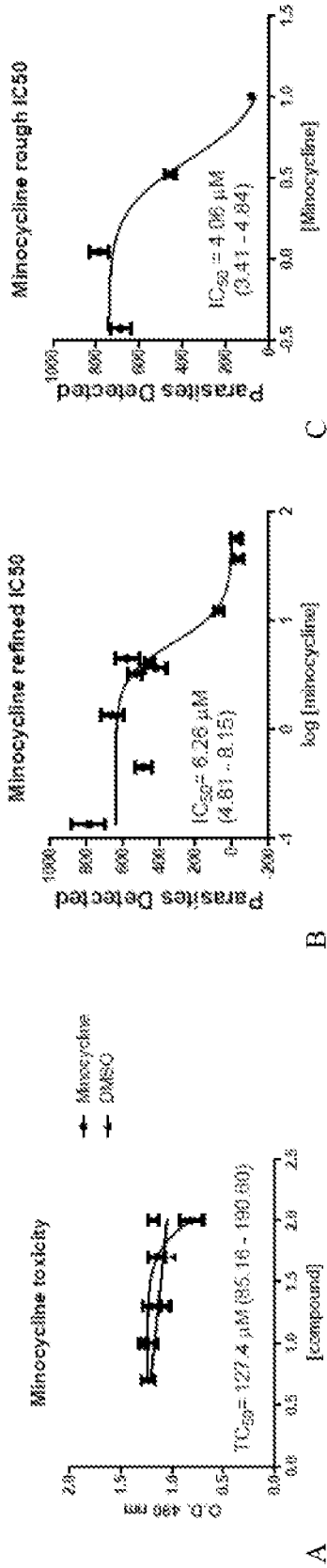


Figure 40

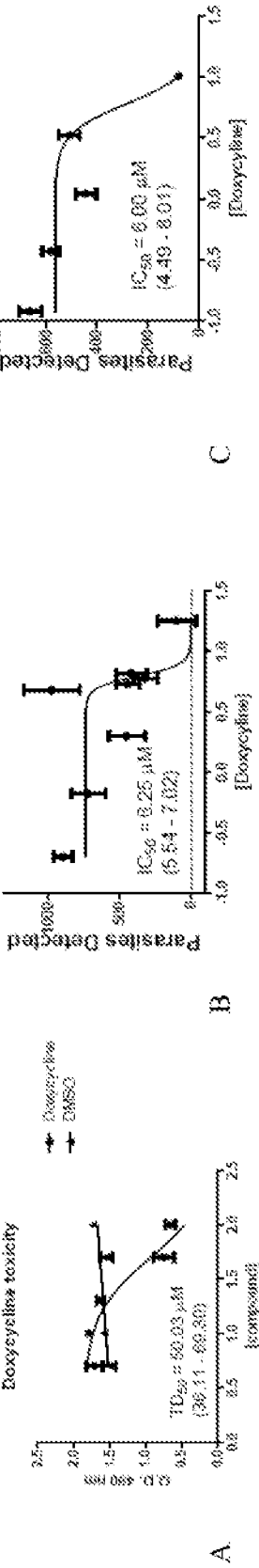


Figure 41

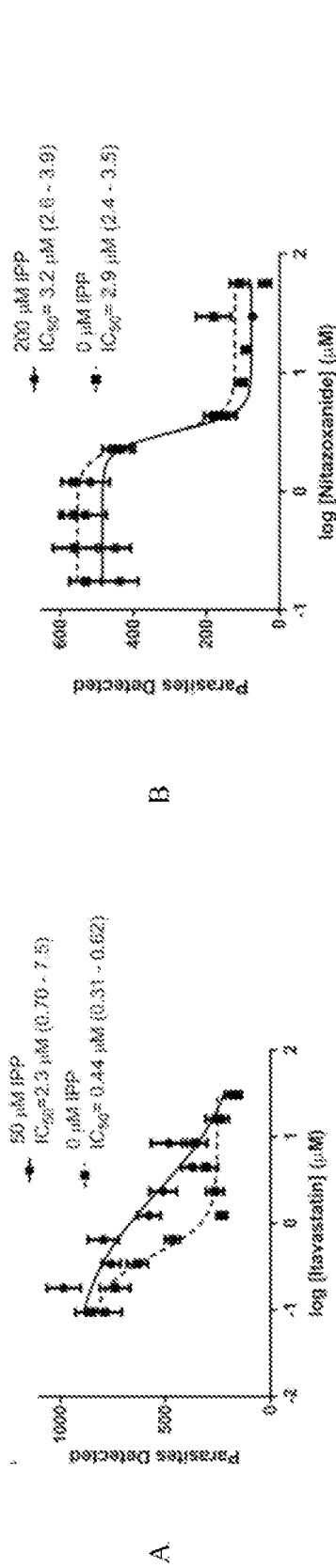


Figure 42

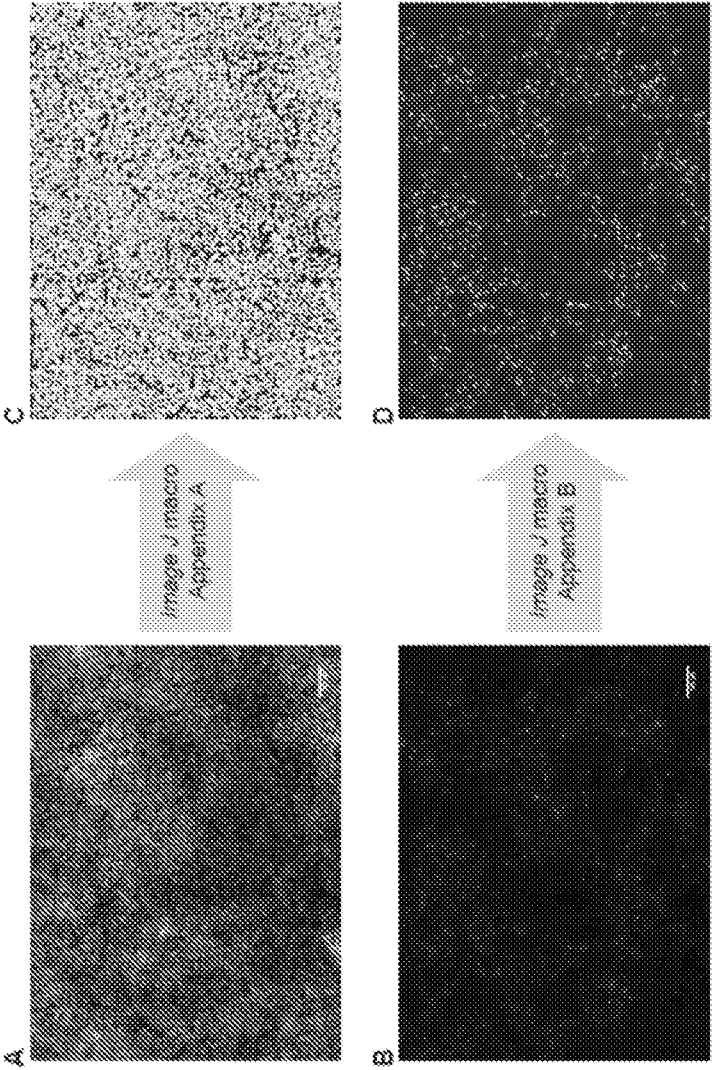


Figure 43

