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INHIBITION OF *TOXOPLASMA GONDII* AND *PLASMODIUM FALCIPARUM* INFECTIONS IN VITRO BY NSC3852, A REDOX ACTIVE ANTIPROLIFERATIVE AND TUMOR CELL DIFFERENTIATION AGENT

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ABSTRACT: We searched the National Cancer Institute (NCI) compound library for structures related to the antitumor quinoline NSC3852 (5-nitroso-8-quinolinol) and used a computer algorithm to predict the antiprotozoan activity for each of 13 structures. Half of these compounds inhibited *Toxoplasma gondii* tachyzoite propagation in human fibroblasts at ≤1 μM. The active compounds comprise a series of low-molecular-weight quinolines bearing nitrogen substituents in the ring-5 position. NSC3852 (EC_{50} 80 nM) and NSC74949 (EC_{50} 646 nM) were the most potent. NSC3852 also inhibited *Plasmodium falciparum* growth in human red blood cells (EC_{50} 1.3 μM). To investigate the mechanism for NSC3852's anti–*T. gondii* activity, we used chemiluminescence assays to detect reactive oxygen species (ROS) formation in freshly isolated tachyzoites and in infected host cells; the absence of ROS generation by NSC3852 in these assays indicated NSC3852 does not redox cycle in *T. gondii*. Inhibitors of enzyme sources of free radicals such as superoxide anion, nitric oxide (NO), and their reaction product peroxynitrite did not interfere with the anti–*T. gondii* activity of NSC3852. However, inhibition of *T. gondii* tachyzoite propagation by NSC3852 involved redox reactions because tachyzoites were protected from NSC3852 by inclusion of the cell permeant superoxide dismutase mimetic, MnTMPyP, or N-acetylcysteine in the culture medium. We conclude that the Prediction of Activity Spectra for Substances (PASS) computer program is useful in finding new compounds that inhibit *T. gondii* tachyzoites in vitro and that NSC3852 is a potent *T. gondii* inhibitor that acts by indirect generation of oxidative stress in *T. gondii*.

Toxoplasma gondii is an obligate intracellular apicomplexan parasite and the etiologic agent of toxoplasmosis (Petersen, 2007). Rapidly proliferating *T. gondii* tachyzoites propagate by host cell lysis, egress, reattachment, and invasion of new host cells. Human toxoplasmosis is transmitted transplacentally, by ingestion of undercooked meats or contaminated soil bearing sporulated oocysts, or, more rarely, by transplantation of an infected organ (Jones et al., 2001; Dubey et al., 2005; Barsoum, 2006; Rogers et al., 2008). Acute infections in the young and in immunosuppressed persons, including HIV-positive individuals, are life threatening (Kasper and Buzoni-Gatel, 1998; Montoya and Rosso, 2005; Subsai et al., 2006). Pregnant women are particularly vulnerable to infection with *T. gondii*, and this may lead to ocular disease and mental impairments in the newborn or become manifest as the child grows older (Jones et al., 2001; Holland, 2004). Recently, it has become apparent that many additional infected persons are at risk for pathologic sequelae. Latent infections persist as encysted *T. gondii* in the brain and other tissues. *Toxoplasma gondii* exposure has been associated with cognitive and behavioral dysfunction, including schizophrenia (Lindova et al., 2006; Flegr, 2007; Mortensen et al., 2007; Torrey et al., 2007; Torrey and Yolken, 2007). *Toxoplasma gondii* infections are also recognized as a risk factor for cardiovascular disease (Portugal et al., 2004; Yazar et al., 2006; Franco-Paredes et al., 2007). Based on antibody titers, it has been estimated that 20–30% of people worldwide have been infected by *T. gondii*, and, regionally, the prevalence can rise to 80% or higher (Jeannel et al., 1988; Saygi, 2001; Francisco Fde et al., 2006; Jones et al., 2007; Torrey et al., 2007). *Toxoplasma gondii* control measures and treatment options are rather limited considering the impact of this pathogen on human health.

Macrophages and drugs that produce oxidative stress limit the pathogenesis of *T. gondii* and related *Plasmodium falciparum* apicomplexan parasites. Immunologic defense against acute toxoplasmosis requires a robust CD8+ T-cell response and interferon-gamma-mediated activation of macrophages (Beaman et al., 1994; Abou-Bacar et al., 2004; Buzoni-Gatel and Werts, 2006; Miller et al., 2006). Activated macrophages exert anti–*T. gondii* activity by producing reactive oxygen species (ROS) and nitric oxide (NO) (Stafford et al., 2002; Shrestha et al., 2006). Redox active enzymes expressed in *T. gondii* tachyzoites and *P. falciparum* have been scrutinized, and the antioxidant enzymes that are likely to maintain parasite survival during intracellular growth, as well as during their extracellular transit prior to infection of new host cells, have been identified as potential new targets for drug development (Ding et al., 2004; Pino et al., 2007). Existing drugs such as atovaquone inhibit apicomplexans through redox mechanisms (Baggish and Hill, 2002). We showed previously that NSC3852 is a novel antiproliferative and breast tumor cell differentiation agent that generates ROS in human breast cancer cells (Martirosyan et al., 2006). Here, we describe the use of the PASS computer program to find anti–*T. gondii* compounds related to NSC3852, and identify NSC3852 as a potent inhibitor of *T. gondii* tachyzoites as well an inhibitor of *P. falciparum* in vitro.

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MATERIALS AND METHODS

Materials

Stock solutions of all NSC compounds (10 mM) were prepared in DMSO and stored at −20 C protected from light. The 2,3-dimethoxy-

1,4-napthoquinone was supplied by VWR International (West Chester, Pennsylvania). Apocynin (acetovanillone), L-NAME (N^G-nitro-L-arginine methyl ester, HCl), N-acetyl-L-cysteine, luminol, horseradish peroxidase, and superoxide dismutase (*Escherichia coli*) were obtained from Sigma (St. Louis, Missouri). MnTMPyP (Mn[III]tetrakis[1-methyl-2-pyridyl]porphyrin pentachloride), MnTBAP (MnIII 5,10,15, 20-tetrakis[4-benzoic acid] porphyrin), NONOate/AM and nitric oxide assay kits were purchased from EMDBiosciences (La Jolla, California). Promega Cell Titer 96 $AQ_{quesous}$ One Solution was purchased from Fisher Scientific (Suwanee, Georgia). HS68 cells were purchased from the American Type Culture Collection (Manassas, Virginia). Tissue culture media and fetal bovine serum (FBS) were purchased from Mediatech (Herndon, Virginia) and Atlanta Biologicals (Lawrenceville, Georgia), respectively.

Growth of *T. gondii* in cell culture

HS68 human foreskin fibroblasts (passage #2–4) were maintained in RPMI 1640 culture medium supplemented with 1 mM sodium pyruvate, 100 U/ml penicillin, 100 U/ml streptomycin, and 10% FBS. Tachyzoites of the RH strain *T. gondii* were propagated in bovine macrophage (BM) cells and purified for infection assays as described previously (Strobl et al., 2007).

Toxoplasma gondii infection assays were performed with HS68 cells replica plated in 48-well tissue culture plates. HS68 cell monolayers were infected with 0.8–1.5 $\times$ 10^5 tachyzoites/well for 2 hr, and then the medium containing the tachyzoites was removed and replaced with RPMI + 2% FBS + the test compounds or a solvent control. All manipulations involving MnTMPyP and MnTBAP were conducted under low light. After 48–52 hr, the tachyzoites released from the cells into media were fixed in phosphate-buffered saline + 5% formaldehyde, collected by centrifugation, and counted using a hemacytometer. The HS68 monolayer in each well was fixed and stained using 0.5% crystal violet–5% formaldehyde–50% ethanol–0.85% NaCl and then photographed under $\times$250 or $\times$400 magnification using MetaVue software to store the images.

Propagation of *Plasmodium falciparum* in vitro

Plasmodium falciparum 3D7 parasites, obtained from the Malaria Resource Center (Manassas, Virginia), MR4, were cultured in type O-positive blood (Interstate Blood Bank, Memphis, Tennessee). Parasite cultures were maintained by the method of Trager and Jensen (1976). Briefly, parasite cultures were maintained at 1% parasitemia, with 2.5% hematocrit in RPMI 1640 supplemented with 0.5% Albumax I (Invitrogen, Carlsbad, California) and 50 μg/ml hypoxanthine (Sigma-Aldrich). On alternate days, the parasites were subcultured and gassed to maintain pH balance.

For the drug inhibition assays, the parasites were synchronized to the ring stage using 5% sorbitol (Lambros and Vanderberg, 1979). The culture was centrifuged at 3,000 rpm for 3 min, and the pellet was resuspended in 5% sorbitol. This suspension was incubated for 10 min at 37 C and washed 2 times with RPMI to remove traces of sorbitol. This treatment yielded >99% of early to mid-ring stage parasites. The parasitemia was adjusted to 0.1% with 2% hematocrit. The drugs were diluted as 2$\times$ stock solutions (50-ul volume) prepared in complete RPMI media and applied to a U-bottom 96-well plates. Parasites were then added (50 μl per well) to yield a final parasitemia of 0.05% in 1% hematocrit. The cultures were incubated for 60 hr at 37 C to allow the parasites to undergo at least 1 replication cycle.

SYBR green assay

For antiplasmodial drug testing, the plates were centrifuged at 300 rpm for 5 min, and the supernatant was removed by aspiration. The pellet was resuspended by gentle vortexing. For the *T. gondii* tachyzoites, the supernatant from the culture plates was centrifuged and aspirated, and the pellet was used to determine the proliferation of the parasites. To determine the growth increase, a DNA-binding fluorescent probe, SYBR green I from Molecular Probes (Carlsbad, California), was utilized. SYBR green at 1:10,000 dilution prepared in lysis buffer, containing 0.01% saponin and 0.1% triton X 100, was added to the parasite pellets (Bennett et al., 2004). The plates were incubated at 37 C for 30 min to allow complete lysis before being read on a spectrum max fluorescence plate reader with excitation at 460 nm and emission at 530 nm.

MTS assay

The MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium) assay, as described previously (Martirosyan et al., 2004), was used to determine the EC_{50} of NSC3852 in HS68 cell monolayers.

ROS assay

Luminol-derived chemiluminescence (CL) was used to measure ROS produced by tachyzoites and tachyzoite-infected cells according to the procedure described previously (Li and Trush, 1998). Freshly isolated tachyzoites were collected by centrifugation (3,000 g, 15 min at 5 C), suspended in phosphate-buffered saline, and assayed within 30 min. The CL response was continuously measured at 37 C for 15 min with a Berthold 953 luminometer upon adding 10 μM luminol and 10 μg/ml horseradish peroxidase to the reaction in 1 ml phosphate-buffered saline. Data are expressed as integrated area under the curve of the CL response.

NO assays

HS68 cells were replica plated in 6-well tissue culture plates and grown to confluence. For NO measurements, the growth medium was replaced with 2 ml of serum-free, phenol-red free DMEM. After 24 hr, the medium was collected and centrifuged to remove cells, and the supernatant was stored at −20 C until assayed. The cells on the monolayer were harvested by trypsinization, fixed in 5% formaldehyde, and counted using a hemacytometer. NO was measured in the supernatants using the protocol provided by the suppliers. The procedure measures NO as nitrate by a colorimetric assay (the Griess method) after reducing nitrate to nitrite by incubation with nitrate reductase and NADH. NO (μmole produced/10^6 cells/24 hr) was determined using a nitrite standard curve.

Statistical analyses

Concentration-response curves were generated using the mean and standard deviation of duplicate or triplicate determinations in at least 3 independent experiments using PrismGraphPad v. 5 (San Diego, California) least-squares regression method to fit the data to a sigmoidal log concentration-response equation. The statistical significance of effects of inhibitors on *T. gondii* tachyzoite proliferation was evaluated using ANOVA and Dunnett's *t*-test analyses (PrismGraphPad v. 5).

RESULTS

Inhibition of *Toxoplasma* by anticancer quinolines

Table I lists 13 small molecule anticancer quinolines from the NCI compound library and a *P*-value, which is a predictive measure of their antiprotozoan activity obtained from the PASS computer program (Poroikov et al., 2003). We identified these using a 2-step process. First, using the NCI search engine, http://cactus.nci.nih.gov/ncib2, we located all the structural analogs of NSC3852 in this compound library, and, second, we ran the PASS program on these 13 compounds. The higher the *P*-value, the more likely it is that a compound will exhibit the designated biological activity. The predicted antiprotozoan activity of the antimalarial quinoline, chloroquine, was 0.701 and that of NSC3852 was 0.414. The predicted antiprotozoan activity of most NSC compounds identified in this search exceeded that of NSC3852.

The analogs identified as possible antiprotozoan agents were tested at 0.5, 1, and 2.5 μM for the ability to reduce plaque formation in HS68 monolayers and decrease release of tachyzoites into the culture medium. Their structures are presented

TABLE I. Chemical names and predicted antiprotozoan activity of selected quinolines.

NSC#	MW	Chemical name	Predicted antiprotozoan activity
130787	174.15	5-nitroso-6-hydroxyquinoline	.395
3852	174.16	5-nitroso-8-hydroxyquinoline	.414
2039	145.16	8-hydroxyquinoline	.422
53940	190.16	5-(hydroxyl[oxido]amino)-2-hydroxyquinoline	.497
55494	204.18	5-(hydroxyl[oxido]amino)-6-methoxyquinoline	.499
57103	235.16	5,7-bis(hydroxyl[oxido]amino)-8-hydroxyquinoline	.544
74947	190.16	5-(hydroxyl[oxido]amino)-8-hydroxyquinoline	.555
33806	190.16	7-(hydroxyl[oxido]amino)-4-hydroxyquinoline	.555
65583	174.16	5-(hydroxyl[oxido]amino)quinoline	.559
109814	189.17	6-amino-5-(hydroxyl[oxido]amino)quinoline	.559
74950	175.2	5,7-diamino-8-hydroxyquinoline	.566
74949	160.18	5-amino-6-hydroxyquinoline	.578
137449	174.16	7-(hydroxyl[oxido]amino)quinoline	.579

in Figure 1, and the names of the most active compounds are indicated in bold type. NSC3852 and NSC74949 were the most potent compounds of this series and were consistently active in n = 15 and n = 9 independent experiments, respectively. In addition, 1 μM NSC68553 (n = 5), NSC74947 (n = 5), NSC74950 (n = 3), and NSC109814 (n = 3) reduced *T. gondii* tachyzoites and plaque formation in HS68 cell monolayers by 50–90%; however, none of these compounds was as potent or consistently active as NSC74949 or NSC3852. All the active compounds bore a nitrogen substituent in the 5-position of the quinoline ring and, with the notable exception of NSC3852, had a predicted antiprotozoan index of 0.55 or greater (Table I). Of the remaining analogs, none achieved reproducible reductions of more than 25% in plaque formation at the highest concentration screened.

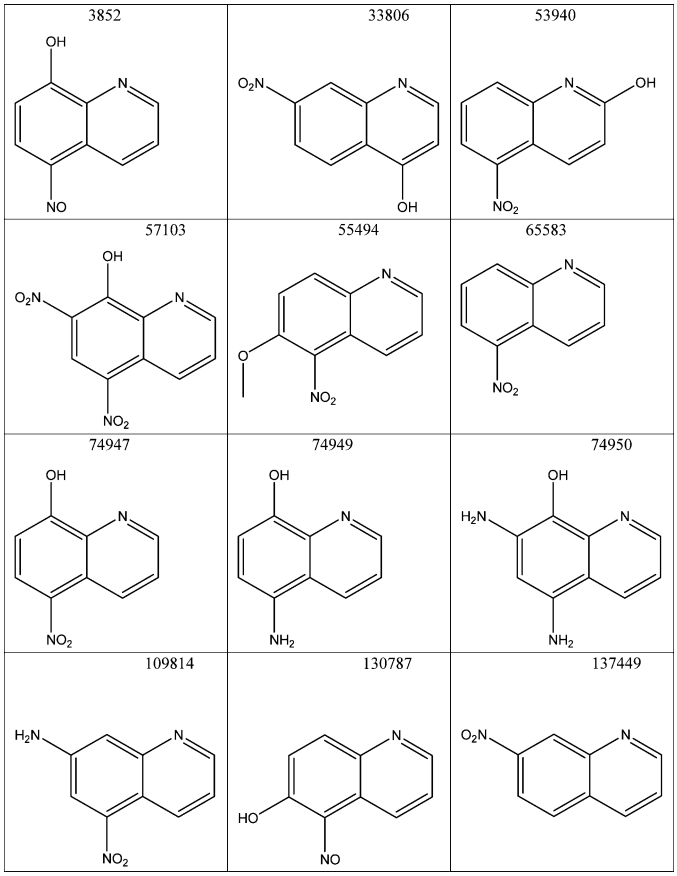

FIGURE 1. Structures of antitumor quinolines. Active anti–*Toxoplasma gondii* compounds are indicated in bold.

Concentration-response experiments with the 2 most active agents were analyzed to estimate IC_{50} for reducing tachyzoite release into the culture medium. The median NSC3852 EC_{50} was 78.6 nM (n = 5) and the median NSC74949 EC_{50} was 646 nM (n = 4). These data indicated that NSC3852 is a potent anti–*T. gondii* agent in vitro. To test whether NSC3852 might have more general anti-apicomplexan activity, its antimalarial activity was investigated. The relative activity of NSC3852 in *T. gondii* and *P. falciparum* was assessed using a SYBR green assay. Quantities of *T. gondii* in the culture medium measured

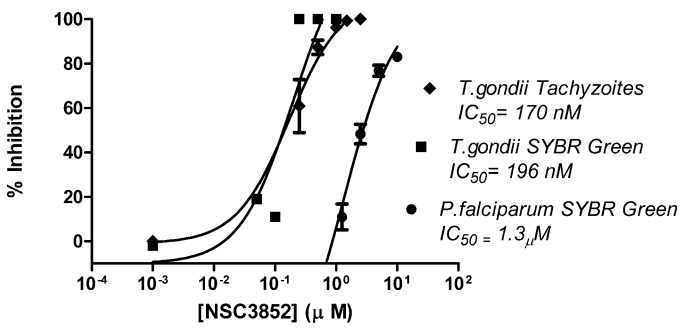

FIGURE 2. Comparison of NSC3852 concentration-response curves in *Toxoplasma. gondii* and *Plasmodium falciparum*. *Toxoplasma gondii*–infected HS68 cells or *P. falciparum*–infected human red blood cells were exposed to NSC3852 for 48 hr or 72 hr, respectively. Inhibition of *T. gondii* tachyzoite numbers (diamonds) or SYBR green fluorescence (squares) and inhibition of *P. falciparum* SYBR green fluorescence (circles) are indicated. The nonlinear regression curves for each data set were generated by PrismGraphPad. The *P. falciparum* data represent n = 3 independent experiments performed in duplicate. The *T. gondii* results shown here are single independent experiments performed with 4 replicates used to compare 2 end points, SYBR green fluorescence and tachyzoite counts.

TABLE II. Nitric oxide production in HS68 fibroblasts.

	Exp. 1	Exp. 2
No production by control HS68 (μmol NO/10^6 cells/24 hr)	25.9 ± 6.8	9.7 ± 1.8
	n = 2	n = 4
NO production relative to control:		
NONOate (30 μM)	3.0	6.7
NSC3852		
250 nM	1.2	0.7
500 nM	0.7	0.9
1,000 nM	1.3	0.9

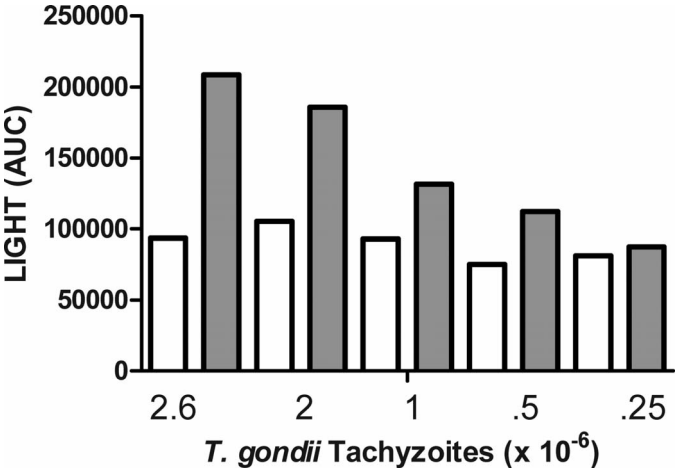

FIGURE 3. ROS production in *Toxoplasma gondii* tachyzoites. Isolated *Toxoplasma gondii* tachyzoites were suspended in 1 ml of phosphate-buffered saline containing 1 μM 2,3-diOMe-1,4 NQ and placed in a luminometer. Reactions were initiated by the injection of luminol (10 μM final concentration) and horseradish peroxidase (10 μg/ml final concentration). Light production was monitored continuously and quantified as the integrated area under the curve using PrismGraphPad. Data shown are representative of 3 experiments. The open bars are *T. gondii* alone, and the gray bars are *T. gondii* stimulated with 1 μM 2,3-diOMe-1,4 NQ.

by tachyzoite counts and SYBR green fluorescence were equivalent (Fig. 2).

The data also show that *P. falciparum* is sensitive to NSC3852 inhibition in vitro as well. The NSC3852 EC_{50} estimated from the SYBR green response curves indicates that *P. falciparum* is ~8 times less sensitive to NSC3852 than *T. gondii*. The reasons for this difference are not known, and we proceeded to investigate the role of oxidative stress in the mechanism of action of NSC38542 using *T. gondii* as a model apicomplexan species.

Detection of ROS production by *T. gondii*

The luminol CL assay is sensitive to ROS production in isolated tachyzoites. We used 1 μM 2,3-diOMe-1,4-NQ, a pure redox cycling quinone (Floreani and Carpenedo, 1991), as a positive control to stimulate isolated tachyzoites (Fig. 3). The tachyzoites generated a clear ROS signal in response to 2,3-diOMe-1,4-NQ that decreased as the number of tachyzoites in the reaction decreased. Unstimulated tachyzoites did not release ROS above background levels; this is reflected in nearly constant CL signals from tachyzoite suspensions between 2.6 × 10^6 and 0.25 × 10^6/ml. The ROS signals were also dependent upon living tachyzoites. Tachyzoites held on ice for more than 1 hr failed to produce ROS signals. Our results show that 1–2 × 10^6 healthy tachyzoites/ml are sufficient to detect ROS production, and this study represents the first demonstration of ROS in isolated *T. gondii* tachyzoites. ROS production by host cells and *T. gondii*–infected host cells was also measurable in response to 2,3-diOMe-1,4NQ (data not shown). However, NSC3852 (10 nM–1 μM) did not stimulate ROS in isolated tachyzoites, and 1 μM NSC3852 did not stimulate ROS in the host cells. We conclude that NSC3852 does not redox cycle in *T. gondii* and that NSC3852 does not stimulate ROS production in normal cells.

NSC3852 anti–*T. gondii* actions are NADPH oxidase- and NOS-independent

NADPH oxidase and nitric oxide synthase (NOS) produce superoxide anion and NO, respectively, which react together to form a highly toxic free radical, peroxynitrite (Heigold et al., 2002). *Toxoplasma gondii*–infected HS68 cells were treated with 250 nM NSC3852 for 48 hr in the presence or absence of the NADPH oxidase inhibitor, apocynin, or the NOS inhibitor, L-NAME, to determine if enzymatic generation of either superoxide anion or NO participated in the mechanism of action of NSC3852. The concentrations of apocynin and L-NAME used were sufficient to inhibit NADPH oxidase and NOS completely (Heigold et al., 2002; Saed et al., 2004).

Toxoplasma gondii–infected cells released tachyzoites and caused disruption of the monolayer (Fig. 4A), and similar results were observed when 1 mM L-NAME or 100 μM apocynin was included in the culture medium (Figs. 4C, E, respectively). The average number of tachyzoites per well released into the culture medium under these 3 conditions did not differ significantly (control HS68, 1.58 × 10^4 ± 0.5; 1 mM L-NAME, 1.89 × 10^4 ± 0.3; 100 μM apocynin, 2.99 × 10^4 ± 0.8, $P > 0.05$). NSC3852 (250 nM) completely inhibited tachyzoite propagation in the HS68 cells alone (Fig. 4B) or in the presence of L-NAME (Fig. 4D) or apocynin (Fig. 4F). Because neither enzyme inhibitor interfered with NSC3852, we concluded that NSC3852's anti–*T. gondii* actions are independent of these enzyme activities. These findings are consistent with the CL assay findings, indicating that NSC3852 does not stimulate ROS in normal *T. gondii* host cells.

Antioxidants interfere with NSC3852 anti–*T. gondii* activity of NSC3852

The cytotoxicity of compounds due to ROS generation can be neutralized by increasing cellular antioxidant capabilities. Superoxide dismutase (SOD) affords protection against oxidative damage by the enzymatic conversion of reactive superoxide anion to hydrogen peroxide. N-acetylcysteine is an antioxidant that raises intracellular levels of the ROS scavenger, glutathione, and also directly binds and inactivates ROS.

Inclusion of a cell permeant SOD mimetic metalloporphyrin,

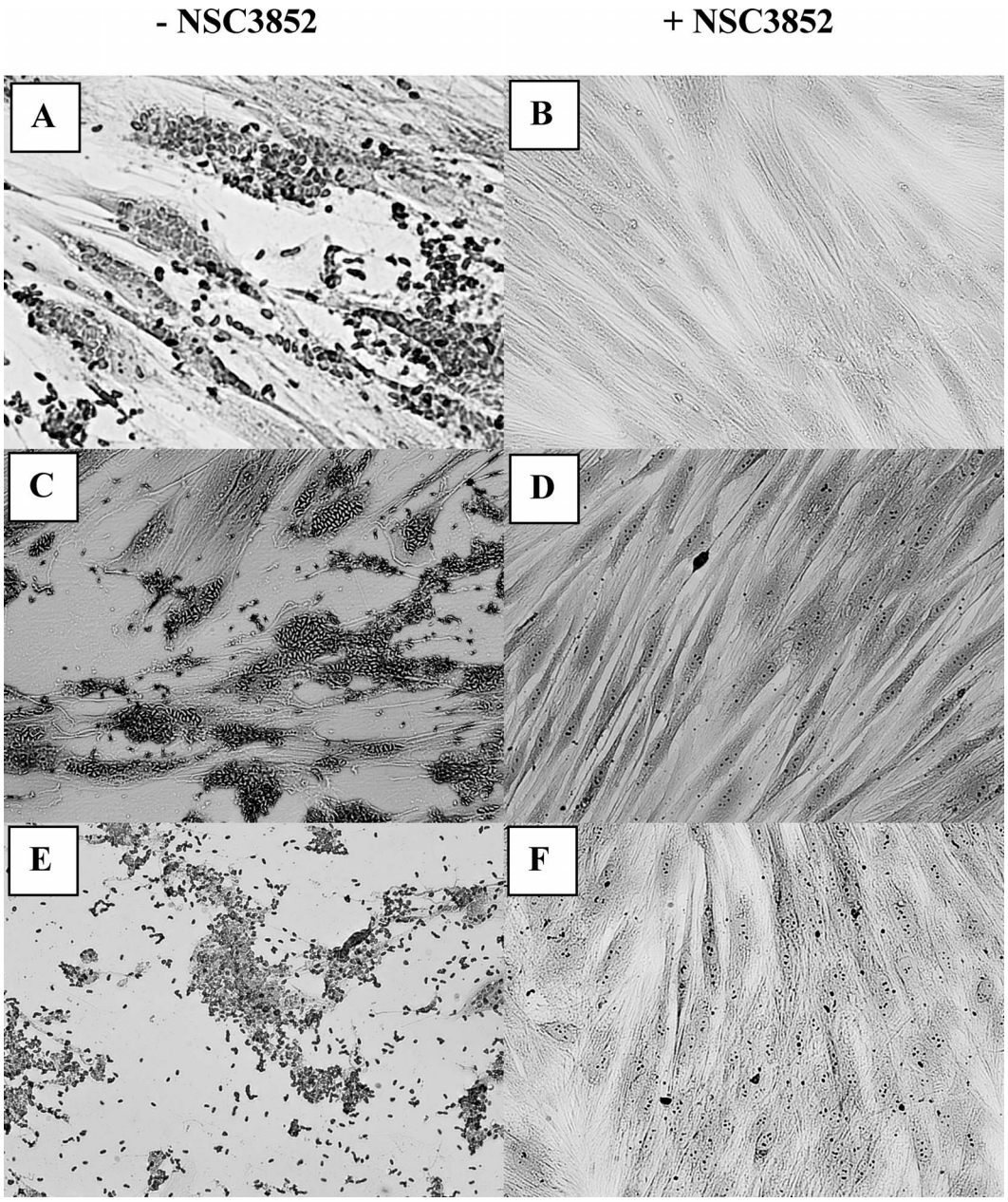

FIGURE 4. Effect of NADPH oxidase or NOS inhibitors on NSC3852 anti–*Toxoplasma gondii* activity. HS68 cells infected with *T. gondii* were treated as follows: (**A**) no additions; (**B**) 250 nM NSC3852; (**C**) 1 mM L-NAME; (**D**) 250 nM NSC3852 + 1 mM L-NAME; (**E**) 100 μM apocynin; and (**F**) 250 nM NSC3852 + 100 μM apocynin.

MnTMPyP, interfered significantly with the anti–*T. gondii* activity of NSC3852. The number of tachyzoites released into the medium in the presence of NSC3852 was increased in a concentration-dependent manner with increasing concentrations of MnTMPyP in the medium (Figs. 5, 6E). Another metalloporphyrin, MnTBAP, recently shown to lack SOD-mimetic activity (Reboucas et al., 2008), had no effect on the anti–*T. gondii* activity of NSC3852 (Fig. 6F). N-acetylcysteine is another antioxidant that interfered with NSC3852 anti–*T. gondii* activity. Both 5 mM N-acetylcysteine (data not shown) and 20 mM N-acetylcysteine in the culture medium increased numbers of tachyzoites released into the culture medium in the presence of NSC3852 (Fig. 6D). Supplementation of the culture medium with the membrane impermeant SOD enzyme itself did not block the activity of NSC3852 (Fig. 6B). These results suggest that NSC3852 produces intracellular oxidative stress, but the reason that *T. gondii* tachyzoites are 43 times more sensitive to this effect (EC_{50} 80 nM) than the host HS68 cells (EC_{50} 3.4 μM) is not yet clear.

NSC3852 bears an organic nitroso group, and a mechanism for catalytic release of NO from aryl-nitroso groups via a porphyrin- and iron-catalyzed reaction has been described (De Biase et al., 2005). It is possible that NSC3852 releases NO. To test this hypothesis, NO levels were measured in the culture

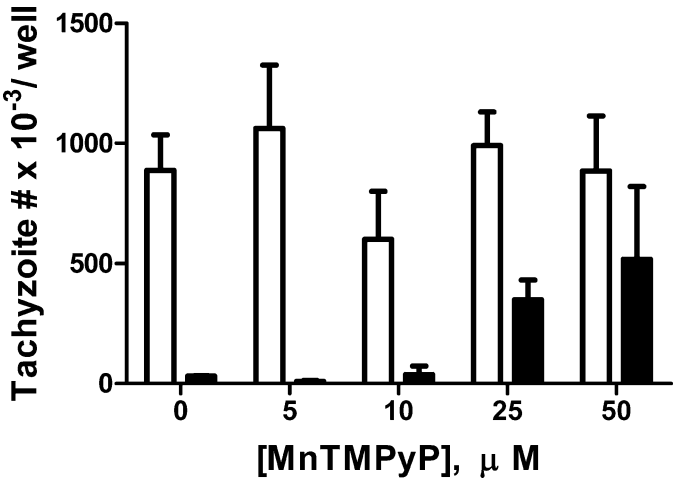

FIGURE 5. Effect of MnTMPyP, a cell permeant SOD mimetic, on NSC3852 anti–*Toxoplasma gondii* activity. *T. gondii*–infected HS68 cells were treated for 48 hr with increasing concentrations (0–50 μM) of MnTMPyP in the absence (open bars) or presence (solid bars) of 250 nM NSC3852.

medium using the sensitive Greiss reaction coupled to a nitrate reductase. HS68 cells produced NO, and elevated NO was detected in response to treatment with a known NO donor, NONOate. We did not detect a statistically significant increase in NO in the medium of HS68 cells treated with anti–*T. gondii* concentrations of NSC3852 (Table II); however, any NO produced by NSC3852 might react within the cells and therefore not be detected in the medium.

DISCUSSION

There is a need for additional drugs to manage *T. gondii* infections. *Toxoplasma gondii* has devastating consequences in various host species, and widespread infections among human and animals provides an opportunity for selection of more pathogenic strains and drug resistance. Dihydrofolate reductase, farnesyl pyrophosphate synthase, and histone deacetylase exemplify drug targets that are shared by human cancer cells and *T. gondii* (Peaslee and Anderson, 2001; Ling et al., 2007; Strobl et al., 2007). Metabolic pathways in intracellular parasites such as *T. gondii* and cancer cells are more dependent upon glycolysis for energy production and more sensitive to oxidative stress than normal cells (Denton et al., 1996; McCarthy and Davis, 2003; Pino et al., 2007). These studies exemplify that anticancer drugs also impact protozoan species and provide a rationale for screening the NCI compound library for new anti–*T. gondii* agents.

The NCI maintains a synthetics library of over 140,000 nonproprietary compounds that are distributed freely for drug-screening purposes. The anticancer and antiretroviral activities of these compounds are known, but their antiprotozoan actions have not yet been annotated (www.nci.nih.gov/dctd/dtp_accomplishments). To begin this process, we utilized the NCI webpage link to the PASS program to predict the antiprotozoan potential of 13 quinolines that we identified as structural analogs of an interesting anticancer compound, NSC3852. The PASS program had been previously validated for the identification of anticancer and antiretroviral activity in the NCI compound library. Poroikov et al (2003) reported that among 250,000 NCI compounds, 12.5% compounds with a PASS *P* value = 0.6 exhibited antiretroviral activity in vitro.

Our work showed 5 of 12 NSC3852 analogs in the NCI compound library had anti–*T. gondii* activity in vitro, and their *P*-values for antiprotozoan activity ranged from 0.555 to 0.578. The most potent NSC3852 analog we found, NSC74949, had a PASS *P*-value of 0.578, indicating that this program can positively identify compounds with in vitro anti–*T. gondii* activity. Four compounds with PASS *P*-values ranging between 0.422 and 0.544 were inactive. However, the parent compound NSC3852, which proved to be the most active among the quinolines tested in vitro, had a PASS *P*-value = 0.414, and NSC137449, which had a PASS *P*-value = 0.579, was inactive. In summary, although the PASS program will generate some false positives and some false negatives, it is useful in screening libraries of antiproliferative chemicals. We recommend using a minimum PASS *P*-value = 0.555 as a screening aid for identifying anti–*T. gondii* compounds in the NCI compound library.

NSC3852 is a potent anti–*T. gondii* agent in vitro. Our results indicate that the mechanism of action of NSC3852 against *T. gondii* differs from its anticancer activity. NSC3852 did not stimulate ROS production in isolated tachyzoites or in normal host cells, and its anti–*T. gondii* activity was apocynin- and L-NAME–insensitive. In contrast, we showed previously that NSC3852 produced superoxide anion in an apocynin-dependent pathway and NO in human breast cancer cells (Martirosyan et al., 2006; J. Strobl, C. Seibert, M. Cassell, A. Rosypal, and D. Lindsay, unpubl. obs.). Thus, despite the ability of human fibroblasts to generate ROS and NO via NADPH oxidase and NOS, respectively (Shen et al., 2006; Sterin-Borda et al., 2007), and the known sensitivity of *T. gondii* to these free radicals (Stafford et al., 2002; Shrestha et al., 2006), NSC3852 works differently against *T. gondii*.

As an alternative mechanism of action for NSC3852 against *T. gondii*, we suggest that NSC3852 produces intracellular oxidative stress by an indirect mechanism. Intracellular antioxidants, MnTMPyP and N-acetylcysteine, protected *T. gondii* from the toxicity of NSC3852. Non–redox cycling quinones, for example 1,4-benzoquinone, cause oxidative stress by arylation of protein sulfhydryls and depletion of intracellular glutathione (Henry and Wallace, 1996). We suggest that in *T. gondii*–infected fibroblasts, NSC3852 is metabolized to a non–redox cycling napthoquinone. This might be mediated by a quinone reductase activity (Maskos and Winston, 1994) or the iron, porphyrin-catalyzed reaction scheme of De Biase et al. (2005). Either reaction would involve the release of NO and the generation of a non–redox cycling napthoquinone capable of depleting glutathione. This model provides a rational explanation for why N-acetylcysteine protects *T. gondii* from NSC3852 toxicity (glutathione repletion), how MnTMPyP protects *T. gondii* from NSC3852 (a free-radical scavenging system), the lack of ROS production in response to NSC3852, and insight into possible mechanisms for the selective toxicity of NSC3852 for *T. gondii*.

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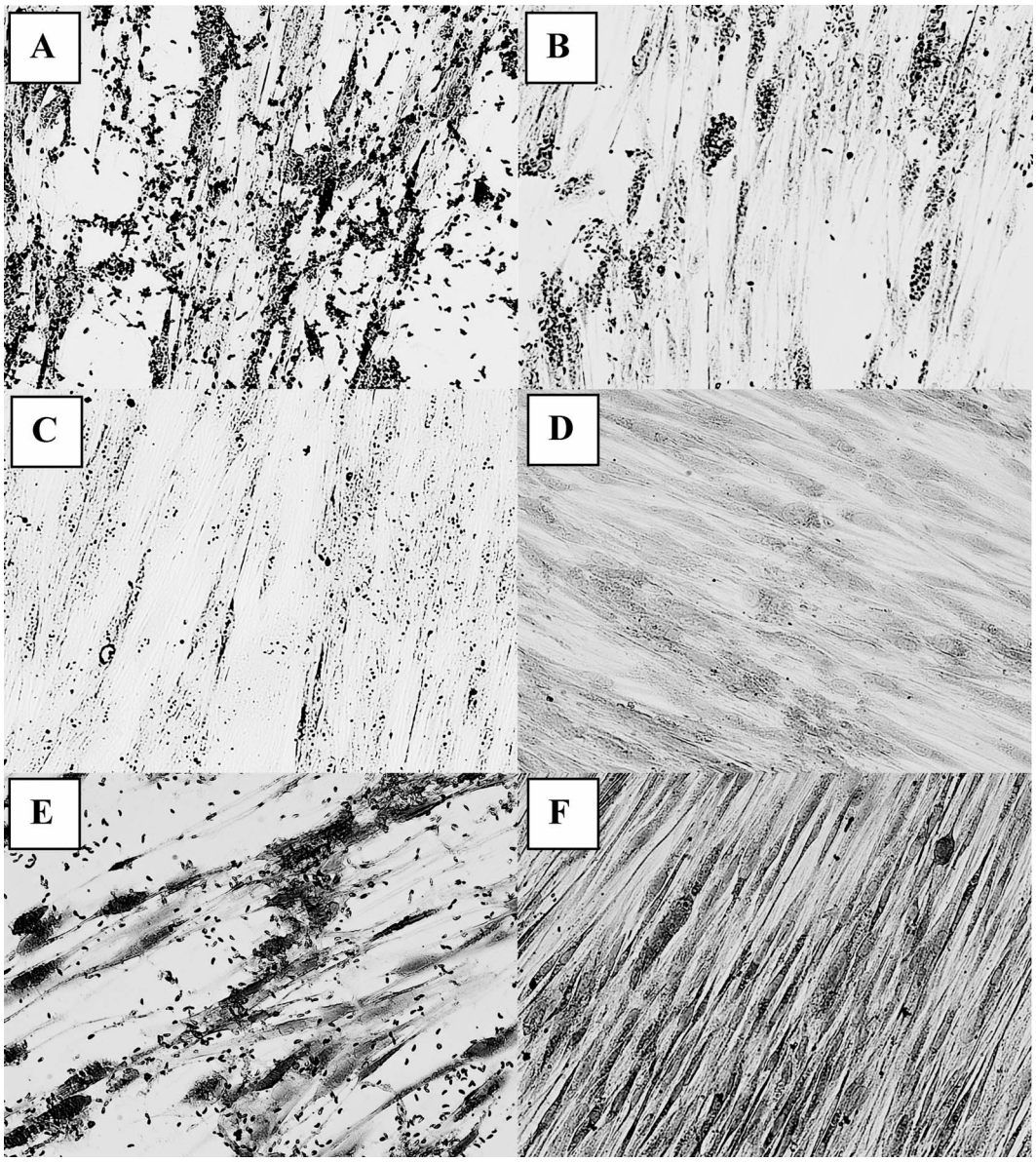

FIGURE 6. Intracellular antioxidants antagonize NSC3852 anti–*Toxoplasma gondii* activity. Panels A–E show HS68 cells infected with *T. gondii*; (**A**) 200 U/ml SOD; (**B**) NSC3852 + 200 U/ml SOD; (**C**) 250 nM NSC3852 only; (**D**) 20 mM N-acetylcysteine + 250 nM NSC3852; (**E**) 25 μM MnTMPyP + 250 nM NSC3852; and (**F**) 25 μM MnTBAP + 250 nM NSC3852.

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