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Discovery of 5-Nitro-6-thiocyanatopyrimidines as Inhibitors of *Cryptococcus neoformans* and *Cryptococcus gattii*

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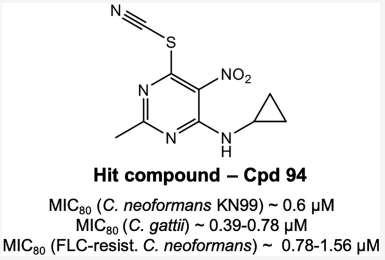
ABSTRACT: Opportunistic infections from pathogenic fungi present a major challenge to healthcare because of a very limited arsenal of antifungal drugs, an increasing population of immunosuppressed patients, and increased prevalence of resistant clinical strains due to overuse of the few available antifungals. Cryptococcal meningitis is a life-threatening opportunistic fungal infection caused by one of two species in the *Cryptococcus* genus, *Cryptococcus neoformans* and *Cryptococcus gattii*. Eighty percent of cryptococcosis diseases are caused by *C. neoformans* that is endemic in the environment. The standard of care is limited to old antifungals, and under a high standard of care, mortality remains between 10 and 30%. We have identified a series of 5-nitro-6-thiocyanatopyrimidine antifungal drug candidates using *in vitro* and computational machine learning approaches. These compounds can inhibit *C. neoformans* growth at submicromolar levels, are effective against fluconazole-resistant *C. neoformans* and a clinical strain of *C. gattii*, and are not antagonistic with currently approved antifungals.



KEYWORDS: *C. neoformans, C. gattii, 4-thiocyano-5-nitropyrimidines, small-molecule antifungal agents, machine learning*

The basidiomycete yeast, *Cryptococcus neoformans*, is a fungal pathogen of immunocompromised people that

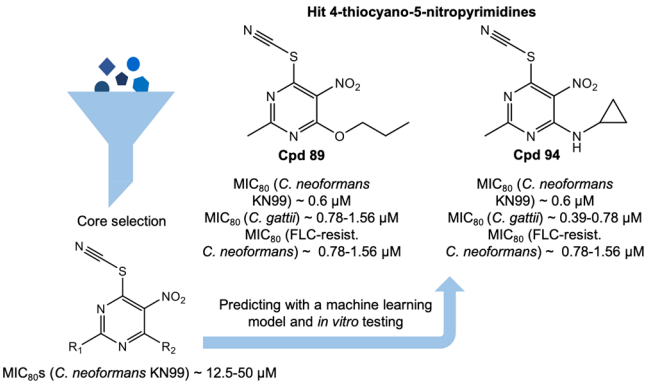


Figure 1. 4-Thiocyano-5-nitropyrimidine core selection and two hit compounds of this class active against *Cryptococcus neoformans* and *Cryptococcus gattii* at submicromolar levels.

each year causes up to 1 million pulmonary infections and meningoencephalitis, which are fatal if untreated and result in up to 250 000 deaths annually.[1] *C. neoformans* was first observed clinically in the 1960s with the advent of organ transplant and aggressive treatment of cancers and other diseases that resulted in immunosuppression.[2] *C. neoformans* is the third leading cause of infections in solid organ transplant patients, where up to 3% develop an invasive fungal infection within the first year, with an overall mortality of 25−40%.[3,4]

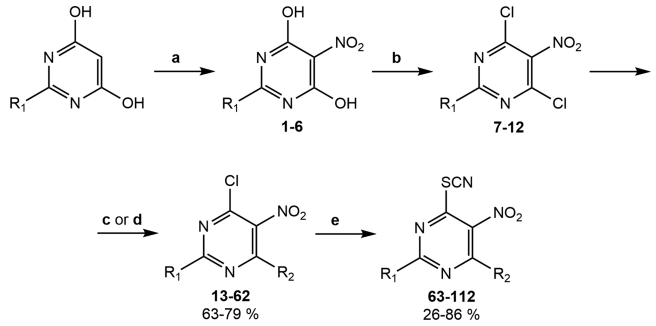


Figure 2. General synthetic scheme of 5-nitro-6-thiocyanatopyrimidines. Reagents and conditions: (a) HNO_3, H_2SO_{4cat}; (b) $POCl_3$, Et_3N, HCl; (c) corresponding amine solution, AcOH, dioxane; or (d) corresponding sodium alkoxide, alcohol; (e) KSCN, alcohol

Transplant patients remain susceptible to *C. neoformans* for 5 years due to its presence in the environment.[5] *C. neoformans* infections can be successfully treated with a combination of amphotericin B (AmB) and flucytosine, but the treatment regimen is long and has significant toxicity. The mortality rate

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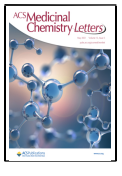

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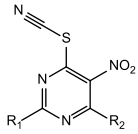
Table 1. *In Vitro* Activity of 2,4-Disubstituted 5-Nitro-6-thiocyanatopyrimidines 63−112 against *Cryptococcus neoformans* and *Cryptococcus gattii*



molecule no.	R_1	R_2	CC_{50} μM (μg/mL)	*C. neoformans* MIC_{80} μM (μg/mL)	*C. gatti*[a] MIC_{80} μM (μg/mL)	*C. neoformans*[a] MIC_{80} (FLC-resistance) μM (μg/mL)
63	H	MeO		9.37 ± 3.12 (1.98 ± 0.66)	>25 (>5.3)	
64	H	PrO	31.8 (7.64)	3.9 ± 0 (0.93 ± 0)	4.68 ± 1.56 (1.12 ± 0.38)	
65	H	iPrO	23.3 (5.6)	1.9 ± 0 (0.45 ± 0)	1.17 ± 0.39 (0.27 ± 0.09)	
66	H	NH_2		4.68 ± 1.56 (0.92 ± 0.3)	1.17 ± 0.39 (0.22 ± 0.07)	
67	H	iPrNH	12 (2.87)	2.34 ± 0.78 (0.55 ± 0.18)	0.58 ± 0.19 (0.13 ± 0.04)	2.34 ± 1.56 (0.55 ± 0.37)
68	H	3-methylbutan-2-yl-NH		3 ± 0 (0.8 ± 0)		
69	H	pentan-3-yl-NH		18.5 ± 0 (4.94 ± 0)	>25 (>6.678)	
70	H	cyclopentyl-NH		4.9 ± 0 (1.29 ± 0)	1.17 ± 0.39 (0.3 ± 0.1)	2.34 ± 1.56 (0.62 ± 0.41)
71	H	cyclohexyl-NH		6 ± 0 (1.67 ± 0)	1.17 ± 0.39 (0.32 ± 0.11)	
72	H	piperidinyl	11.9 (3.16)	2.34 ± 0.78 (0.61 ± 0.2)	1.17 ± 0.39 (0.3 ± 0.1)	4.68 ± 3.13 (1.24 ± 0.83)
73	H	2-Me-piperidinyl		9.37 ± 3.12 (2.61 ± 0.86)	1.17 ± 0.39 (0.32 ± 0.11)	
74	H	2,6-di-Me-piperidinyl		9.37 ± 3.12 (2.74 ± 0.9)	15.62 ± 9.37 (4.58 ± 2.75)	
75	H	3,5-di-Me-piperidinyl	33.6 (9.86)	2.34 ± 0.78 (0.68 ± 0.22)	9.37 ± 3.12 (2.74 ± 0.91)	
76	H	azepanyl	17.7 (4.94)	2.34 ± 0.78 (0.65 ± 0.22)	2.34 ± 0.78 (0.65 ± 0.22)	2.34 ± 1.56 (0.65 ± 0.43)
77	H	azocanyl		4.68 ± 1.56 (1.37 ± 0.46)	2.34 ± 0.78 (0.68 ± 0.23)	
78	H	benzyl-NH	19.6 (5.63)	2.34 ± 0.78 (0.66 ± 0.22)	1.17 ± 0.39 (0.33 ± 0.11)	1.17 ± 0.78 (0.33 ± 0.22)
79	H	(mCF_3-benzyl)-NH	25.91 (9.21)	9.37 ± 12.5 (3.32 ± 4.44)	4.68 ± 1.56 (1.66 ± 0.56)	
80	H	(pCl-benzyl)-NH	22.53 (7.25)	5.85 ± 7.8 (1.88 ± 2.5)	1.17 ± 0.39 (0.37 ± 0.12)	
81	H	Ph-$(CH_2)_2$-NH	15.09 (4.55)	3.51 ± 4.68 (1.05 ± 1.4)	2.34 ± 0.78 (0.7 ± 0.23)	
82	H	Me_2N		4.68 ± 1.56 (1.05 ± 0.34)	1.17 ± 0.39 (0.26 ± 0.09)	
83	H	di-Et-N		9.37 ± 3.12 (2.37 ± 0.78)	0.14 ± 0.04 (0.03 ± 0.01)	
84	H	iPrEtN	10.7 (2.86)	4.55 ± 4.24 (1.4 ± 1.12)	1.17 ± 0.39 (0.3 ± 0.1)	
85	H	cyclohexylMeN	25.3 (7.42)	6 ± 0 (1.76 ± 0)	4.68 ± 1.56 (1.37 ± 0.46)	
86	H	benzylMeN	18.8 (5.66)	2.34 ± 0.78 (0.7 ± 0.22)	1.17 ± 0.39 (0.35 ± 0.12)	
87	H	benzyl-*N*-iPr		>50 (>16.46)	>25 (>8.23)	
88	Me	EtO	19.8 (4.76)	1.17 ± 0.38 (0.27 ± 0.08)	0.29 ± 0.1 (0.06 ± 0.02)	2.34 ± 1.56 (0.56 ± 0.37)
89	Me	PrO	15.8 (4.02)	0.6 ± 0 (0.15 ± 0)	1.17 ± 0.39 (0.29 ± 0.1)	1.17 ± 0.78 (0.29 ± 0.19)
90	Me	iPrO	14.6 (3.71)	1 ± 0 (0.25 ± 0)	1.17 ± 0.39 (0.29 ± 0.1)	1.17 ± 0.78 (0.29 ± 0.19)
91	Me	NH_2	24.5 (5.17)	2.34 ± 0.78 (0.48 ± 0.16)	0.58 ± 0.19 (0.12 ± 0.04)	2.34 ± 1.56 (0.49 ± 0.32)
92	Me	MeNH	>100 (>22.53)	2.34 ± 0.78 (0.52 ± 0.16)	1.17 ± 0.39 (0.26 ± 0.09)	2.34 ± 1.56 (0.52 ± 0.35)
93	Me	pentan-3-yl-NH		9 ± 0 (2.53 ± 0)	9.37 ± 3.12 (2.63 ± 0.88)	
94	Me	cyclopropyl-NH	12.7 (3.19)	0.6 ± 0 (0.15 ± 0)	0.58 ± 0.19 (0.14 ± 0.05)	1.17 ± 0.78 (0.29 ± 0.19)
95	Me	cyclopentyl-NH		9 ± 0 (2.51 ± 0)	2.34 ± 0.78 (0.65 ± 0.22)	

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Table 1. continued

molecule no.	R_1	R_2	CC_{50} μM (μg/mL)	*C. neoformans* MIC_{80} μM (μg/mL)	*C. gatti*[a] MIC_{80} μM (μg/mL)	*C. neoformans*[a] MIC_{80} (FLC-resistance) μM (μg/mL)
96	Me	cyclohexyl-NH		>50 (>24.62)	>25 (>12.31)	
97	Me	pyrrolidinyl		4.68 ± 1.56 (1.23 ± 0.4)	1.17 ± 0.39 (0.3 ± 0.1)	
98	Me	2-Me-piperidinyl		18.75 ± 6.24 (5.49 ± 1.82)	0.58 ± 0.19 (0.16 ± 0.05)	
99	Me	3,5-di-Me-piperidinyl		18.75 ± 6.24 (5.76 ± 1.92)	9.37 ± 3.12 (2.88 ± 0.96)	
100	Me	azepanyl		18.75 ± 6.24 (5.49 ± 1.82)	4.68 ± 1.56 (1.37 ± 0.46)	
101	Me	azocanyl		18.75 ± 6.24 (5.76 ± 1.92)	2.34 ± 0.78 (0.71 ± 0.24)	
102	Me	benzyl-NH	15.87 (4.78)	50 ± 0 (15.06 ± 0)	18.75 ± 6.25 (5.64 ± 1.88)	
103	Me	(pMe-benzyl)-NH	14.73 (4.65)	50 ± 0 (15.76 ± 0)	1.17 ± 0.39 (0.36 ± 0.12)	
104	Me	Ph-$(CH_2)_2$-NH	12.27 (3.87)	50 ± 0 (15.76 ± 0)	2.34 ± 0.78 (0.73 ± 0.24)	
105	Me	Me_2N		4.68 ± 1.56 (1.11 ± 0.36)	2.34 ± 0.78 (0.55 ± 0.18)	
106	Me	di-Et-N		9.37 ± 3.12 (2.5 ± 0.82)	2.34 ± 0.78 (0.62 ± 0.21)	
107	SMe	benzyl-NH	18.59 (6.2)	4.68 ± 4.68 (1.56 ± 1.56)	4.68 ± 1.56 (1.56 ± 0.52)	
108	SMe	(pMe-benzyl)-NH	19.2 (6.67)	9.37 ± 3.12 (3.25 ± 1.08)	4.68 ± 1.56 (1.62 ± 0.54)	
109	SMe	Ph-$(CH_2)_2$-NH	15.39 (5.35)	9.37 ± 9.36 (3.25 ± 3.24)	1.17 ± 0.39 (0.4 ± 0.13)	
110	CF_3	iPrNH	42.8 (13.15)	12.25 ± 6.5 (4.4 ± 2)	>25 (>7.68)	18.75 ± 12.5 (5.76 ± 3.84)
111	Ph	iPrNH		50 ± 0 (15.76 ± 0)		
112	styryl	MeNH		>50 (>15.66)	>25 (>7.83)	

[a]The *C. neoformans* fluconazole-resistant DUMC-158.03, and *C. gattii*, RSA-3615, clinical strains were obtained from Dr. John Perfect, Duke University.[40]

for cryptococcal infections remains 15−30%, even in the context of antiviral treatments for human immunodeficiency virus (HIV).[6−8] The number of AIDS patients infected with *C. neoformans* peaked in the mid-1990s, driven by increased HIV infections. The advent of effective antiretroviral therapies (ART) in 1997 significantly reduced the number of HIV-positive patients with cryptococcosis,[9] with *ca.* 2.9% of AIDS patients in the U.S. positive for the cryptococcal antigen.[10] This reduction has not been observed in resource-limited countries, especially in areas with high disease burden such as sub-Saharan Africa. There, the prevalence of cryptococcal meningitis (CM) in HIV-infected patients is between 25 and 45%.[11] CM causes high mortality among AIDS patients, surpassing tuberculosis deaths in some areas of sub-Saharan Africa.[1]

A closely related *Cryptococcus* species, *C. gattii*, lives in soil and in association with certain trees and can affect the lungs (pneumonia) and nervous system (causing meningitis and focal brain lesions called cryptococcomas) in humans. The main complication of lung infection is respiratory failure. Central nervous system infection may lead to hydrocephalus, seizures, and focal neurological deficit. It can infect immunocompetent people and is endemic in tropical areas. It was first observed in temperate regions in an outbreak on Vancouver Island that has since expanded geographically.[12,13] *C. gattii* causes ongoing infections among immunocompromised patients in southern California and in the southeastern U.S.[2,14−16] Antifungals alone are often insufficient to cure *C. gattii* infections. Cryptococcal infections are therefore difficult to treat, have high mortality, and no new antifungal drugs have been approved since 2003. Hence, there is a dire need to develop new safe and effective drugs for treating cryptococcal species infections.

There are only three approved drugs effective for cryptococcosis, all of which were developed decades ago. AmB, which binds ergosterol, is highly specific for fungi but has very significant toxicity, and its use in resource-poor areas is limited because it is administered intravenously.[17] 5-Fluorocytosine (5-FC) targets pyrimidine biosynthesis, but it is used only in combination with other drugs because of the high rate of resistance evolution. Fluconazole (FLC) also targets ergosterol biosynthesis but is only fungistatic and leaves patients susceptible to relapse. The current standard of treatment is a combination of 5-FC and AmB, but efficacy is limited even in resource-rich settings, where mortality from CM among all patients, regardless of the underlying condition, is between 10 and 35%.[6,7,18,19] Finally, the newest class of antifungals, the echinocandins, is not active against *Cryptococcus*.[20] The current situation has been termed a titanic drug resistance threat.[21] Therefore, there is an urgent need for novel and more effective anticryptococcal therapies.

Multiple research groups are engaged in preclinical studies of anticryptococcal agents and have identified some promising scaffolds.[22−27] In addition, the development of a chemical probe ML407[28] targeting the cell wall may be a useful starting point for drug discovery. Viamet Pharmaceuticals has developed a CYP51 inhibitor, VT-1129, that inhibits the growth of multiple Cryptococcal isolates (as low as 0.015 μg/mL for 50% inhibition and 0.06 μg/mL for 100% inhibition, equivalent to 30−120 nM),[29,30] including *C. neoformans* and *C.*

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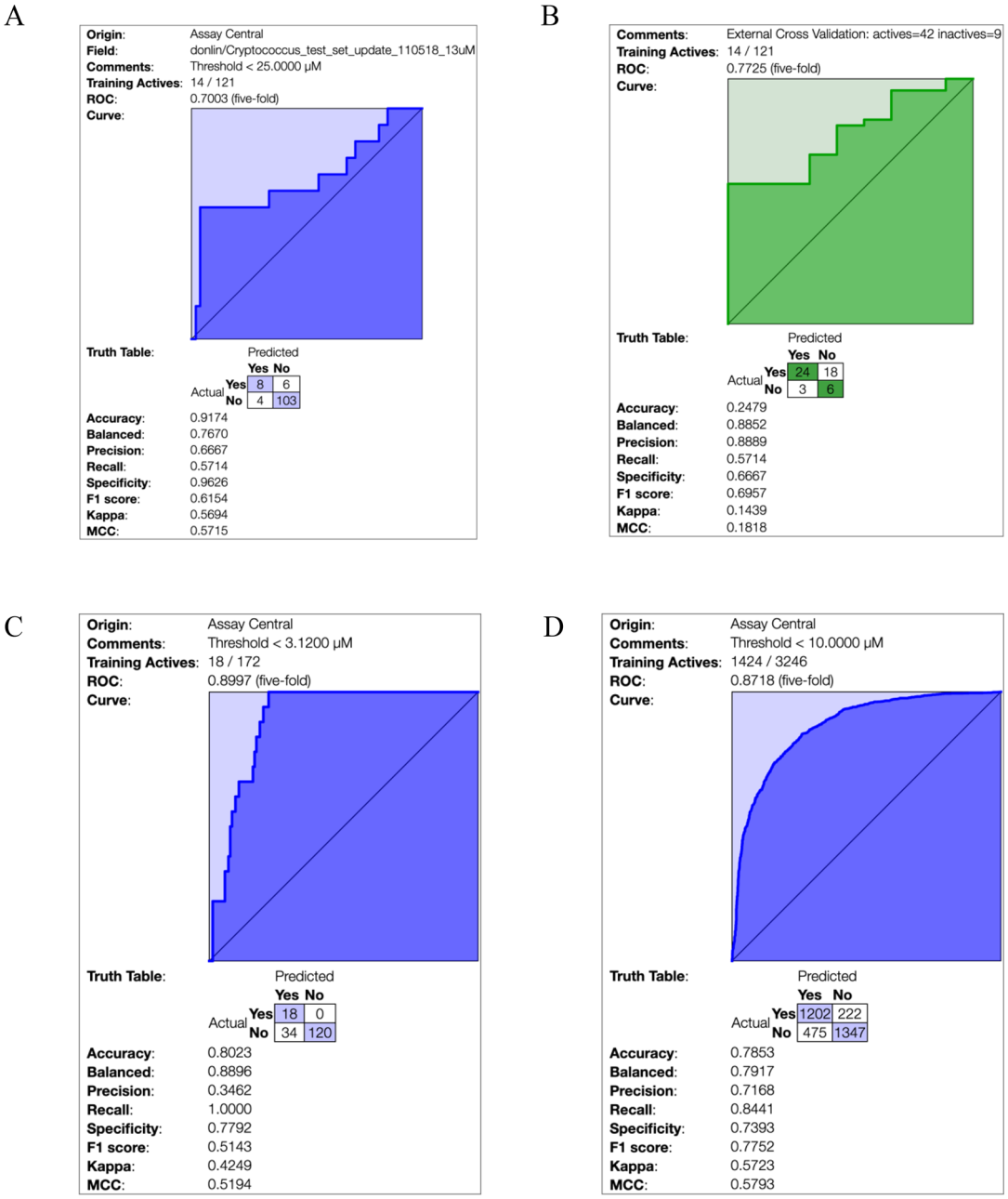

Figure 3. Bayesian model statistics for (A) Cryptococcus model with an MIC_{80} threshold for activity threshold of MIC_{80} 12.5−25.0 μM. (B) "Subvalidation" model statistics for Cryptococcus model using new activity data as a test set. (C) Updated model statistics with new compounds/activity data added with an increased threshold for activity of MIC_{80} 1.52−3.12 μM. (D) Literature cryptococcus model derived from the NIAID ChemDB database.

gattii. VT-1129 also shows activity in a mouse model of CM.[31] VT-1129 has been granted Qualified Infectious Disease Product designation and is currently in phase I clinical trials for treatment of cryptococcal meningitis. However, since the recent acquisition of this company by NovaQuest Capital, the status of this program is uncertain. Among active phase II or phase III clinical trials for treating cryptococcosis, there are only two exploratory treatments that use drugs other than FLC, AmB, or 5-FC. A phase III trial to determine the efficacy of a repurposed serotonin uptake inhibitor, sertraline, for patients with CM (NCT 01802385), was completed in 2019 but saw no improvement in mortality over placebo controls, possibly due to insufficient duration of therapeutic sertraline concentrations.[32] A phase II study to examine efficacy of combinations of tamoxifen, an estrogen receptor agonist, and FLC or AmB (NCT 03112031) was completed in 2019, but no results have yet been posted.[23] No other novel compounds for treatment of cryptococcosis and CM have reached IND status. Thus, it is imperative that we increase the number of new classes of antifungals drug candidates and stimulate the pipeline for treatment of cryptococcosis in general.

In the current study, we have utilized a Bayesian machine learning approach, which classifies data as active or inactive based on user-defined thresholds using a simple probabilistic classification model based on Bayes' theorem. Three early studies by us used Bayesian machine learning models to identify molecules with promising *in vitro* activity for *Mycobacterium tuberculosis*,[33] *Trypanosoma cruzi*,[34] and Ebola

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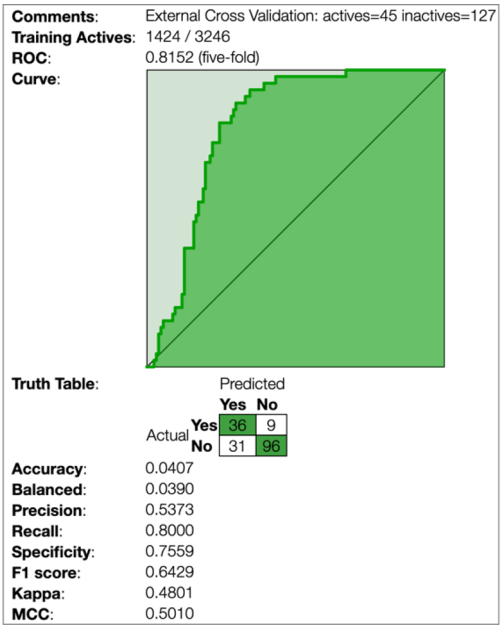

Figure 4. Statistics for the Cryptococcus data from this study when used as an external test set using the literature cryptococcus model derived from the NIAID ChemDB database as a training set (10 μM activity threshold).

virus.[35] Most recently, we have used this software to assist in drug discovery for chordoma,[36] *Neisseria gonorrheae*,[37] HIV,[38] and *Staphylococcus aureus*.[39] This study represents our first attempt at applying this approach to antifungal drug discovery.

We have now identified a series of 5-nitro-6-thiocyanatopyrimidine antifungal drug candidates using *in vitro* and computational machine learning approaches that have been shown to inhibit *C. neoformans* and *C. gattii* growth at submicromolar levels (Figure 1). These may represent a starting point for further hit-to-lead optimization and target identification.

Synthesis of 5-Nitro-6-thiocyanatopyrimidines. 2,4-Disubstituted 5-nitro-6-thiocyanatopyrimidines studied here were synthesized in four steps according to Figure 2. The starting 2-substituted 4,6-dihydroxypyrimidines were nitrated at the 5-position using fuming nitric acid and sulfuric acid in a catalytic amount. Further treatment of these intermediates with phosphorus oxychloride resulted in the formation of 2-substituted 4,6-dichloro-5-nitropyrimidines **7**−**12**. At the next stage, 4,6-dichloropyrimidines were reacted with the corresponding amines in the acetate form in dioxane (Figure 2c) or with the corresponding sodium alkoxides in alcohol (Figure 2d), which led to the replacement of only one chlorine atom in the 4-position to amines or alcohols. The final 5-nitro-6-thiocyanatopyrimidines **63**−**112** (Table 1) were obtained by nucleophilic substitution of the second chlorine atom with potassium thiocyanate in good yields.

In Vitro Assays and Preliminary Structure−Activity Relationships Study. Using a whole-cell phenotypic screen approach, we tested a set of 121 chemically diverse compounds from our laboratory library and measured the MIC_{80} of these compounds against the pathogenic *C. neoformans* laboratory strain KN99.[28] We identified that 17 out of 121 hits show activity with the MIC_{80} values less than or equal to 50 μM. Among them, the 5-nitro-6-thiocyanatopyrimidines represent the most interesting series for further research. Next, we synthesized a number of compounds based on the scaffold for the primary study of the structure−activity relationship. We found that only compounds with small substituents, such as a hydrogen atom or a methyl group at position 2 of the scaffold, exhibit favorable *in vitro* activity, while compounds with bulky groups at the same position were less active. Moreover, the volume and length of the substituents at position 4 also influence the antifungal activity. So, compound **94** with 2-methyl group and 4-cyclopropyl-NH substituent appears to have the most promising MIC_{80} of 0.6 μM for *C. neoformans* and MIC 0.39−0.78 μM for *C. gattii*. The MIC_{80} was 0.79−1.56 μM against a fluconazole (FLC) resistant strain with a CC_{50} of 9.6 μM in human hepatoma cells resulting in a specificity index, SI = 16, for *C. neoformans*. Compound **94** was also tested for synergy in a checkerboard assay with AmB and FLC, where the FICIs were calculated to be 1.25 and 1.5, respectively (data not shown). These data suggest an indifference but, importantly, no antagonism between compound **94** and either AmB or FLC.

Machine Learning. Assay Central was used to generate machine learning models that used a Bayesian algorithm and ECFP6 fingerprints alone, for the 121 compounds with *C. neoformans* data. The threshold for actives was MIC_{80} 12.5−25.0 μM. The 5-fold cross validation ROC was 0.70, and other statistics (Figure 3A, Supporting Information (SI), Table S1) were acceptable for such a relatively small data set. This data was then used to score a set of 51 additional analogues (external set test ROC 0.77, Figure 3B). From past experience, as we add more data to the machine learning model the cross-validation statistics generally improve (172 compounds, ROC 0.90, Figure 3C) and generate better predictive models for optimization of the molecules. We are thus able to lower the threshold for an active to MIC_{80} < 3.12 μM in order to predict more potent compounds using this iterative approach.

We have also generated a Bayesian model with over 3000 compounds with data from the NIAID ChemDB HIV, Opportunistic Infection and Tuberculosis Therapeutics Database, with excellent statistics overall at a threshold of 10 μM (ROC 0.87, Figure 3D), which is potentially useful for exploring more chemical diversity because of the broader makeup of the training set. We used these data from the 172 compounds tested as an external test set for this training model, which yielded reasonable statistics (ROC 0.81; Figure 4).

The molecules of most interest highlighted in this study (Table 1) were used with this literature *C. neoformans* model with a cutoff of 10 μM (ROC 0.62, SI, Figure S1A) or 3.2 μM (ROC 0.82, SI, Figure S1B). The predictions and model domains (a measure of applicability) can be seen in more detail in SI, Table S2. As we also generated *in vitro* data for *C. gattii* in this study, we used this set from Table 1 to construct a preliminary model with a cutoff at MIC 1.52−3.12 μM (ROC 0.62, SI, Figure S2). These machine learning models will be put to further use for virtual screening of compound libraries of commercial molecules in order to find additional molecules for testing against these fungi. These Bayesian models can also be used to help us further optimize the current chemical series alongside models for ADME/Tox properties.

During recent patent preparation, we became aware of a Russian patent describing 2-nitroheterylthiocyanates with activity against *Candida*, *Aspergillus*, and *Fusarium* strains only.[41] Interestingly, they did not test against *Cryptococcus*

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strains, and we also describe many more derivatives as well as the structure−activity relationship.

We have now described new 5-nitro-6-thiocyanatopyrimidine antifungals including compound **94**, which has a submicromolar MIC_{80} for *C. neoformans*, a FLC-resistant strain of *C. neoformans*, and *C. gattii* as a starting point for future optimization and evaluation. Thiocyanate ions are ubiquitous,[42,43] and our antifungals contain an isothiocyanate group which may be important for this activity and may not be cytotoxic. We have shown that we can separate antifungal activity from cytotoxicity (Table 1) and understanding the mechanism of action of these molecules and whether the thiocyanate represents a leaving group responsible for the activity will be addressed in future studies.

EXPERIMENTAL PROCEDURES

All experimental procedures are described in Supporting Information.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.1c00038.

Full experimental procedures, analytical data of all the compounds, description of machine learning software methods, supplemental references, supplemental figures and tables describe the machine learning model results as well as comparisons with *in vitro* data PDF)

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Author Contributions

O.R., A.L., and V.M. synthesized the molecules. E.X., J.L., and M.D. generated biological data, T.R.L. performed all machine learning work. M.D., V.M., and SE designed the study and wrote the manuscript.

Notes

The authors declare the following competing financial interest(s): S.E. is owner, and T.R.L. is an employee of Collaborations Pharmaceuticals, Inc. All others have no competing interests. We have filed a provisional patent on this work.

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ABBREVIATIONS

(AmB), amphotericin B; (ART), antiretroviral therapies; (CM), cryptococcal meningitis; (FBS), fetal bovine serum; (FICI), fractional inhibitory concentration index; (FLC), fluconazole; (5-FC), 5-fluorocytosine; (HIV), human immunodeficiency virus; (MIC), minimal inhibitory concentration

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