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Structural Determination of NSC 670224, Synthesis of Analogs and Biological Evaluation

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In the search for new lead compounds for drug discovery, small molecule and natural product libraries of known biologically active compounds are being investigated by high-throughput (HT) screening methods for potential new uses. One such screening protocol employs the budding yeast *Saccharomyces cerevisiae* as a platform that is both convenient and broadly applicable. Yeast and mammalian cells have many conserved cellular processes, yeast deletion mutants are widely available and profiling samples across the genome-wide yeast deletion libraries can facilitate target identification.^[1-3]

Previously published work identified a group of 46 known compounds as toxic towards *Saccharomyces cerevisiae* through the use of a HT yeast halo assay^[4] on the National Cancer Institute's Diversity Set I, Mechanistic Set and Natural Products Set comprising a total of 3,104 synthetic compounds and natural products.^[5] Genome-wide chemical sensitivity profiling^[6] of these hits against the library of yeast deletion mutants highlighted two compounds that showed very similar profiles: tamoxifen (NSC-180973) and NSC 670224. In human cells a good correlation between the two compounds in the NCI 60 cell line screen (correlation coefficient = 0.55) was found using the COMPARE algorithm^[7] (Figure S1). These data suggest that the compounds may share a target in human cells that, based on the yeast profiling data, is not necessarily related to the estrogen receptor.

Tamoxifen has been a highly effective treatment for estrogen receptor (ER) positive breast cancer for over 30 years and its mechanism of action in these cases is relatively well understood. However, in some cases, tamoxifen has been shown to be effective against ER negative breast cancer, which seems to indicate an alternative mechanism of action or target. Furthermore, the genome of *Saccharomyces cerevisiae* does not encode any protein with significant homology to the human estrogen receptor. Although there are reports connecting the bioactivity of tamoxifen in yeast to calcium signaling,^[8,9] our analysis pointed toward an alternative pathway that accounts for the activity of both tamoxifen and NSC 670224. To our knowledge, there are no published reports on the bioactivity of NSC 670224.

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Analysis of the library sample containing NSC 670224 indicated a pure isomer that was not stereochemically defined in the structure deposited on the NCI database. Our interest in probing the possible mechanistic link between tamoxifen and NSC 670224 led us to undertake the synthesis of both diastereomers of the nominal structure (Figure 1) for stereochemical determination and biological assay. Herein we describe our results, which have led to 1) identification of the correct structure of the compound found in the NCI library, 2) production of a targeted library of NSC 670224 derivatives for biological study, 3) yeast lethality data for the compounds prepared in this study, and 4) preparation of a compound related to NSC 670224 that is capable of derivative formation for protein target identification.

Initially we explored the reaction of Grignard reagents with ketal **1** as a direct route to the desired structure class, as had been reported in the patent literature.^[10] Commercially available benzylmagnesium chloride was reacted with **1** and provided the expected diastereomeric mixture of primary alcohols *cis*- and *trans*-**2**, which were separated by chromatography (Scheme 1). Single crystal X-ray analysis of the first eluted primary alcohol confirmed the structure to be *cis*-**2**. Mesylation, displacement with aqueous dimethylamine,^[11] and formation of the hydrochloride salt were conducted on each diastereomer of **2** to give *cis*- and *trans*-**3**. However, this synthetic route, which delivered the first compounds for our future structure-activity relationship (SAR) study, could not be employed toward NSC 670224 as the requisite dichlorobenzyl halide suffered from severe homocoupling during the formation of the corresponding Grignard reagent.

A longer, but ultimately more fruitful, procedure was developed from the reaction between 2,4-dichlorobenzyl chloride and magnesium in the presence of LiCl and ZnCl₂, as defined by Knochel and coworkers (Scheme 2).^[12] The resulting organozinc reagent reacted cleanly with 4-*tert*-butylcyclohexanone to afford tertiary alcohols *cis*- and *trans*-**4** (73%, approx. 1:1) which, although separable by column chromatography, were taken through the next steps as a mixture. As with compound **2**, X-ray analysis confirmed that *cis*-**4** was the first isomer to elute. Allylation of the tertiary alcohols with KH, NaI and allyl bromide^[13] gave compounds **5** in 75% yield. The mixture of *cis*- and *trans*-**5** was subjected to catalytic osmylation using OsO₄/NMO, followed by oxidative cleavage of the crude diols with NaIO₄/SiO₂^[14] and reduction of the crude aldehyde with NaBH₄ to give primary alcohols *cis*- and *trans*-**6**, which proved easily separable by column chromatography. Mesylation, displacement, and formation of the hydrochloride salt were conducted on each primary alcohol as described for the benzyl compounds **2** to provide *cis*- and *trans*-**7**.

To our surprise, the analytical data of NSC 670224 matched neither synthetic diastereomer of **7** (Figure S2), although mass spectrometry indicated that compounds **7** were isomers of the NCI library sample. Particularly relevant were the ¹H NMR differences in the aromatic region of the spectrum, which led to the consideration of the 3,4-dichloro isomer as the true structure of NSC 670224. The *cis* isomer of **7**, the structure of which was confirmed by single crystal X-ray analysis, was again the diastereomer that eluted first during chromatography.

Fortunately, the synthetic route devised for the production of **7** could be employed without difficulty for the production of the 3,4-dichloro isomers as depicted in Scheme 2. Thus, *cis*- and *trans*-**8** were formed in 73% yield as a separable 1:1 mixture of isomers, of which *trans*-**8** was highly crystalline. Each individual isomer of **8** was subjected to the sequence of reaction conditions that led to the preparation of **7**, including formation of allyl ethers **9** and primary alcohols **10** prior to installation of the terminal dimethylamine functionality that defines the final targets *cis*- and *trans*-**11**. Single crystal X-ray analysis of *cis*-**10** established

the identity of both isomers. Analytical data supports the true structure of NSC 670224 to be *cis*-**11**.

These synthetic efforts afforded a collection of compounds that varied in stereochemistry and identity of the benzyl substituent. Yeast biological activity studies (Table 1 and Figure S3) verified the greater activity of the original library structure *cis*-**11** as compared to both isomers of **3** and *cis*-**7**. The lack of chlorine substituents on the benzyl group is shown to lower the activity of compounds **3** significantly. Within the most active compounds, the *trans* isomer is consistently more active than the *cis*. Hence, *trans*-**11** was found to be more potent than both NSC 670224 and tamoxifen in yeast, and therefore was given the name “tamoxilog” to identify both its activity profile and its structure as the origin of our further SAR studies.

To that end, the decision was made to probe the importance of the choline chain, since this functionality is common to both NSC 670224 and tamoxifen (Scheme 3). The synthesis of homolog **14** began by mesylation of *trans*-**10** and displacement with KCN in DMSO as depicted in Scheme 3.^[15] Nitrile **12** was partially reduced to the aldehyde with DIBAL and the crude aldehyde reduced to primary alcohol **13** in poor yield over two steps.^[16] Compound **13** was aminated similarly to **6** and **10** to give homolog **14**. The synthesis of derivatives **15**-**18** utilized the respective secondary amines in displacement of the mesylate formed from *trans*-**10**, followed by formation of their hydrochloride salts. Compounds **14**-**18** were subjected to yeast lethality assay (Scheme 3 and Figure S3). None of the derivatives matched the activity of tamoxilog, although both homolog **14** and pyrrolidine analog **15** are quite active. This activity profile is supported by growth inhibition studies, which measure yeast growth at a set concentration of agent (5 μ M) over a particular time course (Figure 1). Morpholino-derivative **17** is nearly devoid of activity, showing yeast growth on a par with the DMSO standard. Tamoxilog, homolog **14** and pyrrolidine derivative **15** all present highly active profiles.

The activity data clearly indicate important contributions made by both the choline chain and the chlorinated benzyl substituent. Consequently, it seemed reasonable to suggest that modifications of the *tert*-butyl group would not affect the biological activity substantially. This was important since there is great interest in determining the cellular target of tamoxilog, which would require a biologically active derivative for molecular probe synthesis. An alcohol functionality was chosen as the handle on tamoxilog (*trans*-**11**) from which future probe molecules would be prepared through an ester linkage which, if necessary, could be cleaved under mildly basic conditions.^[17] To test this hypothesis, a simple hexanoate ester derivative of tamoxilog was prepared and subjected to LC₅₀ biological analysis. Synthesis of the potential probe molecule is outlined in Scheme 4.

Using the method of Engel and Schexnayder,^[18] deprotonation of methyl isobutyrate with LDA and reaction with mono-ethylene glycol protected 1,4-diketocyclohexane gave the corresponding alcohol. After work-up, the crude alcohol was dehydrated with thionyl chloride in pyridine to give alkene **19** in 73% yield over two steps. Attempts to reduce the tri-substituted double bond of compound **19** via catalytic hydrogenation were unsuccessful; even at elevated pressures. However, deprotection of the ketal with catalytic PTSA gave a mixture of the deprotected trisubstituted alkene together with the corresponding Δ^2 -unsaturated isomer, which could be reduced at atmospheric pressure with hydrogen and Pd/C. Due to the appearance of a significant amount of an unknown side-product under catalytic PTSA conditions, **19** was deprotected with PPTS in refluxing acetone/water.^[19] The crude alkene was isomerized in refluxing dichloromethane with DBU^[20] to give Δ^2 -unsaturated ketone **20** in 88% yield over two steps. Finally, reduction of **20** with Pd/C and hydrogen at 1 atm gave keto-methyl ester **21** in 96% yield.

It was originally envisioned that the methyl ester would mask the alcohol through the remaining steps. However, introduction of the aryl substituent using Knochel's conditions^[12] provided the desired tertiary alcohols as an inseparable mixture.

Therefore, the crude mixture was reduced with LiAlH_4 to give the mixture of diols **22**, which could be separated efficiently by column chromatography. The *cis* and *trans* diastereomers of **22** were mono-protected with MOM-Cl to give *cis*-**23** and *trans*-**23** in 84% and 71% yield respectively. Although it was envisioned that only the *trans* diastereomer would be taken through to the desired material, *cis*-**23** was found to give X-ray quality crystals, thus allowing for the stereochemical determination of each species. Allylation of *trans*-**23** with KH, allyl bromide and catalytic NaI gave allyl ether **24** in 96% yield. Conversion of the allyl ether to primary alcohol **25** was conducted over three steps in 90% yield in accordance to a published procedure by Cossy et al.^[21] Mesylation of **25** and displacement of the product with dimethylamine gave crude MOM protected amine, which was deprotected in a mixture of HCl/methanol/water. The oil that remained after deprotection was passed through a deactivated silica gel column (1% triethylamine in eluent) to give the free amine. The amine was converted to the hydrochloride salt with HCl gas in ether, and after recrystallization, the desired alcohol **26** was obtained in 72% over four steps. Alcohol **26** could be coupled with commercially available hexanoic acid in DMF with EDCI and catalytic 4-DMAP. Purification with preparative HPLC gave the desired hexanoate ester **27** in 32% yield. Biological evaluation of compound **27** showed the compound to be active, with an LC_{50} of $13.0 \mu\text{M}$ (Figure S4).

The synthetic work described in this paper has clarified the erroneous structural annotation of NSC 670224 in the NCI library. In addition, the preparation of a focused library of derivatives and a molecule capable of use for affinity probe synthesis (**26**) set the stage for biological evaluation and target identification of tamoxilog. Probe synthesis and full biological studies are ongoing and will be disclosed in due course.

Experimental Section

Yeast Studies. Growth Assays

Wild type cells (strain BY474)^[22] were grown in YPD media at 30 °C. Cells were counted with a hemocytometer and cultures were diluted to 8×10^5 cells/mL. The desired concentration of tamoxifen, tamoxilog and related compounds ($5 \mu\text{M}$) or DMSO was added and cultures were incubated at 30 °C with rotation. Optical density at 600 nm was recorded every 1.5-2h, starting at the time of dilution.

LC_{50} Values

Strain BY4741 (195 μL) was plated into a 96 well plate at an OD_{600} of 0.1. Desired compound (5 μL) was added to each well and two-fold serial dilutions of each compound were tested. OD_{600} readings were taken every 30 min with an EnVision plate (PerkinElmer). After 16 h of incubation the OD_{600} was plotted against concentration in Prism (GraphPad) to generate a dose response curve and calculate the LC_{50} .

Full details of methods and experimental conditions are provided in the supplementary information. CCDC 862851-862855 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif, or by contacting The Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

a) Antibreast cancer drug, tamoxifen (NSC 180973) and b) the nominal structure of NCI compound NSC 670224.

Figure 2. Average growth of wildtype yeast cells over 9.5 h at 5 μ M concentration of agent, as measured by optical density of 600 nm.

Scheme 1.

Grignard Addition to Ketal. Conditions: a) MsCl, NEt₃, DCM; b) aq. HNMe₂, refluxing THF; c) HCl gas/ether, 55% over 3 steps.

Scheme 2.

Synthesis of *cis/trans* **7** and **11**; Conditions: a) KH, NaI, 1,2-DME, 0 °C; then allyl bromide, 0 °C to rt; b) OsO₄/NMO, dioxane (**5**) or AD-mix-β, *t*-BuOH/H₂O (**9**); c) NaIO₄, SiO₂, DCM; d) NaBH₄, MeOH, 0 °C; e) MsCl, NEt₃, DCM; f) aq. HNMe₂, refluxing THF; g) HCl gas/ether.

Scheme 3.

Synthesis and activity against *S. cerevisiae* of analogs **14-18**. Conditions: a) MsCl, NEt₃, DCM; b) KCN, DMSO, 65 °C; c) DIBAL, hexanes, -78 °C; d) NaBH₄, MeOH, 0 °C; e) MsCl, NEt₃, DCM; f) aq. HNMe₂, refluxing THF; g) HCl gas/ether.

Scheme 4.

Synthesis of modified tamoxifen. Conditions: a) LDA, -78°C ; c) SOCl_2 , pyridine; d) PPTS, acetone/water (9:1) reflux; e) DBU, DCM, reflux; f) Pd/C, H_2 , 1 atm; h) LAH, 0°C to rt; i) MOMCl, Hunig's base; j) KH, NaI, 1,2-DME, 0°C ; then allyl bromide, 0°C to rt; k) OsO_4 /NMO; NaIO_4 ; l) NaBH_4 , MeOH, 0°C ; m) MsCl , NEt_3 , DCM; n) aq. HNMe_2 , refluxing THF; o) HCl, MeOH, H_2O ; p) HCl gas/ether; q) EDCI, 4-DMAP; $\text{C}_5\text{H}_{12}\text{CO}_2\text{H}$, 0°C to rt.

Table 1Tamoxifen and Synthetic Derivative Lethality Studies on Wild Type *S.cerevisiae*

Compound	LC ₅₀ (μ M)
<i>cis</i> - 3	26
<i>trans</i> - 3	29
<i>cis</i> - 7	6.7
<i>trans</i> - 7	2.5
<i>cis</i> - 11	(NSC 670224) 3.2
<i>trans</i> - 11 ('tamoxilog')	2.3
Tamoxifen	4.1