AD_____

Award Number: DAMD17-00-1-0412

TITLE: Novel Thioredoxin Inhibitors for Breast Cancer Therapy

PRINCIPAL INVESTIGATOR: John S. Lazo, Ph.D.

CONTRACTING ORGANIZATION: University of Pittsburgh
Pittsburgh, Pennsylvania 15260

REPORT DATE: July 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2001	Annual (1 Jul 00 - 30 Jun 01)

4. TITLE AND SUBTITLE
Novel Thioredoxin Inhibitors for Breast Cancer Therapy

5. FUNDING NUMBERS
DAMD17-00-1-0412

6. AUTHOR(S)
John S. Lazo, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Pittsburgh
Pittsburgh, Pennsylvania 15260

E-Mail: lazo@pitt.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color photos.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

The hypothesis being tested is that the thioredoxin redox signaling system is essential for the growth of some human breast cancers and that drugs inhibiting this system will block oncogenesis and cause selective growth inhibition and/or apoptosis. The specific objectives of the IDEA proposal are to generate and identify selective inhibitors of thioredoxin using target-array chemistry methodologies, *in vitro* assays and cell-based screening approaches. The scope of the research activity demanded that we develop semi-automated synthetic methodology. We ultimately intend to select one or more lead compounds that could be optimized as candidates for clinical development, which would encompass a Clinical Translational Research (CTR) proposal.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast cancer	21
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Cover..... 1

SF 298..... 2

Table of Contents 3

Introduction..... 4

Body..... 4

Key Research Accomplishments..... 11

Reportable Outcomes..... 12

Conclusions..... 12

References..... 12

Appendices..... 14

Introduction

The hypothesis being tested is that the thioredoxin redox signaling system is essential for the growth of some human breast cancers and that drugs inhibiting this system will block oncogenesis and cause selective growth inhibition and/or apoptosis. The specific objectives of the IDEA proposal are to generate and identify selective inhibitors of thioredoxin using target-array chemistry methodologies, *in vitro* assays and cell-based screening approaches. The scope of the research activity demanded that we develop semi-automated synthetic methodology. We ultimately intend to select one or more lead compounds that could be optimized as candidates for clinical development, which would encompass a Clinical Translational Research (CTR) proposal.

Body

Technical Objective #1. Synthesize libraries of small molecule compounds designed on the spiroketal naphthodecalin structure.

Our total synthesis approach toward palmarumycin CP_1 is now capable of providing gram quantities of this natural product as well as "unnatural" natural products. After protection of the carbonyl group in **1** with ethylene glycol, Ullmann coupling [1] between the readily available [2] tetralone and 8-iodo-1-methoxynaphthalene (**2**) [3] provides the biaryl ether **3** in 78% yield in refluxing pyridine in the presence of 0.2 equiv of Cu_2O and 1 equiv of K_2CO_3 (Scheme 1). Stepwise cleavage of the acetal in aqueous acetone with TsOH followed by double demethylation with BBr_3 gives **5** in 95% overall yield. The oxidative spirocyclization of phenol **5** is best performed after reduction of the tetralone with LAH by treatment with $PhI(OAc)_2$ in trifluoroethanol for 10 min [4] The linchpin spiroketal intermediate **6** can thus be obtained in high overall yield and is oxidized with Dess-Martin reagent [5], followed by aromatization of the resulting decalin dione with activated MnO_2 to give palmarumycin CP_1. Synthetic palmarumycin, which is spectroscopically in all aspects identical to the data reported for the fermented compound, is thus obtained in 35% overall yield in 8 steps from tetralone **1**. The same strategy provided (±)-deoxypreussomerin A after epoxidation of **6** with cumene hydroperoxide anion in THF in 15% overall yield based on a 9 step conversion from **1.** It should also be noted that we have been able to prepare racemic as well as enantiomerically pure dipoxin σ via related routes and consequently gained unique experience in the synthesis of these structurally unusual metabolites. Using the above-mentioned synthetic approach, we generated 21 diepoxin σ/palmarumycin CP1 analogs in 10 mg quantities (Figure 1). Another 13 analogs were synthesized using an alternate approach: the **TH-39** through **TH-140** series of analogs was prepared in a similar fashion in 10-70% yield from synthetic palmarumycin CP_1 by Mitsunobu reactions with polystyrene-supported triphenylphosphine. **TH-169** was prepared by hypervalent iodine oxidation of diol **1** followed by transketalization with ethylene glycol and 2-step aromatization (Scheme 2). For the preparation of **TH-223**, the cesium salt of diol **3 [6]** was alkylated and cyclized under oxidative conditions. All of these compounds have been fully characterized by melting point, IR, ^1H NMR, ^{14}C NMR, HRMS and HPLC for purity and authenticity. We have also synthesized another 20 analogs that are currently being evaluated. These syntheses are discussed in our submitted manuscript (Appendix). Thus, we completed one of the main components of our first Technical Objective; namely to synthesize an initial library of approximately 50 compounds with maximal diversity in 5-20 mg quantities and to evaluate the purity and confirm the chemical structures of the library members. We are now in the process of deciding if we want to synthesize gram quantities of any of these compounds for additional cellular and/or animal studies. We are also designing second-generation library structures around the core pharmacophore structure (Figure 2).

Scheme 1.

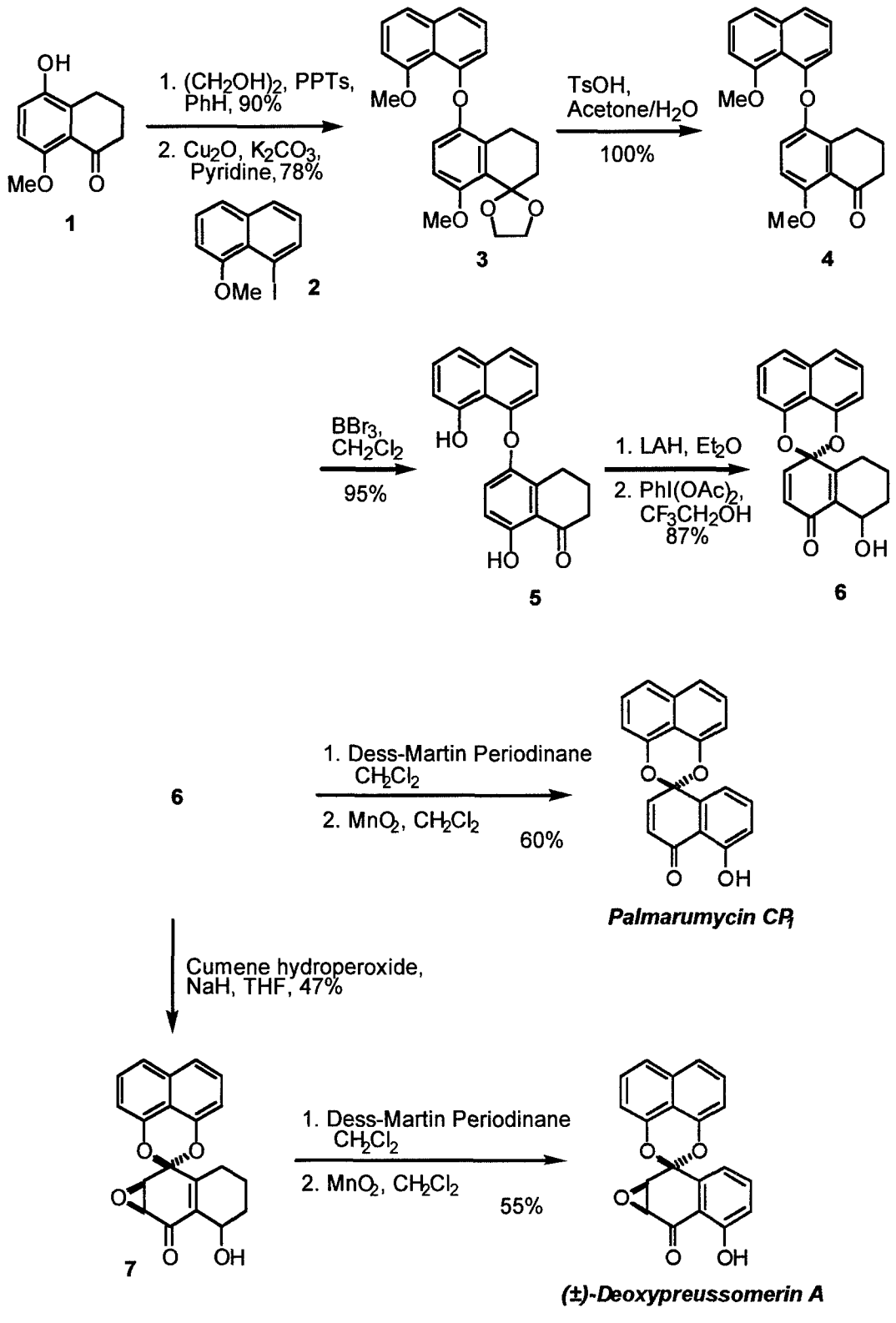
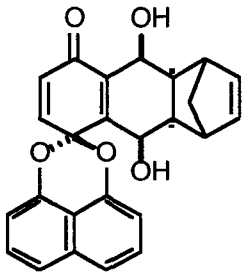
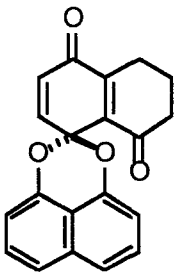

OH
MeO
O
1
1. (CH2OH)2, PPTs, PhH, 90%
2. Cu2O, K2CO3, Pyridine, 78%
OMe
I
2
MeO
O
MeO
O
O
3
TsOH, Acetone/H2O
100%
MeO
O
MeO
O
4
BBr3, CH2Cl2
95%
HO
O
HO
O
5
1. LAH, Et2O
2. PhI(OAc)2, CF3CH2OH
87%
O
O
O
OH
6
6
1. Dess-Martin Periodinane CH2Cl2
2. MnO2, CH2Cl2
60%
O
O
O
OH
Palmarumycin CP1
Cumene hydroperoxide, NaH, THF, 47%
O
O
O
O
OH
7
1. Dess-Martin Periodinane CH2Cl2
2. MnO2, CH2Cl2
55%
O
O
O
O
OH
(±)-Deoxypreussomerin A

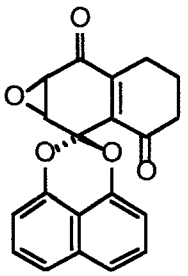
Figure 1. Structures of synthetic Trx-1/TrxR inhibitors.



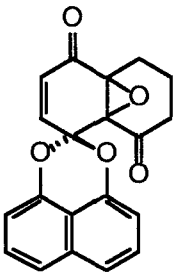
JK-2



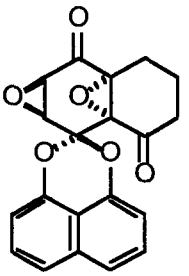
JK-3



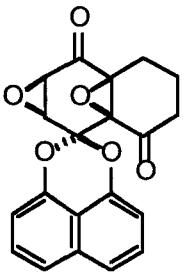
JK-4



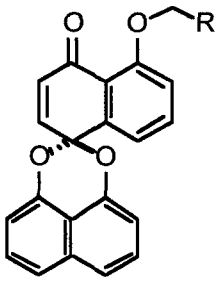
JK-5



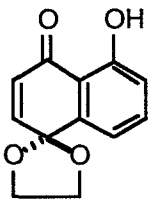
JK-6



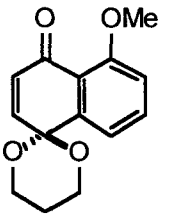
JK-7



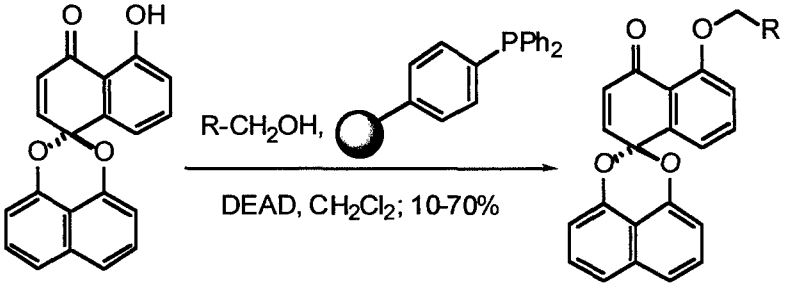
SR-1, R = (*E*)-HC=CHPh
SR-2, R = (*E*)-HC=CHMe
SR-3, R = *n*-C_5H_{11}
SR-4, R = (*E*)-CH_2HC=CHEt
SR-5, R = (*m*)-MeOPh
SR-6, R = Bn
SR-9, R = (*E,E*)-HC=C(Me)CH_2CH_2CH=CMe_2
SR-10, R = 3-furyl
SR-11, R = 2-pyridyl
SR-12, R = 3-pyridyl
SR-13, R = 4-pyridyl
SR-14, R = HC=CH_2



TH-169



TH-223



palmarumycin CP1

TH-39, R = (*p*)-MeOPh
TH-40, R = C(Me)=CH_2
TH-44, R = C≡CH
TH-48, R = (*p*)-BrPh
TH-49, R = (*p*)-ClPh
TH-62, R = 2-furyl(5-(*p*)-BrPh)
TH-63, R = 2-furyl(5-(*o*)-ClPh)
TH-64, R = 2-furyl(5-(*m*)-ClPh)
TH-65, R = 2-furyl(5-(*o*)-CF_3Ph)
TH-66, R = 2-furyl(5-(*m*)-CF_3Ph)
TH-126, R = HC=CMe_2
TH-139, R = (*E*)-C(Me)=CHMe
TH-140, R = (*Z*)-C(Me)=CHMe

Scheme 2.

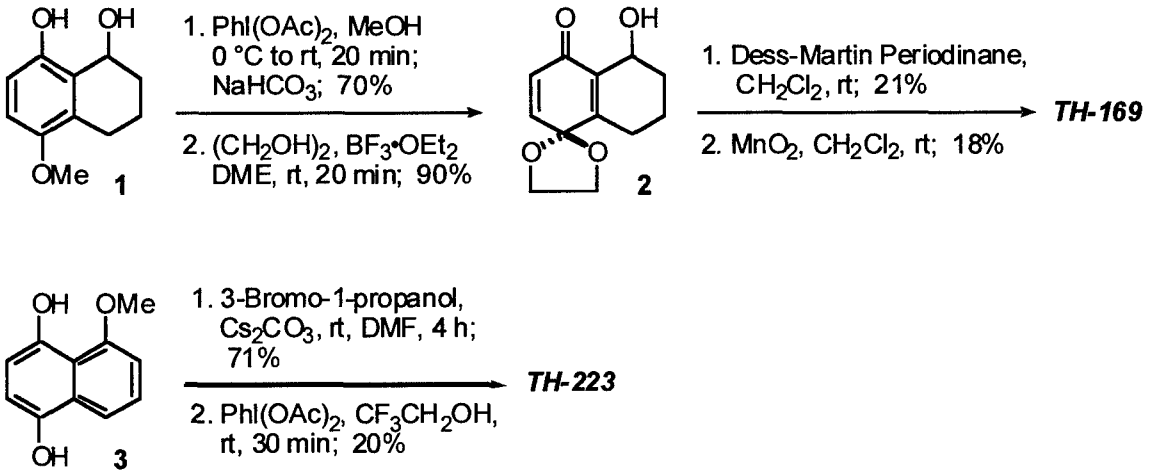
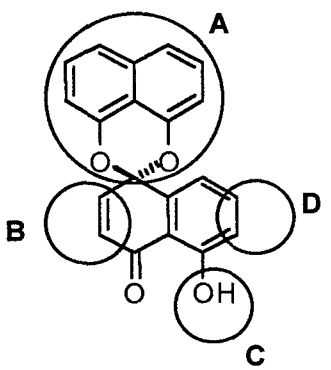


Figure 2. Targeted site for alteration for future palmarumycin SAR studies.



Technical Objective #2. Evaluate the biochemical and cellular activity of library compounds.

During the last funding period, we were able to generate sufficient human thioredoxin, thioredoxin reductase, and Cdc25 to allow us to perform complete *in vitro* evaluations on the above-mentioned compounds. Unfortunately, we were unable to measure PTP1B and glutathione reductase *in vitro* as planned due to difficulties in isolating protein. We believe we have resolved these protein isolation problems. Thus, we measured the ability of each compound (0.1 to 30 μM) to inhibit thioredoxin, thioredoxin reductase, and Cdc25.

Cdc25. The preparation of plasmid DNA and GST-fusion protein has previously been described [7]. The activity of the GST-fusion $Cdc25B_2$ was measured as previously described [7] in a 96-well microtiter plate using the substrate *o*-methyl fluorescein phosphate (OMFP) (Molecular Probes, Inc., Eugene, OR), which is readily metabolized to the fluorescent *o*-methyl fluorescein. All samples were prepared with a Biomek 2000 automated workstation and the OMFP concentration approximating the K_m for $Cdc25B_2$: 40 μM. All analogs were resuspended in DMSO and all reactions including controls were performed at a final concentration of 7% DMSO. The final incubation mixture (150 μl) was optimized for enzyme activity and comprised 30 mM Tris (pH 8.5), 75 mM NaCl, 1 mM EDTA, 0.033% bovine serum albumin, and 1 mM DTT. Reactions were initiated by adding 1 μg of Cdc25 phosphatase and had 30 μM as the initial concentration tested. Fluorescence emission

from the product was measured over a 20-60 min reaction period at ambient temperature with a multiwell plate reader (PerSeptive Biosystems Cytofluor II; Framingham, MA; excitation filter, 485/20; emission filter, 530/30). For all enzymes the reaction was linear over the time used in the experiments and was directly proportional to both the enzyme and substrate concentration. We found none of the compounds caused even a 25% inhibition of enzyme activity.

Table 1. IC_{50} values [μM] for TrxR, Trx-1/TrxR and cell growth inhibition.[a]

Entry	Compound	TrxR	Trx-1/TrxR	MDA-MB-231	MCF-7
1	**palmarumycin CP_1**	12.0	0.35	2.4	1
2	**JK-2**	Nd[b]	8.0	2.1	1.3
3	**JK-3**	Nd	2.1	6.4	3.8
4	**JK-4**	Nd	12.2	23	4.6
5	**JK-5**	Nd	44.0	3.4	1.3
6	**JK-6**	Nd	>50	8.2	4.6
7	**JK-7**	Nd	13.5	2.9	2.8
8	**diepoxin σ**	13.5	4.5	2	1.5
9	**SR-1**	Nd	>50	7.5	7.9
10	**SR-2**	Nd	>50	2.9	1.3
11	**SR-3**	Nd	>50	13.6	13.4
12	**SR-4**	Nd	>50	9.2	>30
13	**SR-5**	Nd	>50	2.7	2.3
14	**SR-6**	Nd	>50	4.6	3.9
15	**SR-7**	Nd	>50	2.5	1.1
16	**SR-9**	Nd	>50	2	4.6
17	**SR-10**	>50	23.2	2	2
18	**SR-11**	>50	41.8	2.8	2
19	**SR-12**	>50	>50	1.4	1.5
20	**SR-13**	>50	>50	7.3	8
21	**SR-14**	>50	23.2	2.7	2
22	**TH-39**	>50	>50	2.2	0.7
23	**TH-40**	>50	4.8	8.2	7.8
24	**TH-44**	>50	13.4	4.7	4.3
25	**TH-48**	>50	>50	9.3	>10
26	**TH-49**	>50	>50	8	>10
27	**TH-62**	20.1	10.2	7.8	5.7
28	**TH-63**	>50	>50	>10	>10
29	**TH-64**	>50	>50	>10	>10
30	**TH-65**	>50	>50	4.9	5
31	**TH-66**	>50	42.4	5.2	5.5
32	**TH-126**	Nd	>50	3.6	2
33	**TH-139**	Nd	>50	5.3	4.3
34	**TH-140**	Nd	>50	4.5	1.9
35	**TH-169**	8.8	3.4	4.3	4.2
36	**TH-223**	>50	40.2	5	4.4

[a] IC_{50} values were calculated from 5 concentrations (0.1 to 50 μM) for both the Trx-1/TrxR and TrxR assays. nd = not determined.

Thioredoxin and thioredoxin reductase. Thioredoxin reductase was purified from human placenta as previously described [8] and recombinant human Trx-1 was prepared as previously described [9]. TrxR and Trx-1/TrxR activities were measured spectro-photometrically using previously published microtiter plate colorimetric assays, based on the increase in absorbance at 405 nm, which occurs as dithionitrobenzoic acid (DTNB) is reduced by the enzyme-mediated transfer of reducing equivalents from NADPH [9]. Trx-1/TrxR-dependent insulin reducing activity was measured in an incubation with a final volume of 60 μL containing 100 mM HEPES buffer, pH 7.2, 5 mM EDTA (HE buffer), 1 mM NADPH, 1.0 μM TrxR, 0.8 μM Trx-1 and 2.5 mg/mL bovine insulin. Incubations were for 30 min at 37 °C in flat-bottom 96-well microtiter plates. The reaction was stopped by the addition of 100 μL of 6 M guanidine-HCl, 50 mM Tris, pH 8.0, and 10 mM DTNB, and the absorbance measured to 405 nm. TrxR activity was measured in a final incubation volume of 60 μL containing HE buffer, 10 mM DTNB, 1.0 μM TrxR and 1 mM NADPH. Compounds were diluted in HE buffer and added to the wells as 20 μL aliquots, and TrxR (20 μL HE buffer) was then added. Analogs were tested at 0.1 to 50 μM concentration. To start the reaction NADPH and DTNB were added as a 20 μL aliquot in HE buffer and the plate was moved to the plate reader that had been preheated to 37 °C. The optical density at 405 nm was measured every 10 s and initial reaction rates were measured.

Table 1 summarizes Trx-1/TrxR assay data. The most active compounds inhibited Trx-1/TrxR with IC_{50} values from 0.35 to low micromolar. In particular, palmarumycin CP_1 rivaled the most active known inhibitor of the thioredoxin system, pleurotin, in activity (entry 1). Palmarumycin CP_1 also demonstrated considerable (>30 fold) selectivity for Trx-1 over TrxR. Alkylation at the phenol as shown in the SR-series of analogs mostly abolished activity, with the exception of **SR-10**, a 3-furylmethyl derivative (entry 17), and **SR-14**, an allylated phenol (entry 21) which were nonetheless >50 fold less active. For the most part, this trend is continued in the TH-series, but several derivatives show more significant affinity to the thioredoxin – thioredoxin reductase system. Specifically, **TH-40** (entry 23), **TH-44** (entry 24), and **TH-62** (entry 27) have IC_{50} values from 4.8 to 13.4 μM. The former two are closely related to **SR-14**, but the activity of **TH-62** is unexpected given the lack of activity of the closely related **TH-63-66**. The beneficial effects of the free phenol group in the palmarumycin pharmacophore for Trx-1/TrxR inhibition are most strikingly demonstrated in the comparison of **TH-169** and **TH-223**. Only the free phenol **TH-169** maintains significant activity (entry 35) while the methyl ether **TH-223** is practically inactive. The comparison between palmarumycin CP_1 and **TH-169** also demonstrates the contribution to activity by the naphthalenediol ketal; a replacement with the 1,3-dioxolane group decreases activity ~10 fold and, most significantly, reduces the Trx-1 selectivity from >30 to ~2 fold. The presence of the conjugated enone is not crucial, as comparisons in the JK-series demonstrate. The diepoxyketones **JK-7** and, in particular, diepoxin σ, maintain respectable levels of inhibition of Trx-1/TrxR (entries 7 and 8), even though successive replacements of the double bonds in **JK-4**, **JK-5**, and **JK-6** with epoxide rings leads to noticeable decreases in activity vs. the parent dienone **JK-3**. These results are described in more detail in our appended manuscript that is under review.

Antiproliferative activity. Antiproliferative activity was examined with estrogen receptor positive, p53-replete, MCF-7 and estrogen receptor negative, p53-deficient, MDA-MB-231 human breast cancer cells using a previously described colorimetric assay [10,11]. Cells were seeded at 4,000 cells/well in 96 well microtiter plates and allowed to attach overnight. Cells were treated with compounds (0.1 to 30 μM) or 0.1% DMSO (vehicle) for 72 h, after which the medium was replaced with serum-free medium containing 0.1% of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide. Plates were incubated for 3 h in the dark and the total cell number was calculated spectrophotometrically at 540 nm. Table 1 indicates that a substantial majority of the compounds evaluated with either MDA-MB 231 or MCF-7 cells were quite active as antiproliferative agents. Generally, MCF-7 cells were slightly more sensitive than the p53-deficient MDA-MB-231 cells, although **SR-4**, **SR-9**, **TH-48** and **TH-49** are notable exceptions. We were, however, unable to make any correlation between *in vitro* inhibition of thioredoxin or thioredoxin reductase and growth inhibition suggesting growth may be the result of other biochemical effects. Thus, these are promising cytotoxic compounds whose mechanism of action for growth inhibition remains to be established. We have some evidence that some of these compounds caused growth inhibition because of apoptotic processes.

We have also begun to evaluate the cell cycle inhibition caused by selected members of this library. We chose SR-7 prior to knowing its lack of an effect on the thioredoxin and thioredoxin reductase system just to refined the assays with these compounds. Using our previously described synchronous tsFT210 mammary carcinoma cells [11], we probed the effects of SR-7 on both G2/M and G1 transition. When incubated at the permissive temperature of 32.0°C, tsFT210 cells had a normal cell cycle distribution (Figure 3A); when incubated at the non-permissive temperature of 39.4°C for 17 h, cells arrested at G2/M phase (4C), due to Cdk1 inactivation (Figure 3B). When G2/M arrested cells were cultured at the permissive temperature for 6 h with DMSO vehicle alone, we saw clear evidence of entry into G1 (2C) (Figure 3C), although a small fraction of cells remained in the G2/M phase. This G2/M retention at 4C is probably due to the extended cell cycle blockage at 39.4°C . Treatment with 1 μM nocodazole blocked cell passage through G2/M (Figure 3D). To determine the effect of SR-7 on G2/M cell cycle transition, we treated cells with 2.5 to 10 μM SR-7 for 6 h after releasing cells at 32.0°C. As indicated in Figure 3E-H, SR-7 caused a concentration-dependent arrest in the G2/M phase, with obvious blockage even with 2.5 μM SR-7. The G2/M inhibition was similar to that seen with the previously reported and structurally unrelated compound SC-ααδ9 (Figure 3I). SC-ααδ9 is an inhibitor of the Cdc25 family of phosphatases that controls cell cycle checkpoints [7,10].

We also examined G1 transition in tsFT210 cells after SR-7 treatment. We again arrested tsFT210 cells at G2/M by shifting to the non-permissive temperature and then released cells into G1 phase by returning to the permissive temperature. In these experiments, however, we added DMSO vehicle, roscovitine, SR-7 or SC-ααδ9 6 h after G2/M phase release. Cells that were treated with the DMSO vehicle passed through G1 phase as expected and produced the predicted broad S phase peak between diploid (2C) and tetraploid (4C) states (Figure 4D), while cells exposed continuously to 50 μM roscovitine were blocked and did not pass through G1 (Figure 4E). As illustrated in Figures 4F and G, cells treated with 5 or 10 μM SR-7 were not delayed at G1 which might be predicted if SR-7 is affecting a thioredoxin-mediated process because these protein are thought to have a potential role in G2/M transition [11]. As expected from our previous studies (12), the dual phase-specific inhibitor SC-ααδ9 caused a prominent G1 block and also prevented cells that were at the G2/M interphase from progressing, which resulted in two prominent cell cycle peaks (Figure 4H). Thus, we now have in place a system to evaluate cell cycle block.

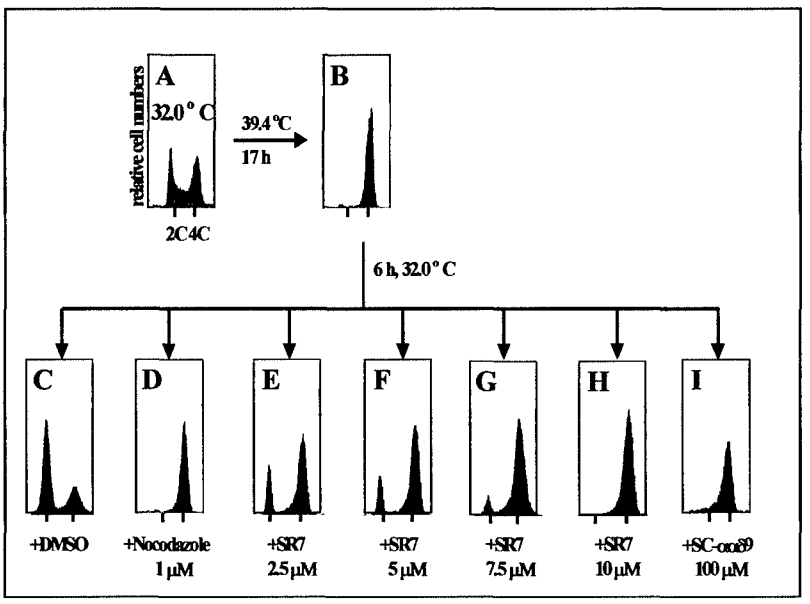


Figure 3. Inhibition of G2/M cell cycle progression by SR-7. tsFT210 cells were cultured at the permissive temperature of 32.0°C (Panel A), and then incubated for 17 h at the non-permissive temperature of 39.4°C (Panel B). Cells were released from cycle arrest by shifting to the 32.0°C medium. The cells were then incubated for 6 h in the presence of DMSO vehicle (Panel C), 1 μM nocodazole (Panel D), 2.5 μM SR-7 (Panel E), 5 μM SR-7 (Panel F), 7.5 μM SR-7 (Panel G), 10 μM SR-7 (Panel H) or, 100 μM SC-ααδ9 (Panel I). Fluorescence corresponding to 2C and 4C DNA content is represented by vertical bars. These results are representative of three independent experiments.

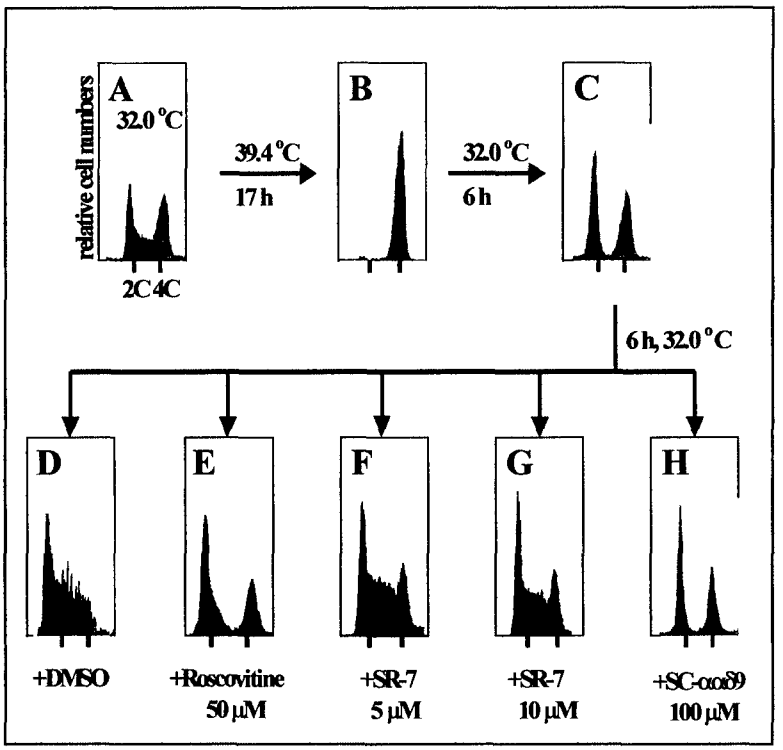


Figure 4. Failure of SR-7 to inhibit G1 cell cycle progression. tsFT210 cells were cultured at the permissive temperature of 32.0°C (Panel A) and then incubated for 17 h at the non-permissive temperature of 39.4°C (Panel B). Cells were released from the G2/M block by incubation at 32.0°C for 6 h (Panel C), and then incubated for an additional 6 h in the presence of various agents. These were: DMSO vehicle (Panel D), 50 μM roscovitine (Panel E), 5 μM SR-7 (Panel F), 10 μM SR-7 (Panel G), or 100 μM SC-ααδ9 (Panel H). Fluorescence corresponding to 2C and 4C DNA contents is represented by vertical bars. These results were replicated in a second independent experiment.

Technical Objective #3. Evaluate the antitumor activity of prioritized compounds.

We have not yet begun to analyze any of these compounds because we want to wait until we have amore potent inhibitor of thioredoxin. If we are unsuccessful in finding such a compound within the next six months, we will selected two of the most potent inhibitors that are also capable to causing growth inhibition of MDA-MB-231 and MCF-7 cells for future *in vivo* studies using the protocol we previously described.

Key Research Accomplishments

Task 1. Specific Aim #1. Synthesize libraries of small molecule compounds designed on the spiroketal naphthodecalin structure.

- Synthesized an initial library of approximately 50 compounds in 5-20 mg quantities
- Evaluated the purity and confirm chemical structure of library members.
- Designed a second-generation library structures that will be synthesized.

Task 2. Specific Aim #2. Evaluate the biochemical and cellular activity of library compounds.

- Generated sufficient thioredoxin, thioredoxin reductase, and Cdc25 for *in vitro* assays.
- Evaluated the inhibitory activity of the initial library against thioredoxin and thioredoxin reductase *in vitro*.
- Determined the growth inhibitory activity of initial library members against MDA-MB-231 and MCF-7 cells.
- Established cell cycle assay with tsFT210 cells and spiroketals.
- Determined the cell cycle actions of initial library members against MDA-MB-231 and MCF-7 cells.
- Examine the structure-activity relationship (SAR) of initial library members and begin to prioritize compounds for further studies and resynthesis.

Reportable Outcomes

Bibliography of Publications:

Wipf P, Hopkins TD, Jung J-K, Rodriguez S, Birmingham A, Southwick EC, Lazo JS, Powis G. (Submitted Bioorg. Med. Chem) New Inhibitors of the Thioredoxin - Thioredoxin Reductase System Based on a Naphthoquinone Spiroketal Natural Product Lead.

Degrees Awarded:
None

Pending Funding:
None

Personnel Supported from the Research Effort:

Name	Degree(s)	[redacted]	Role on Project (e.g., P.I., Res. Assoc.)	[redacted]	Annual % Effort
John S. Lazo	Ph.D.	[redacted]	Principal Investigator	[redacted]	5
Peter J. Wipf	Ph.D.	[redacted]	Co-Investigator	[redacted]	5
Lixia Pu	Ph.D.	[redacted]	Research Associate	[redacted]	50
Tamara D. Hopkins	B.S.	[redacted]	GSR	[redacted]	100

Conclusions

The results of this research have provided several novel cytotoxic agents that may block G2/M progression. Moreover, we have developed a general method for the synthesis of multiple napthoquinone spiroketals. Initial studies indicate several potent inhibitors of thioredoxin that may be useful candidates for future testing in animal models. Moreover, we are being to develop a structure activity relationship that will be useful in our future efforts to make inhibitors of this interesting breast cancer-relevant molecular target.

References

1. Moroz AA, Shvartsberg MS. The Ullmann Ether Condensation. 1974. Russ. Chem. Rev. 43:679-689.

2. Newhall WF, Harris SA, Holly FW, Johnston EL, Richter JW, Walton E, Wilson AN, Folkers K. 1955. Synthesis of 8-Hydroxy-2-Keto-5-Methoxy-4a-Methyl-2,3,4,4a,9,10-Hexahydrophenanthrene. J. Am. Chem. Soc. 77:5646-5652.

3. Graybill BM; Shirley DA. 1996. The Metalation of 1-methoxynaphthalene with *n*-Butyllithium. J. Org. Chem. 31:1221-1225.

4. Pelter A, Elgendy S. 1988. Phenolic Oxidation with (Diacetoxyiodo)Benzene. Tetrahedron Lett. 29:677-680.

5. Dess DB, Martin JC. 1991. A Useful 12-I-5 Triacetoxyperiodinane (the Dess-Martin Periodinane) for the Selective Oxidation of Primary or Secondary Alcohols and a Variety of Related 12-I-5 Species. J. Am. Chem. Soc. 113:7277-7287.

6. Contant P; Haess M; Riegl J; Scalone M, Visnick M. 1999 Synthesis, 821-826.

7. Rice RL, Rusnak JM, Yokokawa F, Yokokawa S, Messner DJ, Boynton AL, Wipf P and Lazo JS. 1997. Biochemistry 36, 15965-15974.

8. Oblong JE, Gasdaska PY, Sherrill K, Powis G. 1993. Biochemistry 32, 7271-7279.

9. Gasdaska PY, Oblong JE, Cotgreave IA, Powis G. 1994. The predicted amino acid sequence of human thioredoxin is identical to that of the autocrine growth factor human adult T-cell derived factor (ADF): Thioredoxin mRNA is elevated in some human tumors. Biochim. Biophys. Acta 1218,292-296.

10. Vogt A, Rice RL, Settineri CE, Yokokawa F, Yokokawa S, Wipf P, Lazo JS. 1998. Disruption of insulin-like growth factor-1 signaling and down-regulation of Cdc2 by SC-ααδ9, a novel small molecule antisignaling agent identified in a targeted array library. J. Pharmacol. Exper. Therap. 287,806-813.

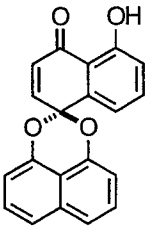
11. Vogt A, Tamura K, Watson S, Lazo J.S. 2000. The antitumor imidazolyl disulfide IV-2 causes irreversible G2/M cell arrest without hyperphosphorylation of the cyclin-dependent kinase, Cdk1. J. Pharmacol. Exper. Therap. 294, 1070-1075.

New Inhibitors of the Thioredoxin - Thioredoxin Reductase System Based on a Naphthoquinone Spiroketal Natural Product Lead

Peter Wipf,*[/] Tamara D. Hopkins,[/] Jae-Kyu Jung,[/] Sonia Rodriguez,[/] Anne Birmingham,[#] Eileen C. Southwick,[f] John S. Lazo,[f] and Garth Powis[#]

[/]*Department of Chemistry, University of Pittsburgh, Pittsburgh, PA 15260;* [#]*Arizona Cancer Center, University of Arizona, 1515 North Campbell Avenue, Tucson, AZ 85724;* [f]*Department of Pharmacology, University of Pittsburgh, Pittsburgh, PA 15261, U.S.A.*

Synthetic analogs of palmarumycin CP_1 were evaluated as new nanomolar to low-micromolar inhibitors of the thioredoxin — thioredoxinreductase redox system. Many derivatives showed significant growth inhibition against two breast cancer cell lines.



palmarumycin CP$_1$

New Inhibitors of the Thioredoxin - Thioredoxin Reductase System Based on a Naphthoquinone Spiroketal Natural Product Lead

Peter Wipf,*[,a] Tamara D. Hopkins,[a] Jae-Kyu Jung,[a] Sonia Rodriguez,[a] Anne Birmingham,[b] Eileen C. Southwick,[c] John S. Lazo[c] and Garth Powis[b]

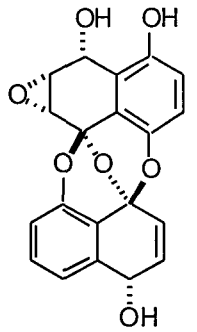
[a]*Department of Chemistry, University of Pittsburgh, Pittsburgh, PA 15260, USA.*

[b]*Arizona Cancer Center, University of Arizona, 1515 North Campbell Avenue, Tucson, AZ 85724, USA.*

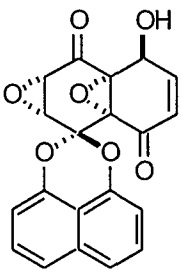
[b]*Department of Pharmacology, University of Pittsburgh, Pittsburgh, PA 15261, USA.*

Abstract—Natural products of the novel naphthoquinone spiroketal structural type served as lead structures for the development of novel inhibitors of the thioredoxin – thioredoxin reductase redox system. The most potent compound in this series inhibited thioredoxin with an IC_{50} of 350 nM, and many derivatives showed low micromolar activities for growth inhibition against two breast cancer cell lines.

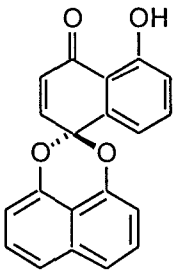
The preussomerin, palmarumycin, and diepoxin families of naphthoquinone spiroketal natural products have attracted considerable synthetic interest.[1] The known biological activities of these novel fungal metabolites span from antifungal antibiotic effects to FTPase, DNA gyrase, and phospholipase D inhibition.[1] Preliminary biological evaluation of 22 naphthoquinone spiroketals against two human breast cancer cell lines revealed several potent and selective growth inhibitors;[1a] in addition, analog **SR-7** was found to arrest mammalian cells in the G2/M phase of the cell cycle.[2] We now report that several natural as well as synthetic members of the naphthoquinone spiroketal class are potent and selective inhibitors of the thioredoxin – thioredoxin reductase cellular redox system.



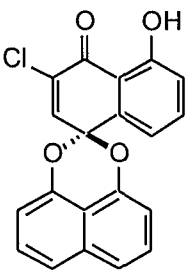
preussomerin A



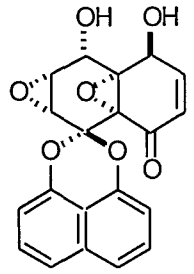
diepoxin σ (Sch 49209)



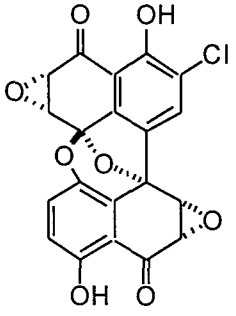
palmarumycin CP$_1$



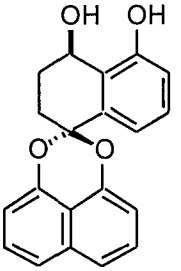
palmarumycin C$_1$



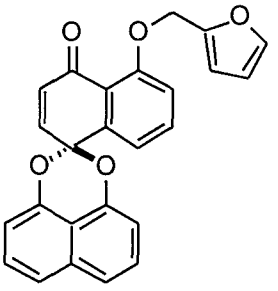
diepoxin ζ (Sch 53514) (palmarumycin C$_{13}$) (cladospirone bisepoxide)



spiroxin A

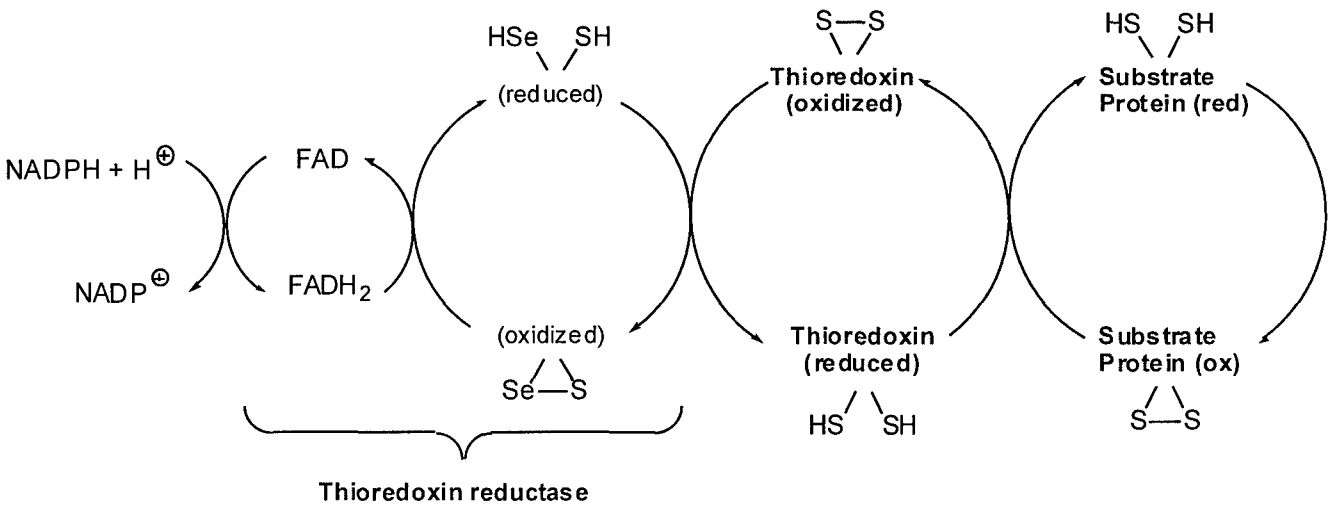


CJ-12,371



SR-7

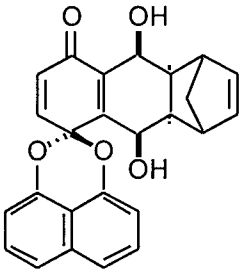
Since its discovery in the early 1960s, the thioredoxin – thioredoxin reductase system has been the subject of intense pharmacological studies.[3] The two redox active proteins have been isolated from many species, and their medical interest is based in part on their value as indicators of widespread diseases such as rheumatoid arthritis, AIDS, and cancer. The cytosolic 12 kDa thioredoxin-1 (Trx-1) is the major cellular protein disulfide reductase and its dithiol-disulfide active site cysteine pair (CXXC) serves as electron donor for enzymes such as ribonucleotide reductase, methionine sulfoxide reductase, and transcription factors including NF-κB and the Ref-1-dependent AP-1.[4] Therefore, thioredoxin-1 is critical for cellular redox regulation, signaling, and regulation of protein function as well as defense against oxidative stress and control of growth and apoptosis.[5] Thioredoxin-1 acts in concert with the glutathione – glutathione reductase system but with a rate of reaction orders of magnitude faster, and lack of cytosolic mammalian thioredoxin is embryonically lethal. Eukaryotic thioredoxin reductases (TrxR) are 112-130 kDa, selenium-dependent dimeric flavoproteins that also reduce substrates such as hydroperoxides or vitamin C.[6] They contain redox-active selenylsulfide-selenolthiol active sites and are inhibited by aurothioglucose and auranofin (K_i 4 nM).[7] NADPH serves as reducing agent of Trx-1 via TrxR.



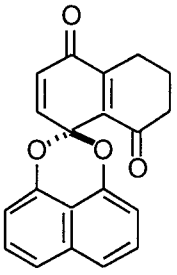
Pathophysiological effects of Trx-1/TrxR are indicated by Trx-1 overexpression in human tumors such as lung, colorectal and cervical cancers and leukemia, and secreted Trx-1 stimulates cancer cell growth and decreases sensitivity to induced apoptosis.[8] The Trx-1/TrxR system is therefore an important target for chemotherapeutic intervention. Alkyl 2-imidazolyl disulfides were found to be inhibitors of Trx-1/TrxR with IC_{50}s of 31/37 μM, respectively; they block MCF-7 human breast cancer cells in the G_2/M phase of the cell cycle and suppress the growth of several human primary tumors in the NCI 60 cancer cell line panel.[8,9] A COMPARE analysis revealed the most potent Trx-1/TrxR inhibitor known to date, the *para*-quinone NSC401005 a.k.a. the natural product pleurotin.[10] The IC_{50} of NSC401005 against Trx-1/TrxR was determined as 0.17 μM; however, the average IC_{50} of this compound for growth inhibition in the NCI 60 tumor cell line panel was only 21.5 μM. While inhibitors of TrxR such as auranofin and nitrosoureas are quite effective, the search for new, more specific, and less toxic compounds is well justified.

Chemistry. Syntheses and characterizations of palmarumycin CP_1, diepoxin σ, the **JK**-series and the **SR**-series of analogs have been reported elsewhere.[1] The **TH-39** through **TH-140** series of analogs was prepared in a similar fashion in 10-70% yield from synthetic palmarumycin CP_1 by Mitsunobu reactions with polystyrene-supported triphenylphosphine (Figure 1).[11] **TH-169** was prepared by hypervalent iodine oxidation of diol **1**[1c] followed by transketalization with ethylene glycol and 2-step aromatization (Scheme 1).[12] For the preparation of **TH-223**, the cesium salt of diol **3**[13] was alkylated and cyclized under oxidative conditions.[14]

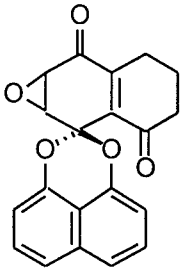
Figure 1. Structures of synthetic Trx-1/TrxR inhibitors.



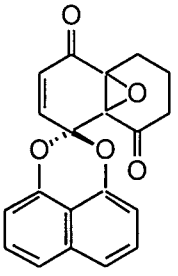
JK-2



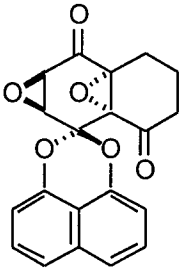
JK-3



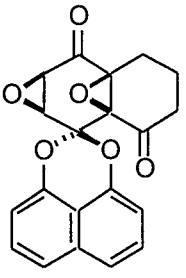
JK-4



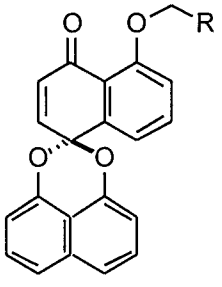
JK-5



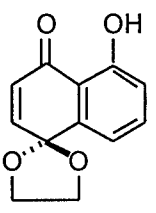
JK-6



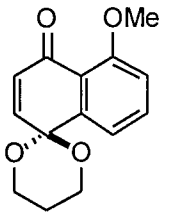
JK-7



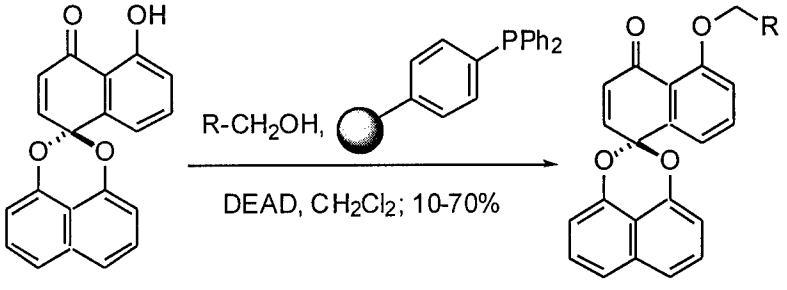
SR-1, R = (*E*)-HC=CHPh
SR-2, R = (*E*)-HC=CHMe
SR-3, R = *n*-C_5H_{11}
SR-4, R = (*E*)-CH_2HC=CHEt
SR-5, R = (*m*)-MeOPh
SR-6, R = Bn
SR-9, R = (*E,E*)-HC=C(Me)CH_2CH_2CH=CMe_2
SR-10, R = 3-furyl
SR-11, R = 2-pyridyl
SR-12, R = 3-pyridyl
SR-13, R = 4-pyridyl
SR-14, R = $HC=CH_2$



TH-169



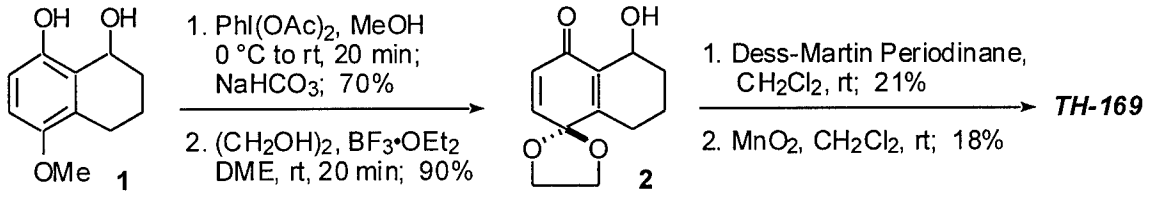
TH-223

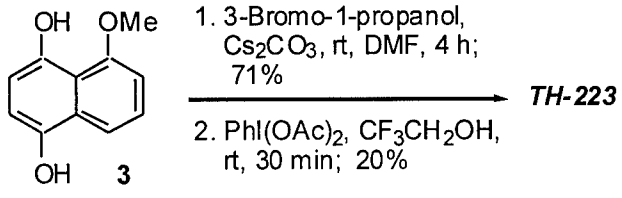


palmarumycin CP1

TH-39, R = (*p*)-MeOPh
TH-40, R = $C(Me)=CH_2$
TH-44, R = C≡CH
TH-48, R = (*p*)-BrPh
TH-49, R = (*p*)-ClPh
TH-62, R = 2-furyl(5-(*p*)-BrPh)
TH-63, R = 2-furyl(5-(*o*)-ClPh)
TH-64, R = 2-furyl(5-(*m*)-ClPh)
TH-65, R = 2-furyl(5-(*o*)-CF_3Ph)
TH-66, R = 2-furyl(5-(*m*)-CF_3Ph)
TH-126, R = $HC=CMe_2$
TH-139, R = (*E*)-C(Me)=CHMe
TH-140, R = (*Z*)-C(Me)=CHMe

Scheme 1.





Biology. Thioredoxin reductase was purified from human placenta as previously described [15] and recombinant human Trx-1 was prepared as previously described.[16] TrxR and Trx-1/TrxR activities were measured spectrophotometrically using previously published microtiter plate colorimetric assays, based on the increase in absorbance at 405 nm which occurs as dithionitrobenzoic acid (DTNB) is reduced by the enzyme-mediated transfer of reducing equivalents from NADPH.[16] Trx-1/TrxR-dependent insulin reducing activity was measured in an incubation with a final volume of 60 μL containing 100 mM HEPES buffer, pH 7.2, 5 mM EDTA (HE buffer), 1 mM NADPH, 1.0 μM TrxR, 0.8 μM Trx-1 and 2.5 mg/mL bovine insulin. Incubations were for 30 min at 37 °C in flat-bottom 96-well microtiter plates. The reaction was stopped by the addition of 100 μL of 6 M guanidine-HCl, 50 mM Tris, pH 8.0, and 10 mM DTNB, and the absorbance measured to 405 nm. TrxR activity was measured in a final incubation volume of 60 μL containing HE buffer, 10 mM DTNB, 1.0 μM TrxR and 1 mM NADPH. Compounds were diluted in HE buffer and added to the wells as 20 μL aliquots, and TrxR was then added, also as 20 μL aliquots in HE buffer. To start the reaction NADPH and DTNB were added as a 20 μL aliquot in HE buffer and the plate was moved to the plate reader which had been preheated to 37 °C. The optical density at 405 nm was measured every 10 s and initial reaction rates were measured.

Antiproliferative activity was examined with estrogen receptor positive, p53 replete MCF-7 and estrogen receptor negative, p53 deficient MDA-MB-231 human breast cancer cells using a previously described colorimetric assay.[1a,2] Cells were seeded at 4,000 cells/well in 96 well microtiter plates and allowed to attach overnight. Cells were treated with compounds (0.1 to 30 μM) or 0.1% DMSO (vehicle) for 72 h, after which the medium was replaced with serum-free medium containing 0.1% of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide. Plates were incubated for 3 h in the dark and the total cell number was calculated spectrophotometrically at 540 nm.

Table 1 summarizes Trx-1/TrxR assay data as well as growth inhibition values for selected compounds. The most active compounds inhibited Trx-1/TrxR with IC_{50} values from 0.35 to <15 micromolar. In particular, palmarumycin CP_1 rivaled the most active known inhibitor of the thioredoxin system, pleurotin, in activity (entry 1). Palmarumycin CP_1 also demonstrated considerable (>30 fold) selectivity for Trx-1 over TrxR. Alkylation at the phenol as shown in the SR-series of analogs mostly abolished activity, with the exception of **SR-10**, a 3-furylmethyl derivative (entry 17), and **SR-14**, an allylated phenol (entry 21) which were nonetheless >50 fold less active. For the most part, this trend is continued in the TH-series, but several derivatives show more significant affinity to the thioredoxin – thioredoxin reductase system. Specifically, **TH-40** (entry 23), **TH-44** (entry 24), and **TH-62** (entry 27) have IC_{50} values from 4.8 to 13.4 μM. The former two are closely related to **SR-14**, but the activity of **TH-62** is unexpected given the lack of activity of the closely related **TH-63-66**. The beneficial effects of the free phenol group in the palmarumycin pharmacophore for Trx-1/TrxR inhibition are most strikingly demonstrated in the comparison of **TH-169** and **TH-223**. Only the free phenol **TH-169** maintains significant activity (entry 35) while the methyl ether **TH-223** is practically inactive. The comparison between palmarumycin CP_1 and **TH-169** also demonstrates the contribution to activity by the naphthalenediol ketal; a replacement with the 1,3-dioxolane group decreases activity ca. 10 fold and, most significantly, reduces the Trx-1 selectivity from >30 to ca. 2 fold. The presence of the conjugated enone is not crucial, as comparisons in the JK-series demonstrate. The diepoxyketones **JK-7** and, in particular, diepoxin σ, maintain respectable levels of inhibition of Trx-1/TrxR (entries 7 and 8), even though successive replacements of the double bonds in **JK-4**, **JK-5**, and **JK-6** with epoxide rings leads to noticeable decreases in activity vs. the parent dienone **JK-3**.

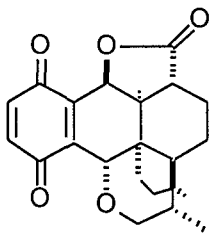
Table 1. IC_{50} values [μM] for TrxR, Trx-1/TrxR and cell growth inhibition.[a]

Entry	*Compound*	*TrxR*	*Trx-1/TrxR*	*MDA-MB-231*	*MCF-7*
1	**palmarumycin CP_1**	12.0	0.35	2.4	1
2	**JK-2**	Nd[b]	8.0	2.1	1.3
3	**JK-3**	Nd	2.1	6.4	3.8
4	**JK-4**	Nd	12.2	23	4.6
5	**JK-5**	Nd	44.0	3.4	1.3
6	**JK-6**	Nd	>50	8.2	4.6
7	**JK-7**	Nd	13.5	2.9	2.8
8	**diepoxin σ**	13.5	4.5	2	1.5
9	**SR-1**	Nd	>50	7.5	7.9
10	**SR-2**	Nd	>50	2.9	1.3
11	**SR-3**	Nd	>50	13.6	13.4
12	**SR-4**	Nd	>50	9.2	>30
13	**SR-5**	Nd	>50	2.7	2.3
14	**SR-6**	Nd	>50	4.6	3.9
15	**SR-7**	Nd	>50	2.5	1.1
16	**SR-9**	Nd	>50	2	4.6
17	**SR-10**	>50	23.2	2	2
18	**SR-11**	>50	41.8	2.8	2
19	**SR-12**	>50	>50	1.4	1.5
20	**SR-13**	>50	>50	7.3	8
21	**SR-14**	>50	23.2	2.7	2
22	**TH-39**	>50	>50	2.2	0.7
23	**TH-40**	>50	4.8	8.2	7.8
24	**TH-44**	>50	13.4	4.7	4.3
25	**TH-48**	>50	>50	9.3	>10
26	**TH-49**	>50	>50	8	>10
27	**TH-62**	20.1	10.2	7.8	5.7
28	**TH-63**	>50	>50	>10	>10
29	**TH-64**	>50	>50	>10	>10
30	**TH-65**	>50	>50	4.9	5
31	**TH-66**	>50	42.4	5.2	5.5
32	**TH-126**	Nd	>50	3.6	2
33	**TH-139**	Nd	>50	5.3	4.3
34	**TH-140**	Nd	>50	4.5	1.9
35	**TH-169**	8.8	3.4	4.3	4.2
36	**TH-223**	>50	40.2	5	4.4

[a] IC_{50} values were calculated from 5 concentrations (0.1 to 50 μM) for both the Trx-1/TrxR and TrxR assays.
[b] nd = not determined.

It is quite intriguing to compare the structures of the most active palmarumycin analogs with pleurotin, a 0.17 μM inhibitor (Figure 2). The polycyclic perhydroanthracene core of pleurotin is most closely related to **JK-2**, but the latter has considerably lower Trx-1/TrxR activity (entry 2). This difference is likely due to the presence of a *para*-quinone in pleurotin, which is a significantly more electrophilic moiety than the cyclohexadienone in **JK-2**. It is therefore noteworthy that the IC_{50} of palmarumycin CP_1 almost matches that of pleurotin, since the presence of the phenol ring attached to the cyclohexenone further reduces its electrophilicity and intrinsic reactivity toward thiols. Both **JK-2** and palmarumycin CP_1 demonstrate 10-20 times greater cell growth inhibition against MCF-7 and MDA-MB-231 cancer cell lines compared with pleurotin. Interestingly, the parent natural products as well as >80% (29/35) of the synthetic analogs show IC_{50} values <10 μM against estrogen receptor positive and negative breast adenocarcinoma cells. Thus, this series is quite enriched for cytotoxic compounds directed toward human malignancies. All six compounds that inhibit Trx-1 *in vitro* with an IC_{50} <10 μM, namely palmarumycin CP_1, **JK-2** (entry 2), **JK-3** (entry 3), diepoxin σ, **TH-40** (entry 23) and **TH-169** (entry 35), have an IC_{50} for growth inhibition with the two human breast cancer cell lines of <10 μM. Several of the compounds that lacked TrxR or Trx-1/TrxR inhibitory activity, such as **TH-48** (entry 25), **TH-49** (entry 26), **TH-63** (entry 28) and **TH-64** (entry 29), have IC_{50} values for growth inhibition in excess of 10 μM. Nonetheless, we do not currently have any direct evidence that the cytotoxicity was caused by inhibition of the Trx-1/TrxR system at this time. This will require addition investigation. In general, however, there is no obvious direct correlation between cell growth inhibitory activity and Trx-1/TrxR inhibition in the palmarumycin series. This could reflect undesirable pharmacological attributes of some of the good *in vitro* inhibitors of Trx-1, such as poor cellular uptake, rapid cellular efflux, metabolism, or binding to multiple macromolecular targets, which limits their cellular toxicity. The substantial cytotoxicity of compounds lacking obvious *in vitro* activity against the Trx-1/TrxR system is most likely due to interactions with other, as yet, undefined molecular targets.

Figure 2. Structure of pleurotin.



pleurotin

In summary, we have discovered a new, nanomolar lead structure for the development of inhibitors of the thioredoxin – thioredoxin reductase system. Palmarumycin CP_1 rivals the enzyme inhibitory activity reported for the structurally much more complex and more electrophilic quinone pleurotin and shows 50% cancer cell growth inhibition at 10-20 times lower concentrations. Our SAR studies on synthetic derivatives demonstrate that both the phenol group and the enone moiety in palmarumycin CP_1 are important for maximizing enzymatic activity, and that the presence of the naphthalenediol ketal enhances Trx-1 over TrxR selectivity. It is possible that the antifungal and antibacterial effects found for palmarumycins[17] are a consequence of *in vivo* inhibition of the cellular redox state; certainly, the palmarumycins represent an attractive new opportunity for the development of chemotherapeutic agents that target the Trx-1/TrxR system.

Acknowledgment. This work was supported in part by grants from the National Science Foundation (CHE-9453461), the Department of Defense (DAMD 17-00-1-0412), and by the National Institutes of Health (U19CA-52995). We thank Professor Billy Day (University of Pittsburgh) for stimulating discussions and Ms. Angela Wang for technical support.

References

1. (a) Wipf, P.; Jung, J.-K.; Rodriguez, S.; Lazo, J. S. *Tetrahedron* **2001**, *57*, 283. (b) Coutts, I. G. C.; Allcock, R. W.; Scheeren, H. W. *Tetrahedron Lett.* **2000**, *41*, 9105. (c) Wipf, P.; Jung, J.-K. *J. Org. Chem.* **2000**, *65*, 6319, and references cited therein.
2. Lazo, J. S.; Tamura, K.; Vogt, A.; Jung, J.-K.; Rodriguez, S.; Balachandran, R.; Day, B. W.; Wipf, P. *J. Pharm. Exp. Ther.* **2001**, *296*, 364.

3. Williams, C. H. *Eur. J. Biochem.* **2000**, *267*, 6101.
4. Arnér, E. S. J.; Holmgren, A. *Eur. J. Biochem.* **2000**, *267*, 6102.
5. Davis, W.; Ronai, Z.; Tew, K. D. *J. Pharm. Exp. Ther.* **2001**, *296*, 1.
6. Williams, C. H.; Arscott, L. D.; Müller, S.; Lennon, B. W.; Ludwig, M. L.; Wang, P.-F.; Veine, D. M.; Becker, K.; Schirmer, R. H. *Eur. J. Biochem.* **2000**, *267*, 6110.
7. Becker, K.; Gromer, S.; Schirmer, R. H.; Müller, S. *Eur. J. Biochem.* **2000**, *267*, 6118.
8. Powis, G.; Kirkpatrick, D. L.; Angulo, M.; Baker, A. *Chem.-Biol. Interactions* **1998**, *111*, 23.
9. Vogt, A.; Tamura, K.; Watson, S.; Lazo, J. S. *J. Pharm. Exp. Ther.* **2000**, *294*, 1070.
10. Kunkel, M. W.; Kirkpatrick, D. L.; Johnson, J. I.; Powis, G. *Anti-Cancer Drug Des.* **1997**, *12*, 659.
11. *General procedure for Mitsunobu reactions.* **8-(4-Methoxy-benzyloxy)-1-oxo-1,4-dihydronaphthalene-4-spiro-2'-naphto[1",8"-de][1',3']dioxin (TH-39)**. A solution of palmarumycin CP_1 (20.1 mg, 0.0635 mmol), diphenylphosphino-polystyrene (230 mg, 1.41 mmol/g, 0.230 mmol) and 4-methoxybenzyl alcohol (39.6 μL, 0.318 mmol) in dry CH_2Cl_2 (2 mL) was stirred for 45 min at room temperature and cooled to 0 °C. Then, DEAD (50.0 μL, 0.318 mmol) was added dropwise to the reaction mixture at 0 °C. The solution was warmed to room temperature, stirred for 35 h, diluted with additional CH_2Cl_2, and washed with 5% aqueous KOH solution followed by 5% HCl. The organic extracts were filtered. The resin was washed further with CH_2Cl_2 and the combined extracts were concentrated *in vacuo*. Chromatography on SiO_2 (Hexanes/EtOAc, 25:1 → 10:1 → 4:1) gave 6.1 mg (69%) of **TH-39**: ^{1}H NMR ($CDCl_3$) δ 7.70-7.45 (m, 8 H), 7.21 (dd, 1 H, J = 8.1, 0.8 Hz), 6.98 (t, 4 H, J = 8.2 Hz), 6.87 (d, 1 H, J = 10.5 Hz), 6.31 (d, 1 H, J = 10.5 Hz), 5.26 (s, 2 H), 3.84 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 182.7, 159.2, 158.8, 147.4, 141.0, 135.1, 134.7, 134.1, 132.2, 128.4, 128.3, 127.6, 121.2, 120.4, 115.9, 114.1, 109.8, 93.4, 70.7, 55.3; HRMS (EI) calcd for $C_{28}H_{20}O_5$ 436.1311, found 436.1323.
12. *Spectroscopic data for **TH-169**:* Mp 96.2-100.5 °C; IR (neat) 2956, 2919, 2852, 1662, 1617, 1460, 1393, 1344, 1296, 1240, 1157, 1083, 967, 843, 806, 746 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 12.16 (s, 1 H), 7.54 (t, 1 H, J = 8.7 Hz), 7.12 (d, 1 H, J = 7.6 Hz), 7.01 (d, 1 H, J = 8.3 Hz), 6.85 (d, 1 H, J = 10.3 Hz), 6.33 (d, 1 H, J = 10.3 Hz), 4.4-4.2 (m, 4 H); ^{13}C NMR ($CDCl_3$) δ 189.6, 161.8, 144.1, 141.4, 136.2, 128.3, 118.9, 118.0, 114.6, 99.9, 65.9; HRMS (EI) calcd for $C_{12}H_{10}O_4$ 218.0579, found 218.0571.
13. Contant, P.; Haess, M.; Riegl, J.; Scalone, M.; Visnick, M. *Synthesis* **1999**, 821.
14. *Spectroscopic data for **TH-223**:* Mp 147.5-152.1 °C; IR (neat) 2960, 2919, 2840, 1670, 1636, 1595, 1475, 1322, 1258, 1281, 1094, 1060 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.69-7.55 (m, 3 H), 7.03 (dd, 1 H, J = 7.8, 1.5 Hz), 6.37 (d, 1 H, J = 10.8 Hz), 4.33 (td, 2 H, J = 12.6, 2.5 Hz), 4.09 (dd, 2 H, J = 7.2, 4.6 Hz), 3.95 (s, 3 H), 2.5-2.2 (m, 1 H), 1.65-1.60 (m, 1 H); ^{13}C NMR ($CDCl_3$) δ 183.4, 159.4, 145.0, 134.6, 134.5, 130.9, 119.3, 118.6, 112.5, 90.8, 61.3, 56.2, 25.1; HRMS (EI) calcd for $C_{14}H_{14}O_4$ 246.0892, found 246.0896.
15. Oblong, J. E.; Gasdaska, P. Y.; Sherrill, K.; Powis, G. *Biochemistry* **1993**, *32*, 7271.
16. Gasdaska, P. Y.; Oblong, J. E.; Cotgreave, I. A.; Powis, G. *Biochem. Biophys. Acta* **1994**, *1218*, 292.
17. Krohn, K.; Michel, A.; Flörke, U.; Aust, H.-J.; Draeger, S.; Schulz, B. *Liebigs Ann. Chem.* **1994**, 1093.