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Synthesis and biological activity of prodrug inhibitors of the thioredoxin–thioredoxin reductase system

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A series of palmarumycin prodrugs and water-soluble analogs has been synthesized and assayed for inhibition of the thioredoxin–thioredoxin reductase system. Increased aqueous solubility led to an improved *in vivo* activity profile.

Regulation of the thioredoxin–thioredoxin reductase (Trx-1–TrxR) system is attracting increasing interest due to its implication in cancer, HIV-AIDS and rheumatoid arthritis along with other medical conditions.[1,2] Compounds such as auranofin (**1**),[3] palmarumycin CP_1 (**2**),[4,5] BCNU (**3**),[6] and AW464 (**4**, NSC 706704)[7] have been shown to inhibit either thioredoxin, thioredoxin reductase, or both (Fig. 1). We have previously reported that the naphthoquinone spiroketal pharmacophore of the palmarumycin family of fungal metabolites holds promising biological activity against the Trx-1–TrxR system, and we have proceeded to investigate various analogues. These compounds were tested against two human breast cancer cell lines and several members displayed potent effects in inhibiting cell proliferation.[4,5] A second generation series of palmarumycin CP_1 analogues showed increased *in vitro* activity,[8,9] but failed to reduce tumor growth *in vivo*. We attributed the lack of correlation between enzyme and animal assays to the low water solubility and limited bioavailability of our natural product lead structure. This prompted us to investigate the synthesis of more polar prodrug molecules, with hopes of attaining both improved solubility and significant antitumor activity.

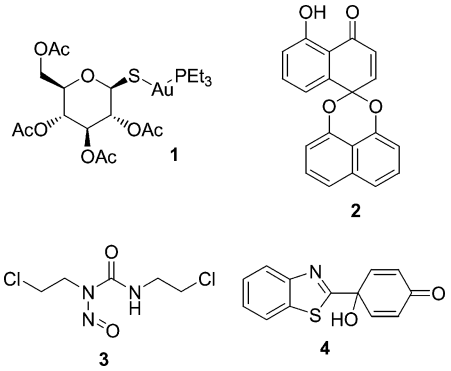
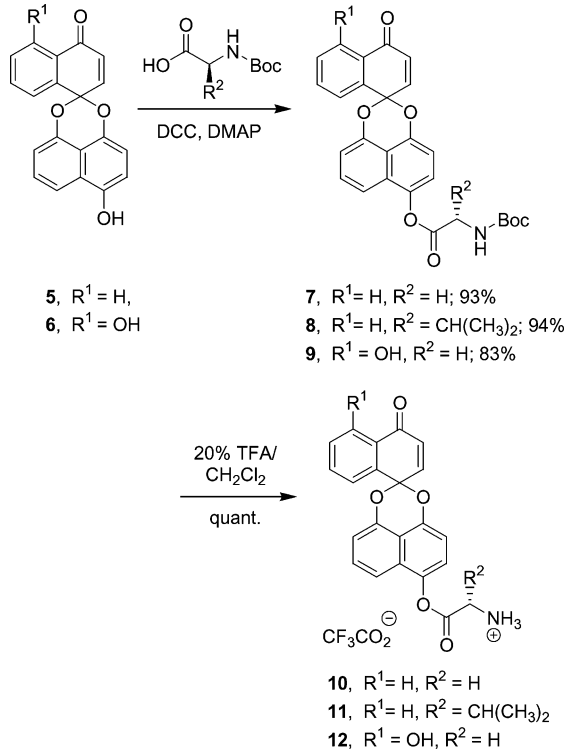


Fig. 1 Structures of Trx–TrxR inhibitors.

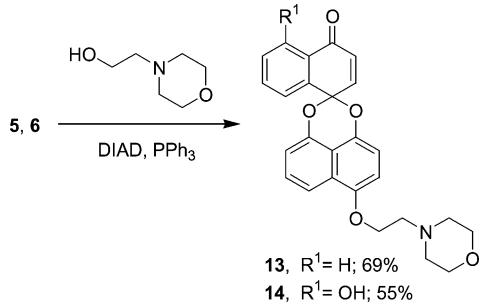
Typical solubilizing functions in prodrug derivatives include phosphonate or phosphate esters,[10] amino acid esters,[11] phenolic acetates,[12] and various other acyl groups.[13] In order to potentially increase the *in vivo* activity of the promising but very hydrophobic naphthoquinone spiroketal scaffolds **5**, and, particularly, **6**,[8] we decided to use the phenolic groups to introduce charged, hydrolytically cleavable functions. Coupling of compounds **5** and **6** with various Boc-protected amino acids proceeded in good yield and high regioselectivity (Scheme 1).[14] Only the phenolic



Scheme 1 Preparation of glycine and valine-derived analogs.

hydroxy group distal from the carbonyl functionality in **6** was acylated under the DCC mediated esterification conditions. This regioselectivity can be attributed to strong hydrogen bonding between the phenol and the carbonyl oxygen as well as the inductive attenuation of the nucleophilicity at this site. Following esterification, the carbamate was removed with 20% TFA in dichloromethane to afford a series of TFA salts[15–17] which were tested for their ability to inhibit thioredoxin reductase along with general cytotoxicity.

In addition to amino esters, the introduction of a tertiary amine in the form of a morpholine heterocycle was likewise investigated (Scheme 2).[18,19] Once again, we observed selective



Scheme 2 Preparation of morpholine-derived analogs.

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Table 1 IC_{50} values (μM) for TrxR and human breast cancer cell growth inhibition (SEM = standard error of the mean from 3 separate experiments)

Entry	Compound	TrxR inhibitory activity (±SEM)	MCF-7 growth inhibition (±SEM)
1	**6**	0.20 ± 0.01	2.6 ± 0.1
2	**10**	1.8 ± 0.1	2.4 ± 0.1
3	**11**	0.62 ± 0.06	2.2 ± 0.1
4	**12**	0.28 ± 0.04	3.1 ± 0.3
5	**13**	1.6 ± 0.1	1.2 ± 0.3
6	**14**	4.2 ± 0.3	2.6 ± 0.6

monoetherification at the hydroxyl group distal from the carbonyl group. Compounds **13** and **14** were also subjected to biological evaluation.[9] In contrast to the cleavable acyl functionalities in prodrugs **10**–**12**, the ether-linked **13** and **14** do not readily release the parent compounds; however, we had learned from our previous SAR studies[4,5,8] that the naphthaline diol substructure of **5** and **6** was mainly solvent exposed and tolerated modifications that were primarily aimed at increasing water-solubility and decreasing plasma protein binding.

As expected, all naphthoquinone spiroketal derivatives **10**–**14** showed fundamentally equivalent low micromolar IC_{50} values for MCF-7 cell growth inhibition compared to the parent phenols **5** and **6** (Table 1).[20] *In vitro* inhibition of the thioredoxin–thioredoxin reductase system was more variable, and most prodrugs did not reach the nanomolar level of activity of **6**. The TrxR IC_{50} values of permanently ether-linked **13** and **14** increased notably; in contrast, glycyl prodrug **12** was approximately equipotent. Presumably, different amounts of active drug are being released from the acyl prodrugs during the enzyme assays by spontaneous hydrolysis. Glycyl ester **12** was also considerably more water soluble than the parent compound (0.7 mg mL^{-1} *vs.* <0.1 mg mL^{-1} for **6**), and furthermore use of 20% β-cyclodextrin increased its solubility to 2 mg mL^{-1}. The half-life for the conversion of **12** to **6** in ethanol at room temperature was $t_{1/2} > 5$ d. In water at room temperature and pH 4, **12** had a $t_{1/2} = 37$ h, but it was rapidly broken down at pH 7 and above ($t_{1/2} < 1$ h). The relative lability of the prodrug at alkaline pH does not present a problem for formulation since pH 4 media can readily be administered to patients. The chemical stability of **12** in mouse plasma at room temperature was $t_{1/2} <$ 2 min, and the prodrug was indeed converted to **6** according to HPLC analysis. Upon release from its *O*-acyl protective function, **6** had a $t_{1/2}$ of *ca.* 31 min in plasma. Accordingly, glycine-derivative **12** met all the requirements that we had set forth for further development as a lead compound in *in vivo* tumor xenograft models.

Because of the promising biological profile of prodrug **12**, we were also interested in resolving the enantiomers of the parent spirocycle **6** to test if they exhibited differential biological activities. The most direct approach would be a separation *via* chiral HPLC. The low solubility that plagued the biological testing of **6** likewise hampered our efforts towards chiral separation on a multimilligram scale. However, small quantities (*ca.* 1 mg) of enantiomerically pure **6** could be obtained with a Chiralcel AD-H column,[21] and both enantiomers demonstrated comparable *in vitro* activity. Since the difference in the absolute configuration of **6** relates to the spatial orientation of the naphthaline ketal, the lack of enantioselectivity in the biological assay supports the hypothesis that this group is not primarily involved in any activity-determining interactions.[8] Accordingly, the naphthaline ketal represents a preferred site for chemical changes that target the optimization of physicochemical properties.

In conclusion, water soluble and reversible prodrug derivatives of potent inhibitors of the thioredoxin–thioredoxin reductase system were synthesized in a convergent fashion. The *O*-glycyl naphthoquinone spiroketal **12** demonstrated equivalent biological activity compared to the previous lead structure **6** in the MCF-7 tumor model as well as in the thioredoxin reductase inhibition assay. Moreover, **12** had 1–2 orders of magnitude improved aqueous solubility and, while stable at pH 4, rapidly released the active compound under physiological conditions. Prodrug **12** represents an attractive chemical probe for the study of the cellular pathways that are subject to regulation by the Trx–TrxR complex. It is possible that **12** or related agents may become useful leads for therapeutic intervention in diseases such as AIDS, rheumatoid arthritis, and certain forms of cancer.

Acknowledgements

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14 General procedure for coupling reactions. To a partial suspension of spirocycle **6** (92 mg, 0.28 mmol) in CH_2Cl_2 (6 mL) was added *N*-(*tert*-butoxycarbonyl)glycine (58 mg, 0.33 mmol), DCC (74 mg, 0.36 mmol) and DMAP (7 mg, 0.06 mmol). The reaction mixture was stirred at room temperature for 1 h as the starting material gradually dissolved and a white precipitate was formed. The cloudy solution was filtered, rinsed with CH_2Cl_2 and concentrated under reduced pressure. The residue was purified by chromatography on SiO_2 (hexanes–EtOAc, 7 : 3) to afford 112 mg (83%) of 4′-(*N*-*tert*-butyloxycarbonylamino)acetic acid ester **9** as a yellow solid: mp 182–185 (dec., EtOAc–hexanes); IR 3375, 2980, 1772, 1662, 1609, 1506, 1419 cm^{-1}; ^1H NMR (300 MHz, $CHCl_3$) δ 12.15 (s, 1 H), 7.65 (t, 1 H, J =8.0 Hz), 7.58–7.48 (m, 2 H), 7.44 (d, 1 H, J = 7.5 Hz), 7.25 (d, 1 H, J = 8.4 Hz), 7.14 (d, 1 H, J = 8.4 Hz), 7.03 (d, 1 H, J = 7.8 Hz), 7.01 (d, 1 H, J = 10.5 Hz), 6.95 (d, 1 H, J = 8.2 Hz), 6.37 (d, 1 H, J = 10.5 Hz), 5.26 (brs, 1 H), 4.34 (d, 2 H, J = 5.7 Hz), 1.50 (s, 9 H); ^{13}C NMR (75 MHz, $CHCl_3$) δ 188.8, 169.5, 162.1, 156.1, 147.6, 145.4, 141.1, 139.5, 138.8, 136.8, 130.2, 128.7, 127.4, 120.0,

119.9, 119.5, 115.8, 114.0, 113.7, 111.0, 109.6, 93.4, 80.6, 42.9, 28.6; MS (EI) *m/z* (rel. intensity) 416 ([M–O–*t*-Bu]$^+$, 48), 389 (15), 332 (100); HRMS (EI) calcd for $C_{23}H_{14}NO_7$ (M–O–*t*-Bu) 416.0770, found 416.0776.

15 General procedure for deprotection. To a solution of glycine ester **9** (95 mg, 0.19 mmol) in CH_2Cl_2 (4 mL) was added trifluoroacetic acid (1 mL). The reaction mixture was stirred at room temperature for 30 min, and concentrated under reduced pressure to afford 98 mg (100%) of **12** as a yellow solid: IR 3200, 1772, 1665, 1610, 1420, 1205 cm^{-1}; ^{1}H NMR (300 MHz, CD_3CN) δ 12.09 (brs, 1 H), 7.70 (dd, 1 H, J = 8.4, 7.7 Hz), 7.64 (dd, 1 H, J = 8.6, 1.1 Hz), 7.58 (dd, 1 H, J = 8.5 Hz, 7.4 Hz), 7.45 (dd, 1 H, J = 7.7, 1.0 Hz), 7.33 (d, 1 H, J = 8.3 Hz), 7.14 (dd, 1 H, J = 8.4, 1.0 Hz), 7.07 (dd, 1 H, J = 7.3, 1.0 Hz), 7.06 (d, 1 H, J = 10.5 Hz), 7.00 (d, 1 H, J = 8.3 Hz), 6.35 (d, 1 H, J = 10.5 Hz), 4.30 (s, 2 H); ^{13}C NMR (75 MHz, CD_3CN) δ 189.8, 167.4, 162.7, 160.8, (q, J = 37.4 Hz), 148.5, 146.6, 141.5, 140.6, 139.7, 137.8, 130.9, 129.9, 128.0, 121.0, 120.6, 120.4, 117.1 (q, J = 287.6 Hz), 116.4, 114.7, 114.4, 111.9, 110.4, 94.4, 41.8; MS (ESI) *m/z* (rel. intensity) 390 ([M–OCOCF$_3$]$^+$, 100) 359 (47); HRMS (ESI) calcd for $C_{22}H_{16}NO_6$ (M–OCOCF$_3$) 390.0978, found 390.0975.

16 Spectral data for **10**: ^{1}H NMR (300 MHz, CD_3CN) δ 8.11 (dd, 1 H, J = 7.8, 1.1 Hz), 8.00 (dd, 1 H, J = 7.8, 0.9 Hz), 7.84 (td, 1 H, J = 7.6, 1.4 Hz), 7.71 (td, 1 H, J = 7.6, 1.2 Hz), 7.68–7.57 (m, 2 H), 7.36 (d, 1 H, J = 8.3 Hz), 7.09 (dd, 1 H, J = 7.3, 1.0 Hz), 7.08 (d, 1 H, J = 10.6 Hz), 7.02 (d, 1 H, J = 8.3 Hz), 6.38 (d, 1 H, J = 10.6 Hz), 4.28 (s, 2 H); MS (ESI) *m/z* (rel. intensity) 374 ([M–OCOCF$_3$]$^+$, 37), 317 (100), 299 (30); HRMS (ESI) calcd for $C_{22}H_{16}NO_5$ (M–OCOCF$_3$) 374.1028, found 374.1034.

17 Spectral data for **11**: ^{1}H NMR (300 MHz, CD_3CN) δ 8.09 (dd, 1 H, J = 7.8, 1.3 Hz), 7.96 (d, 0.5 H, J = 7.8 Hz), 7.94 (d, 0.5 H, J = 7.8 Hz), 7.79 (t, 1 H, J = 7.6 Hz), 7.69 (d, 1 H, J = 7.7 Hz), 7.64 (d, 1 H, J = 8,5 Hz), 7.55 (t, 1 H, J = 8.1 Hz), 7.33 (d, 1 H, J = 8.2 Hz), 7.06–7.00 (m, 2 H), 6.97 (dd, 1 H, J = 8.2, 1.7 Hz), 6.33 (d, 0.5 H, J = 10.6 Hz), 6.31 (d, 0.5 H, J = 10.6 Hz), 4.44 (d, 1 H, J = 4.2 Hz), 2.65–2.59 (m, 1 H), 1.22 (d, 6 H, J = 6.9 Hz); MS (ESI) *m/z* (rel. intensity) 416 ([M-OCOCF$_3$]$^+$, 100), 307 (12), 225 (18), 199 (18); HRMS (ESI) calcd for $C_{25}H_{22}NO_5$ (M-OCOCF$_3$) 416.1498, found 416.1485.

18 General procedure for attachment of morpholine tether. To a solution of spirocycle **6** (30 mg, 0.090 mmol) in THF (2 mL) was added *N*-(2-hydroxyethyl)morpholine (11 μL, 0.090 mmol), PPh_3, (26 mg, 0.10 mmol) and DIAD (20 μL, 0.10 mmol). The reaction mixture was stirred at room temperature for 3 h then concentrated under reduced pressure. The residue was purified by chromatography on SiO_2 (EtOAc–MeOH, 19 : 1) to afford 22 mg (55%) of **14** as a yellow solid film: ^{1}H NMR (300 MHz, $CHCl_3$) δ 12.17 (s, 1 H), 7.90 (d, 1 H, J = 8.5 Hz), 7.66 (t, 1 H, J = 8.0 Hz), 7.50–7.45 (m, 2 H), 7.14 (dd, 1 H, J = 8.4, 0.9 Hz), 7.04 (d, 1 H, J = 7.3 Hz), 7.00 (d, 1 H, J = 10.5 Hz), 6.89 (d, 1 H, J = 8.3 Hz), 6.80 (d, 1 H, J = 8.3 Hz), 6.35 (d, 1 H, J = 10.5 Hz), 4.30 (t, 2 H, J = 5.5 Hz), 3.80–3.75 (m, 4 H), 2.98 (t, 2 H, J = 5.5 Hz), 2.70–2.65 (m, 4 H, *J*); MS (ESI) *m/z* (rel. intensity) 446 ([M + 1]$^+$, 100), 359 (65), 331 (20), 272 (12); HRMS (ESI) calcd for $C_{26}H_{24}NO_6$ (M + H) 446.1604, found 446.1581.

19 Spectral data for **13**: ^{1}H NMR (300 MHz, $CHCl_3$) δ 8.18 Hz (dd, 1 H, J = 7.8, 1.2 Hz), 7.98 (d, 1 H, J = 7.8 Hz), 7.90 (d, 1 H, J = 8.3 Hz), 7.77 (td, 1 H, J = 7.5, 1.3 Hz), 7.67 (td, 1 H, J = 7.5, 1.1 Hz), 7.48 (t, 1 H, J = 8.1 Hz), 7.03 (d, 1 H, J = 7.0 Hz), 7.01 (d, 1 H, J = 10.5 Hz), 6.89 (d, 1 H, J = 8.3 Hz), 6.80 (d, 1 H, J = 8.3 Hz), 6.39 (d, 1 H, J = 10.5 Hz), 4.30 (t, 2 H, J = 5.6 Hz), 3.80–3.75 (m, 4 H), 2.97 (t, 2 H, J = 5.6 Hz), 2.70–2.65 (m, 4 H); MS (ESI) *m/z* (rel. intensity) 430 ([M + 1]$^+$, 100), 343 (12), 279 (8); HRMS (ESI) calcd for $C_{26}H_{24}NO_5$ (M + H) 430.1654, found 430.1568.

20 IC_{50} values for TrxR and human breast cancer cell growth inhibition for **5** were 0.34 μM and 2.8 μM, respectively (see ref. 8).

21 Retention times for the two enantiomers on an AD-H column in 14% *i*-PrOH–hexanes were 8.27 and 10.07 min, respectively.