**The synthesis and evaluation of thymoquinone analogues as anti-ovarian cancer and antimalarial agents**

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**Abstract**

Thymoquinone (TQ), 2-isopropyl-5-methyl-1,4-benzoquinone, a natural product isolated from *Nigella sativa* L., has previously been demonstrated to exhibit antiproliferative activity *in vitro* against a range of cancers as well as the human malarial parasite *Plasmodium falciparum*. We describe here the synthesis of a series of analogues of TQ that explore the potential for nitrogen-substitution to this scaffold, or reduction to a hydroquinone scaffold, in increasing the potency of this antiproliferative activity against ovarian cancer cell lines and *P. falciparum*. In addition, alkyl or halogen-substituted analogues were commercially sourced and tested in parallel. Several TQ analogues with improved potency against ovarian cancer cells and *P. falciparum* were found, although this increase is suggested to be moderate. Key aspects of the structure activity relationship that could be further explored are highlighted.

**Keywords:** Thymoquinone; ovarian cancer; malaria; synthesis

The seeds of *Nigella sativa* L., belonging to the Ranunculaceae family, are commonly known as ‘black cumin’ or ‘black onion’. As well as a common spice, these seeds have been widely used as a traditional medicine in the treatment of a range of diseases such as cancer, diabetes, hypertension, fever, arthritis, inflammation, and gastro-intestinal disturbances.1 Studies that have explored the anti-inflammatory, antidiabetic, antimicrobial, antioxidant, immunomodualtory and antitumor activities of the essential oil from these seeds have identified thymoquinone (TQ, **1**), 2-isopropyl-5-methyl-1,4-benzoquinone (Figure 1) as a key bioactive component.2-5 Further, studies specifically investigating the cytotoxic activity of TQ have shown this compound to inhibit proliferation of several cancer cell lines, including ovarian, prostate, colon, breast, pancreatic cancers, leukaemia and osteosarcoma.2, 4, 6 Recently, TQ has been shown to block substrate recognition by the Polo-Box domain of Polo-like-kinase 1 (Plk1), a mitotic regulator that when overexpressed causes cancer.7 TQ-based inhibitors of Plk1, such as poloxin, have been developed and validate the potential of targeting Plk1 using non-peptide inhibitors of protein-protein interactions at the Polo-Box domain.8 Further modifications to TQ, including; TQ-fatty acid9 and -terpene10 conjugates, 6-alkyl thymoquinone11, 2, 5-bis (alkyl/aryl-amino) 1,4-benzoquinone12, TQ-gallate conjugate13, and TQ-artesunic acid hybrid14 have similarly demonstrated the utility of TQ-analogues as antiproliferatives in cancer cell lines. In addition to the anticancer potential of TQ, it has also been shown to have *in vitro* anti-plasmodial activity with an IC50 of 0.2 µg/ml.15 *P. falciparum* lacks polo-like kinases, although a number of mitotic kinases have been described.16, 17

Whilst TQ and TQ-analogues appear relatively safe, efforts have been focussed on improvement of the antiprolifertive activity and increased solubility to enhance its bioavailability.18 For example, only a preliminary structure-activity relationship (SAR) of TQ analogues against pancreatic cancer cell lines explored the potential of amino-substituted 2-methyl-naphthoquinone and 1,4-benzoquinone12. Thymoquinone has been demonstrated to be effective against ovarian cancer cells *in vitro* and *in vivo*19-22. In particular, treatment of syngeneic mice of ovarian cancer by TQ alone resulted in a 2-fold increase in ascites volume after 60 days compared to vehicle-treated mice. A further combination of TQ and cisplatin caused increased reduction in peritoneal implants and mesenteric tumors compared to either drug alone.19 TQ has been shown to induce apoptosis by regulation of Bcl-2 and Bax21 and increase of reactive oxygen species in ovarian cancer cells20. However, there is no SAR study on TQ against ovarian cancer and plasmodial parasites. In particular, the effect of the two alkyl substitution groups (methyl and 2-isopropyl groups) on the quinone ring, the additional substitution of the quinone ring of TQ by amine or halogen groups, and the reduction of quinones to quinol forms on the *in vitro* anti-ovarian cancer and antiplasmodial activities has not been investigated.

This study aims to discover potent cytotoxic/antiplasmodial analogues of TQ. Eleven TQ analogues including six nitrogen-substituted TQ analogues (**2**-**4**, **6**-**8**) and five reduced hydroquinones (**18**-**22**) were synthesized (Supporting information), and ten others (**5**, **9**-**17**) with different substituted groups (e.g. variation of length of alkyl chains and halogen atoms) were procured (Figure 1). Both the synthetic and procured TQ analogues were investigated for their growth inhibition in three human ovarian cancer cell lines and immortalized human ovarian epithelial cell line (HOE) by determining their IC50 values using sulforhodamine B (SRB) cytotoxicity assay23, 24 (Table 1). Eleven TQ analogues (**6**, **9**, **10-12**, **14**, **18-22**) showed IC50 less than 10 µM as TQ in A2780 cell line; they also showed potent cytotoxicity in OVCAR-8 and CIS-A2780 (cisplatin resistant) cancer cell lines with the former one being more resistant. Compound **10** with a *tert*-butyl group (an additional methyl group to the isopropyl group of TQ) and a methyl group on the quinone ring does not increase its cytotoxicity, however its SI increased from 2.3 of TQ to 7.5. Further increase of the bulkiness and hydrophobicity of the side chains either at the 2-methyl or 5-isopropyl group significantly decreases their cytotoxicity as seen for compounds (**8, 13, 16**, and **17**). These findings are consistent with loss of cytotoxicity in pancreatic cancer cell lines of the synthetic TQ analogues with bulkier substitution groups via amino addition as previously reported.12



**Fig. 1** Structure and schemes for the synthesis of TQ analogues.

**Table 1 Evaluation of inhibition of growth in ovarian cancer cell lines, HOE, and *P. falciparum* (Dd2 strain), selectivity index (SI), and ClogP values of the TQ analogues.** The SI values are calculated by comparing the activity against the non-cancer HOE cell line with that against A2780 ovarian cancer cells. The results are expressed as mean ± SEM, n=3.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Compound | Clog P | A2780  (µM) | OVCAR-8 (µM) | CIS-A2780 (µM) | HOE (µM) | SI against A2780 | *P. falciparum* (µM) |
| 1 | 1.04 | 7.9 ± 0.2 | 11.6 ± 0.3 | 7.8 ± 0.2 | 17.8±0.4 | 2.3 | 25.0±5.3 |
| 2 | 0.01 | 14.0 ±0.4 | 12.9 ±0.6 | 7.3 ± 0.3 | 36.8±3.0 | 5.0 | 52.7±10.8 |
| 3 | 0.35 | 12.6±0.2 | 16.0±0.7 | 9.6 ± 0.6 | 30.3±2.0 | 3.1 | 11.1±1.3 |
| 4 | 0.51 | 19.9±0.6 | 35.1±0.4 | 19.3±1.5 | 138.9±5.0 | 7.1 | 19.3±0.8 |
| 5 | 0.06 | 12.1±0.4 | 13.4±2.5 | 11.8±0.5 | 6.0±0.3 | <1 | - |
| 6 | 0.71 | 6.2 ± 0.2 | 5.6 ± 0.4 | 4.7 ± 0.5 | 7.9±0.9 | 1.7 | 9.9±1.4 |
| 7 | 0.71 | 16.7±2.0 | 35.6±6.6 | 20.5±.3.0 | 28.2±3.9 | 1.3 | 191.1±32.3 |
| 8 | 0.74 | 15.0±0.5 | 32.0 ± 3.9 | 21.8 ± 0.3 | 20.2±2.1 | 1.3 | 100.3±22.5 |
| 9 | 1.66 | 4.9 ± 0.2 | 3.6 ± 0.3 | 5.3 ± 0.5 | 7.3±0.4 | 1.5 | 30.0±5.1 |
| 10 | 1.51 | 7.5±1.2 | 13.5±1.5 | 7.9±2.0 | 55.8±6.2 | 7.5 | 19.7±2.8 |
| 11 | 1.59 | 4.5±0.5 | 6.0±0.4 | 4.2±0.6 | 3.1±0.3 | <1 | 16.2±1.1 |
| 12 | 1.16 | 5.7±0.5 | 10.0±0.5 | 4.9±1.2 | 10.3±1.1 | 1.8 | 15.6±1.8 |
| 13 | 2.73 | 34.0±5.6 | 51.2±0.4 | 42.5±1.9 | 117.4±1.6 | 3.4 | 4.2±0.9 |
| 14 | 0.29 | 3.2±0.2 | 5.2±0.2 | 2.9±0.3 | 5.3±0.5 | 1.7 | 10.4±1.9 |
| 15 | 2.73 | 24.6±0.6 | 37.4±1.5 | 31.1±4.1 | 51.5±0.5 | 2.1 | 29.5±2.7 |
| 16 | 3.56 | 36.1±0.1 | 54.2±1.6 | 48.8±5.4 | >200 | >5 | 78.8±0.8 |
| 17 | 6.06 | 44.2±0.3 | 56.5±0.6 | 51.8±3.1 | >200 | >4.6 | 116.3 ±15.8 |
| 18 | 2.98 | 3.1±0.2 | 8.9±0.7 | 9.8±0.3 | 14.0±2.4 | 4.5 | 15.9±2.4 |
| 19 | 4.63 | 3.4±0.4 | 11.6±0.5 | 4.8±0.4 | 11.0±2.1 | 3.2 | 25.6±1.1 |
| 20 | 3.45 | 6.4±1.5 | 12.2±0.5 | 11.5±0.3 | 85.6±5.2 | 13.4 | 17.9±1.0 |
| 21 | 2.23 | 3.6±0.4 | 8.3±0.7 | 3.6±0.6 | 7.8±1.6 | 2.1 | 15.4±0.8 |
| 22 | 1.74 | 5.7±0.4 | 6.2±0.2 | 4.7±0.2 | 6.3±0.7 | 1.1 | 133.7 ± 23.3 |
| Carboplatin | -0.34 | 16.0±1.0 | 10.8±1.3 | > 100 | 15.2±3.0 | 1.0 | 12.0 25 |
| Paclitaxel | 2.50 | 0.009± 0.001;  0.05±0.01a 26 | 0.01 ± 0.004 | -  0.003-0.004 27 | -  0.03±0.01a 26 | -  0.6 | -  0.003-0.006 28 |
| Chloroquinine | 5.00 | - | - |  | - | - | 0.17 ± 0.02 |

1. The IC50 reported was determined using the MTT method26.

Substitution of CH of isopropyl group of TQ by a single nitrogen atom in **6** results in a 2-fold increase in cytotoxicity against the cancer cell lines although the SI is similar to that of **1**. Addition of a free amine group (**4**) or an alkylamino (methylamine (**2**) or ethylamino (**3**)) group slightly reduces their activity. Substitution of TQ (**1**) and compound **10** by bromine and chlorine, respectively, shows a similar potency as TQ. Reduction of five quinones (**1**, **9**, **10**, **14** and **5**) to their reduced hydroquinones (**18-21**)29, 30 generally increases their potency and selectivity (Table 1). The SIs of TQ (**1**) and most of their synthetic analogues are slightly better than that of the clinically used carboplatin for the treatment of ovarian cancer with a similar potency in cytotoxicity as TQ (Table 1). They are also better than that of the extremely cytotoxic paclitaxel (IC50 1-50 nM) which show little difference here in selectivity between A2780 and HOE cell lines26.

The IC50 of antiplasmodial activity of TQ analogues against human malarial parasite *P. falciparum* Dd2 using Sybr Green l fluorescence assay were determined (Table 1). TQ shows a moderate anti-plasmodial activity with IC50 of 25.0±5.3 µM being greater than that previously reported15. Ten compounds (**3**, **6**, **10-14**, **18**, **20**, and **21**) show more potent antiplasmodial activity than TQ itself, particularly compounds **6** and **13** showed their IC50 values less than 10 µM. Preliminary structure antimalarial activity relationship of TQ analogues is drawn as follows. Compound **10** with a single *tert*-butyl substitution and a methyl group slightly increases its antiplasmodial activity compared to TQ (**1**). Compound **12** with single *tert*-butyl substitution group without a methyl group is also active, and compound **13** with two *tert*-butyl groups at 2 and 5 positions of quinone ring shows the most potent activity with IC50 of 4.2 µM. However, compound **15** with also two *tert*-butyl groups but at 2 and 6 positions of quinone ring show decreased activities; further increase of their hydrophobicity in the side chains in compounds **8, 16, 17** causes further decrease of their activities. These indicate that the number of *tert*-butyl groups and their position on the quinone ring are crucial for their *in vitro* antiplasmodial activity. Introducing a basic nitrogen atom to TQ has also a great impact on the antiplasmodial activity as their cytotoxicity described above. The antiplasmodial activity of compound **6** was found to be 2-fold more potent than **1**. Compounds **3** and **4** but not **2** show a greater antiplasmodial activity than TQ. Halogen-substituted quinones like **9** and **11** demonstrate similar activity. Hydroquinones (**18-21**) show similar or slightly more potency compared to their oxidized quinones.

Based on the calculated partition coefficient (ClogP) values of 1.04 and 0.29 for **6** and **14**, respectively (Table 1), they were predicted to be more water-soluble than TQ (**1**). As expected, the water solubility of compound **1**, **6** and **14** were determined to be 467.8±2.2, 8320.7±0.7 and 5043.0±6.5 µg/ml in a phosphate buffer (pH7.4) at 37°C using high-performance liquid chromatography, respectively (Supporting Information). Compound **6** and **14** possess 18 and 11-fold more water-soluble than TQ. The obtained solubility of TQ is consistent with the previously documented solubility of TQ.31 This finding is significant as poor aqueous solubility is a major deterrant to the clinical advancement of almost 50% of potent compounds classed as new chemical entities.32 However, the most anti-plasmodial compound **13** has a larger ClogP of 2.73 (Table 1), its water solubility was not further determined and is expected to be less water-soluble.

Key observations of SAR of TQ are summarized as follows:

Ovarian cancer cell lines:

* Except the promising compound **6**, there appears to be no real improvement when nitrogen containing substitutions are tested
* When halogen substitutions are used, we see an improvement in **9** and **11**
* Bulky alkyl substations, like in pancreatic cancer, are less potent
* A reduction of TQ to produce thymoquinols generally increases potency, and appears to be additive when halogens are included in **19**, but no improvement in selective activity
* **4** and **20** are of note, they are less active against HOE and this increases their SI, but for **4** this is only due to lower potency against HOE and for **20** it is improved activity against cancer cell line and reduced toxicity against HOE

*P. falciparum:*

* Introduction of halogen, nitrogen and larger alkyl substitutions in general have no effect on potency
* The same is also true for the thymoquinols
* Of note are **6** and **13** – the 2,5 arrangement may be important based on comparisons between **6** and 7
* SI is not appropriate here – HOE is not really an effective comparison to make

In summary, we have synthesized a series of TQ analogues and evaluated their antiproliferative activities against ovarian cancer cell lines and human malaria parasite. The TQ analogues **6** and **14** had significant inhibitory activities that doubled that of TQ. Compound **6** and 2,5-di-tert-butyl-1,4-benzoquinone (**13**) show the most potent antiplasmodial activity. Furthermore, the improved solubility of compound **6** together with its synthetic tractability warrants additional SAR studies of other analogues (e.g. monoalkylamino, dialkylamino, and cyclicamino groups) in order to find lead compounds with significantly improved antiproliferative activities against ovarian cancer cell lines.

**Acknowledgments:**

Okiemute Rosa Johnson-Ajinwo is funded by the Education Tax Fund (ETF), Nigeria for a PhD studentship. Imran Ullah was supported by a Keele University ACORN PhD award and the Charles Wallace Pakistan Trust.

**A. Supplementary data**

Supplementary data (synthesis of the compounds and characterizations, and biological assay of the compounds) associated with this article can be found in the online version.

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