



EXTRACTION AND SPECTROSCOPIC AUTHENTICATION OF THYMOQUINONE FROM NIGELLA SATIVA AND THE SYNTHESIS, CHARACTERISATION AND IN VITRO HIV-1 REVERSE TRANSCRIPTASE INHIBITION OF PYRIDINONE ANALOGUES

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ABSTRACT

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) remain central to HIV-1 therapy, but their durability is limited by resistance mutations in the reverse transcriptase (RT) non-nucleoside binding pocket. Thymoquinone (TQ), the principal quinone of *Nigella sativa*, has a broad pharmacological profile but no established anti-HIV pharmacophore. We report the experimental arm of an integrated programme that uses TQ as a seed for RT-directed pyridinone leads. TQ was isolated from cold-pressed *N. sativa* seed oil by silica-gel column chromatography and recrystallisation, and authenticated by proton and carbon NMR and high-resolution mass spectrometry (HRMS): crude oil yield 32% w/w of seed, TQ content 1.1% w/w, isolated TQ 0.9% w/w of oil at 96% purity, melting point 42–44 °C; HRMS confirmed $C_{10}H_{12}O_2$. Three leads were prepared and characterised: a virtual-screening pentacenedione (Ligand 1), a rationally designed pyrazolopyridinyl pyridin-2(1H)-one (Ligand 2, TQ-PZP-03) and a 3D-QSAR-benchmark pyridinone (Ligand 3, VC1). Recasting the quinone as a pyridin-2(1H)-one removed the reactive Michael-acceptor motif and installed a lactam N–H hydrogen-bond donor. In a colorimetric HIV-1 RT inhibition assay benchmarked against efavirenz (EC_{50} 4.1 $\mu\text{g mL}^{-1}$), TQ and Ligand 3 were the most active test compounds (EC_{50} 11.7 and 19.4 $\mu\text{g mL}^{-1}$; rated high), while Ligands 1 and 2 were weaker (105 and 67.1 $\mu\text{g mL}^{-1}$). The results establish TQ and the adamantyl-pyridinone as tractable, chemically validated scaffolds for optimisation as broad-spectrum RT inhibitors. Thiazolidine, Biological activities, etc.

KEYWORDS: Thymoquinone, *Nigella sativa*, HIV-1 reverse transcriptase, NNRTI, pyridin-2(1H)-one, enzyme

1. INTRODUCTION

HIV-1 reverse transcriptase (RT) is the enzyme that copies the viral RNA genome into DNA and is one of the most productive targets in antiretroviral therapy.^[1] Non-nucleoside reverse transcriptase inhibitors (NNRTIs) act allosterically at a hydrophobic pocket adjacent to the polymerase active site and are a mainstay of first-line regimens.^[3] Their weakness is durability: a small set of pocket mutations, chiefly K103N, Y181C and Y188L, confers broad cross-resistance across the class.^[2,11]

Sustaining the value of the NNRTI concept therefore depends on new chemotypes that combine drug-like properties with binding that tolerates these mutations.

Natural products offer chemical starting points that are underexploited for this target. Thymoquinone (TQ; 2-methyl-5-(propan-2-yl)cyclohexa-2,5-diene-1,4-dione), the major bioactive constituent of *Nigella sativa* (black seed), is a small p-benzoquinone with documented anticancer, anti-inflammatory, hepatoprotective and neuroprotective activities.^[5–8] It has, however, two

obvious shortcomings for RT-directed drug design: it lacks a defined NNRTI pharmacophore, and its quinone ring is a reactive Michael acceptor that raises medicinal-chemistry alerts and complicates developability. These very shortcomings define a design opportunity — TQ can be used as a seed structure whose quinone is recast as a pyridin-2(1H)-one, the hydrogen-bond-donor scaffold that anchors clinical pyridinone NNRTIs in the binding pocket.^[10]

This paper reports the experimental realisation of that strategy: the extraction and rigorous spectroscopic authentication of TQ from *N. sativa* seed, the retrosynthetic design and laboratory synthesis of three RT-directed leads, their structural confirmation by NMR and HRMS, and their head-to-head evaluation against efavirenz in a validated in-vitro HIV-1 RT inhibition assay. The complementary computational work — construction and molecular-dynamics validation of a triple-mutant (K103N/Y181C/Y188L) RT model and structure-based docking of these compounds across the resistance panel — is reported in the companion paper.^[19] Together the two studies close the discovery loop from natural-product source to enzyme-level assay.

2. MATERIALS AND METHODS

2.1 Plant Material and Authentication

Nigella sativa L. seeds were procured from a certified supplier and botanically authenticated; a voucher specimen was retained. Seeds were stored at 4 °C in airtight, light-protected containers, with a representative sample retained for HPTLC/HPLC-based thymoquinone-content screening.

2.2 Extraction and Purification of Thymoquinone

Seed oil was obtained by mechanical cold pressing and used directly as the source matrix. The crude oil was dried over anhydrous sodium sulfate and stored at 4 °C protected from light. It was loaded onto a silica-gel (60–120 mesh) column and eluted with a graded n-hexane:ethyl acetate gradient (98:2 → 90:10 v/v); fractions were monitored by TLC (n-hexane:ethyl acetate 9:1) with UV_{254/366} visualisation, TQ appearing as a diagnostic yellow spot. Target fractions were pooled, concentrated under reduced pressure, polished on a second silica column to remove co-eluting terpenoids, and recrystallised from cold n-hexane to afford TQ as yellow crystalline needles.

2.3 Purity Assessment and Structural Characterisation

Purity was confirmed by reverse-phase HPLC (C18; methanol:water mobile phase; UV detection at 254 nm) against a certified reference standard and by a single sharp melting point.^[4] Identical instrumental protocols were applied to the isolated TQ and to the synthesised leads to allow direct comparison: ¹H NMR (400/500 MHz, CDCl₃, referenced to residual solvent/TMS); ¹³C NMR (100/125 MHz); high-resolution mass spectrometry (molecular ion and diagnostic

fragmentation compared with calculated exact mass); and open-capillary melting point as a final identity check.

2.4 Synthesis of the Leads

Ligand 1 (6,13-dihydropentacene-6,13-dione). 1,4-Cyclohexanedione (1.0 eq) and o-phthalaldehyde (2.2 eq) were dissolved in ethanol; ethanolic NaOH was added and the mixture refluxed under nitrogen with TLC monitoring to effect the double aldol condensation. The crude bis-adduct was isolated by filtration and recrystallised, then aromatised with DDQ in refluxing toluene and purified by recrystallisation from a high-boiling aromatic solvent.^[13]

Ligand 2 (TQ-PZP-03). The heteroaromatic building block (pyrazolo[1,5-a]pyridin-3-yl)methanol was prepared in two steps from commercially available pyrazolo[1,5-a]pyridine (formylation followed by borohydride reduction of the aldehyde to the alcohol). The pyridinone core was elaborated and the pyrazolopyridinylmethyl ether installed by a Mitsunobu reaction (with mesylation used in the alternative route), followed by introduction of the (2H-tetrazol-2-yl)methyl substituent, to give the target pyridin-2(1H)-one.^[12]

Ligand 3 (VC1). The 4-(adamantan-1-yloxy)-3-iodo-6-methyl-5-[(2H-tetrazol-2-yl)methyl]pyridin-2(1H)-one benchmark was prepared following the disconnections implied by the reference 3D-QSAR study, introducing the adamantyloxy ether and the ring iodo substituent onto the functionalised pyridinone scaffold.^[9] All final compounds were characterised as in Section 2.3.

2.5 In Vitro HIV-1 Reverse Transcriptase Inhibition Assay

A non-radioactive, colorimetric (PicoGreen, ELISA-format) HIV-1 RT activity assay was used.^[17] This format was selected over radiolabelled dNTP-incorporation and cell-based p24/replication assays because it directly and unambiguously measures RT-catalysed DNA polymerisation — a mechanism-proximal readout uncomplicated by cell permeability, efflux or off-target antiviral effects — while retaining quantitative, plate-reader sensitivity and avoiding radioisotope handling; it is well precedented for head-to-head comparison of novel chemotypes, including natural-product compositions, against approved NNRTIs.^[18] RT was diluted to its assay-day working concentration after a preliminary enzyme-ladder titration. Template and primer were annealed; a reaction mixture was assembled in the plate; test substances (prepared at 2× final concentration in DMSO/nuclease-free water) were added; the reaction was run and then stopped, and PicoGreen detection dye was added. Fluorescence was read on a plate reader, activity plotted against inhibitor concentration, and EC₅₀ (the concentration reducing activity by 50%) determined for each compound. Efavirenz served as the reference NNRTI, and compounds with EC₅₀ < 50 µg mL⁻¹ were rated high.

Cell-based antiviral confirmation is reserved for future work.

3. RESULTS AND DISCUSSION

3.1 Extraction, Purification and Yield of Thymoquinone

Thymoquinone was isolated from cold-pressed *N. sativa* seed oil by graded silica-gel column chromatography (n-hexane:ethyl acetate, 98:2 → 90:10 v/v), TLC-guided pooling of the characteristic yellow fraction, a second polishing column to remove co-eluting terpenoids, and final recrystallisation from cold n-hexane. The material was obtained as a bright-yellow crystalline solid with a mild pungent aromatic odour. Yield and purity data are summarised in Table 1. The isolated TQ was insoluble in water and freely soluble in ethanol, methanol, acetone, ethyl acetate and chloroform, and gave a sharp melting point of 42–44 °C consistent with the reference compound.

Table 1: Extraction and purity data for thymoquinone isolated from *Nigella sativa* seed.

3.2 Spectroscopic Authentication of Thymoquinone

The isolated compound was confirmed as thymoquinone ($C_{10}H_{12}O_2$, 164.20 g mol⁻¹, CAS 490-91-5) by three orthogonal techniques. The ¹H NMR spectrum (CDCl₃) showed five environments accounting for all 12 protons: two olefinic ring =CH signals at δ 6.59 (fine q, H-6) and 6.52 (fine d, H-3), the isopropyl methine septet at δ ~3.0, the ring 5-CH₃ singlet at δ 2.05, and the isopropyl gem-dimethyl doublet at δ 1.21 (6H). Key ¹H assignments are given in Table 2. The ¹³C NMR spectrum showed nine signals, including the diagnostic p-quinone carbonyl pair near δ 187/187, two quaternary and two protonated olefinic ring carbons, and three aliphatic carbons — exactly the count expected for TQ. HRMS gave [M+H]⁺ at m/z 165.091 (calc. 165.0910; mass error -0.04 ppm) with an A+1 isotope intensity of 11.04% (calc. 10.93% for a 10-carbon ion), unambiguously confirming the molecular formula. No extraneous signals were observed, consistent with a clean, pure isolate.

Table 2: Selected ¹H NMR assignments for isolated thymoquinone (CDCl₃).

The overall integration ratio (2H olefinic : 1H methine : 3H ring-CH₃ : 6H gem-dimethyl = 12 H) matches $C_{10}H_{12}O_2$, and the ¹H, ¹³C and HRMS data are mutually consistent, confirming the identity and purity of the natural-product seed material used to nucleate the design work.

3.3 Design and Synthesis of the Pyridinone Leads

Three leads were carried into synthesis. Ligand 1 (6,13-dihydropentacene-6,13-dione) emerged from structure-based virtual screening and was prepared by a double aldol condensation of o-phthalaldehyde (2.2 eq) with 1,4-cyclohexanedione (1.0 eq) under ethanolic NaOH and reflux, followed by DDQ oxidative aromatisation in

refluxing toluene and recrystallisation. Ligand 2 (TQ-PZP-03; 6-methyl-3-(propan-2-yl)-4-[(pyrazolo[1,5-a]pyridin-3-yl)methoxy]-5-[(2H-tetrazol-2-yl)methyl]pyridin-2(1H)-one) was assembled to satisfy the four field requirements of the pyridinone pharmacophore, using (pyrazolo[1,5-a]pyridin-3-yl)methanol as a key building block introduced by a Mitsunobu etherification. Ligand 3 (VC1; 4-(adamantan-1-yloxy)-3-iodo-6-methyl-5-[(2H-tetrazol-2-yl)methyl]pyridin-2(1H)-one), the top virtual compound from a published 3D-QSAR study, was prepared in parallel as a benchmark.^[9] Critically, exchanging the TQ benzoquinone for a pyridin-2(1H)-one removed the reactive Michael-acceptor liability and introduced the lactam N–H that hydrogen-bonds to Lys101 in clinical pyridinone NNRTIs.^[10]

3.4 Spectroscopic Characterisation of the Leads

All three structures were confirmed by ¹H/¹³C NMR and HRMS (Table 3). For Ligand 1, the high molecular symmetry (D_{2h}) collapsed 12 aromatic protons into three 1:1:1 multiplets (δ 8.75, 7.97, 7.67) and 22 carbons into six signals, including the acene-quinone carbonyl at δ 182.5 — a stringent symmetry test that a lower-symmetry isomer would fail. Ligand 2 showed the diagnostic isopropyl and pyrazolopyridine signals expected for the designed connectivity. Ligand 3 showed the adamantyl envelope together with the two individually diagnostic carbons of the substituted pyridinone — the iodine-shielded ring carbon at δ ~94 ppm and the oxygenated adamantyl quaternary at δ ~82 ppm — and, tellingly, lacked both the isopropyl and pyrazolopyridine signals, confirming the distinct substitution pattern. Exact-mass values fixed all molecular formulae.

3.5 In Vitro HIV-1 Reverse Transcriptase Inhibition

Inhibitory potency was measured in a non-radioactive, colorimetric (PicoGreen, ELISA-format) HIV-1 RT activity assay, in which RT-catalysed synthesis of double-stranded DNA generates a fluorescent signal that is attenuated by inhibitors; results are expressed as EC₅₀, the concentration halving enzyme activity. Compounds with EC₅₀ < 50 µg mL⁻¹ were rated as showing high probability of real-time effectiveness. Efavirenz was the reference NNRTI. The results are given in Table 4. Efavirenz was, as expected, the most potent (EC₅₀ 4.1 µg mL⁻¹). Among the test compounds, thymoquinone (11.7 µg mL⁻¹) and the adamantyl-pyridinone Ligand 3 (19.4 µg mL⁻¹) fell within the high-activity band, whereas Ligand 2 (67.1 µg mL⁻¹) and the pentacenedione Ligand 1 (105 µg mL⁻¹) were substantially weaker.

Table 4: In vitro HIV-1 RT inhibition (colorimetric assay). Compounds with EC₅₀ < 50 µg mL⁻¹ are rated high.

3.6 Interpretation

The experimental results deliver a coherent, if qualified, verdict on the natural-product-seeded design strategy.

Thymoquinone, isolated cleanly and authenticated unambiguously, showed genuine enzyme-level activity (EC_{50} 11.7 $\mu\text{g mL}^{-1}$) despite having no prior anti-HIV credentials — validating its selection as a seed pharmacophore. Its practical limitations are not potency but developability: poor aqueous solubility, erratic absorption, photothermal instability and a reactive quinone that triggers PAINS and Brenk alerts.^[14–16]

The scaffold hop from the TQ benzoquinone to a pyridin-2(1H)-one is chemically sound: it eliminated the Michael-acceptor liability and installed the lactam N–H hydrogen-bond donor characteristic of clinical pyridinone NNRTIs. That the adamantyl-pyridinone Ligand 3 retained high-band activity (19.4 $\mu\text{g mL}^{-1}$) shows the pyridinone framework can carry meaningful potency. The virtual-screening leads were less convincing in the assay: the pentacenedione Ligand 1 was the weakest inhibitor (105 $\mu\text{g mL}^{-1}$) and, being a planar polycyclic aromatic quinone of organic-electronics provenance with irritant and specific-target-organ hazards, is de-prioritised on both potency and safety grounds. Ligand 2, though rationally designed, was only moderately active (67.1 $\mu\text{g mL}^{-1}$), indicating that satisfying the pharmacophore field requirements in silico does not by itself guarantee enzyme-level potency and that the substituent pattern needs optimisation.

Two points frame the interpretation. First, none of the test compounds matched efavirenz, so the immediate value of TQ and Ligand 3 is as validated, chemically tractable starting scaffolds for structure-guided optimisation rather than as finished candidates. Second, this is an enzyme-level, mechanism-proximal assay; it establishes direct RT inhibition uncontaminated by cell-permeability or off-target effects, but cell-based antiviral confirmation (p24/reporter assays) and evaluation against additional HIV targets remain necessary next steps. Read together with the companion computational study — in which these same compounds occupied the non-nucleoside pocket and retained their docking scores across the K103N/Y181C/Y188L resistance panel — the enzymatic activity of TQ and Ligand 3 supports advancing the pyridinone series toward broader-spectrum RT inhibitors.^[19]

4. CONCLUSION

Thymoquinone was extracted from *Nigella sativa* seed and unambiguously authenticated by $^1\text{H}/^{13}\text{C}$ NMR and HRMS, and three RT-directed leads were synthesised and characterised. In a validated enzyme-level HIV-1 RT inhibition assay, thymoquinone and the adamantyl-pyridinone (Ligand 3) were the most active test compounds, both within the high-activity band, though less potent than efavirenz; the virtual-screening pentacenedione and the designed pyrazolopyridinyl pyridinone were weaker. Recasting the reactive thymoquinone quinone as a pyridin-2(1H)-one is chemically validated as a route to remove the Michael-acceptor liability and install the NNRTI hydrogen-bond

donor. Together with the resistance-panel docking of the companion study, these findings identify thymoquinone and the pyridinone framework as tractable, experimentally supported scaffolds for optimisation toward broad-spectrum reverse transcriptase inhibitors, with cell-based antiviral testing as the immediate next step.

AUTHOR'S CONTRIBUTION

Conceptualisation, Kamil R. F. and Mohale D. S.; extraction and synthesis, Chandewar Anil; spectroscopic characterisation, Chandewar Anil and Kamil R. F.; assay, Mohale D. S.; writing — original draft, Chandewar Anil; writing — review and editing, all authors; supervision, Kamil R. F. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author on reasonable request.

INSTITUTIONAL REVIEW BOARD STATEMENT

Not applicable. The study used commercially sourced enzyme and plant material and involved no human participants or animals.

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