Novel Thioureido Derivatives Carrying Thione and Sulfonamide Moieties Induce the Cytoprotective Enzyme NAD(P)H:Quinone Oxidoreductase 1

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Plant-derived molecules have played an important role for the prevention and treatment of cancer. On the account of the reported anticancer activity of quinazolines containing the biologically active sulfonamide moieties, a novel series of thioureido and quinazoline derivatives were synthesized using methyl 2-isothiocyanatobenzoate (**2**) as a strategic starting material. The present work reports the synthesis and NAD(P)H:quinone oxidoreductase 1 (NQO1) inducer activity of three novel thioureido and quinazoline derivatives **3**, **4** and **5**. The thioureido derivative **3** was obtained in good yield *via* reaction of compound **2** with sulfaphenazole in absolute ethanol at room temperature. Reaction of compound **2** with sulfaphenazole and/or sulfadimethoxazine in absolute ethanol containing a catalytic amount of triethylamine furnished the corresponding sulfonamide derivatives **4** and **5**, respectively. The structure of the newly synthesized compounds was confirmed on the basis of elemental analyses, IR, ¹H NMR and ¹³C NMR spectra. Evaluation of the NQO1 enzyme activity in murine cells exposed to the new compounds revealed that introduction of the thioureido moiety confers inducer activity. Furthermore, incorporating the thioureido moiety within a heterocyclic ring system increases the inducer potency.

Keywords: Synthesis, Thioureido, Quinazoline, NQO1, Electrophilicity, Cytoprotection.

INTRODUCTION

Considerable attention has been devoted to the construction of new derivatives of thioureido and quinazolines, on account of their reported biological activities¹⁻⁸. Several methods have been described for the elaboration of substituted sulfonamides⁹⁻¹¹, which as a class have been reported to have cytotoxic and anticancer activity. Although tremendous progress has been achieved in the development of novel cancer treatments. Most of the current drugs exhibit high toxicity and drug resistance by tumor cells frequently develops. This dilemma is particularly true for DNA-damaging agents, the mainstay of cancer treatment¹². Quinazolines have been reported to possess several pharmacological properties, including anticancer activity¹³⁻¹⁷. It was also found that the acetamide derivatives constitute an important class of drugs, with several types of pharmacological agents possessing anticancer activity¹⁸⁻²¹. A large number of structurally novel quinazolines have been reported to show substantial anticancer activity *in vitro* and *in vivo*²².

Several mechanisms have been proposed for the anticancer activity of the quinazoline sulfonamide compounds²³⁻²⁷. The

most prominent of these mechanisms involves inhibition of carbonic anhydrase (CA) and it was suggested by Chegwidan and Spencer²⁷ that these compounds may reduce the provision of bicarbonate for the synthesis of nucleotides and other essential cellular components, such as membrane lipids. The objective of this study was to design and synthesize sulfonamide compounds which could potentially be developed as cancer chemopreventive agents. We have recently shown that sulfonamide compounds which bear electrophilic Michael acceptor enone or cyanoenone functionalities have the ability to induce NAD(P)H:quinone oxidoreductase 1 (NQO1)²⁸. NQO1 is an enzyme with a broad range of cytoprotective activities^{29,30}. The gene expression of NQO1 can be upregulated by a number of small molecule activators (inducers), through the Keap1/Nrf2 pathway³¹. Under basal conditions, Keap1, a substrate adaptor protein for Cullin3/Rbx1-based ubiquitin ligase, continuously targets transcription factor Nrf2 for ubiquitination and degradation. Inducers react with cysteine sensors of Keap1, which loses its repressor activity, leading to Nrf2 accumulation and activation of transcription of its downstream target genes, including NQO1. Small molecule Nrf2 activators, such as the plant-derived isothiocyanates, which have been identified

based on their ability to induce NQO1, have subsequently shown protective effects in numerous animal models of chemical as well as genetic carcinogenesis, at various organ sites³². Based on the above information and as a continuation of previous work on anticancer agents³³⁻³⁹, we hypothesized that introduction of the sulfhydryl-reactive thioureido moiety will confer NQO1 inducer activity. Indeed, inducers of NQO1 have one common property: reactivity with sulfhydryl groups. As NQO1 is a marker cytoprotective enzyme which is upregulated by chemopreventive compounds, information of the structural features required for NQO1 inducer activity is important for the future development of agents for cancer prevention.

EXPERIMENTAL

The starting material methylanthranilate (**1**) and thiophosgene were purchased from Sigma-Aldrich. Melting points were determined on an electrothermal melting point apparatus (Stuart Scientific, Stone) and were uncorrected. Precoated Silica gel plates (Kiesel gel 0.25 mm, 60 G F 254, Merck) were used for thin layer chromatography (TLC). The developing solvent system was chloroform/methanol (10: 3) and the spot were detected by ultraviolet light. IR spectra (KBr disc) were recorded on FT-IR spectrophotometer (Perkin Elmer) at the Research Center, College of Pharmacy, King Saud University, Saudi Arabia. ¹H NMR spectra were scanned in dimethyl sulfoxide (DMSO-*d*₆) on a NMR spectrophotometer (Bruker AXS Inc.) operating at 500 MHz for ¹H and 125.76 MHz for ¹³C at the aforementioned Research Center. Chemical shifts are expressed in δ values (ppm) relative to tetramethylsilane (TMS) as an internal standard. Exchangeable protons were confirmed by addition of drop of D₂O. Elemental analyses were done on a model 2400 CHNSO analyzer (Perkin Elmer).

Synthesis of methyl 2-(3-(4-(*N*-(1-phenyl-1*H*-pyrazol-5-yl)sulfamoyl)-phenyl)-thioureido)-benzoate (3**):** A mixture of compound **2** (1.93 g, 0.01 mole) and 4-amino-*N*-(1-phenyl-1*H*-pyrazol-5-yl)benzenesulfonamide (3.14 g, 0.01 mole) in absolute ethanol (30 mL) was stirred at room temperature for 5 h. The reaction mixture was poured onto ice water and the solid obtained was recrystallized from dioxane to give **3**. Yield % 88; m.p. 322.9 °C; IR (KBr, $\nu_{\max}$, cm^{-1}): 3448, 3275 (NH), 3039 (CH arom.), 2977, 2863 (CH aliph.), 1662 (C=O), 1620 (C=N), 1342, 1168 (SO₂), 1261 (C=S). ¹H NMR spectrum in (DMSO-*d*₆): 4.21 [s, 3H, OCH₃], 6.60, 7.64 [2d, 2H, 2CH pyrazole, $J = 7.33$ Hz], 7.08- 8.12 [m, 13H, Ar-H], 11.46 [s, 1H, SO₂NH, D₂O-exchangeable], 12.95 [s, 2H, 2NH, D₂O-exchangeable]. ¹³C NMR spectrum (in DMSO-*d*₆): 52.4, 95.6, 121.3 (2), 121.8, 127.9 (2), 128.5 (2), 129.0 (2), 131.4, 132.6, 134.3, 137.8, 139.6, 141.2, 141.8, 144.9, 168.1, 181.2. Anal. Calc. for C₂₄H₂₁N₅O₄S₂ (508): C, 56.79; H, 4.17; N, 13.80; found: C, 56.42; H, 4.34; N, 13.49.

Synthesis of 4-(4-oxo-2-thioxo-1,2-dihydroquinazolin-3(4*H*)-yl)-*N*-(1-phenyl-1*H*-pyrazol-5-yl)benzenesulfonamide (4**):** A mixture of compound **2** (1.93 g, 0.01 mole) and 4-amino-*N*-(1-phenyl-1*H*-pyrazol-5-yl)benzenesulfonamide (3.14 g, 0.01 mole) in absolute ethanol (30 mL) containing a catalytic amount of triethylamine was refluxed for 18 h. The reaction mixture was cooled and poured onto ice water and the solid obtained was recrystallized from dioxane to give **4**.

Yield % 90; m.p. 300.4 °C; IR (KBr, $\nu_{\max}$, cm^{-1}): 3307, 3210 (NH), 3088 (CH arom.), 1688 (C=O), 1610 (C=N), 1365, 1155 (SO₂), 1272 (C=S). ¹H NMR spectrum (in DMSO-*d*₆): 6.44, 7.41 [2d, 2H, 2CH pyrazole, $J = 6.97$ Hz], 7.05-8.23 [m, 13H, Ar-H], 9.35 [s, 1H, NH, D₂O-exchangeable], 11.44 [s, 1H, SO₂NH, D₂O-exchangeable]. ¹³C NMR spectrum (in DMSO-*d*₆): 91.7, 119.8 (2), 120.6 (2), 122.7, 124.6, 125.1, 126.8 (2), 127.6, 127.9, 128.5 (2), 130.6, 134.1, 134.7, 140.4, 141.3, 141.9, 144.8, 162.7, 187.3. Anal. Calc. for C₂₃H₁₇N₅O₃S₂ (476): C, 58.09; H, 3.60; N, 14.73; found: C, 58.32; H, 3.31; N, 14.51.

Synthesis of *N*-(2,6-dimethoxypyrimidin-4-yl)-4-(4-oxo-2-thioxo-1,2-dihydro-quinazolin-3(4*H*)-yl)benzenesulfonamide (5**):** A mixture of compound **2** (1.93 g, 0.01 mole) and 4-amino-*N*-(3,5-dimethoxy-phenyl)benzenesulfonamide (3.08 g, 0.01 mole) in absolute ethanol (30 mL) containing a catalytic amount of triethylamine was refluxed for 15 h. The reaction mixture was cooled and poured onto ice water and the solid obtained was recrystallized from DMF/EtOH to give **5**. Yield % 84; m.p. 219.1 °C; IR (KBr, $\nu_{\max}$, cm^{-1}): 3216, 3194 (NH), 3065 (CH arom.), 1691 (C=O), 1622 (C=N), 1357, 1163 (SO₂), 1265 (C=S). ¹H NMR spectrum (in DMSO-*d*₆): 3.62, 3.74 [2s, 6H, 2OCH₃], 6.26 [s, 1H, CH pyrimidine], 7.13-8.28 [m, 8H, Ar-H], 8.93 [s, 1H, NH, D₂O-exchangeable], 10.90 [s, 1H, SO₂NH, D₂O-exchangeable]. ¹³C NMR spectrum (in DMSO-*d*₆): 56.6 (2), 84.3, 120.8 (2), 122.6, 125.7, 126.3 (2), 126.9, 127.8, 129.8, 134.6, 134.9, 138.4, 161.3, 161.7, 163.2, 174.1, 187.8. Anal. Calc. for C₂₀H₁₇N₅O₅S₂ (472): C, 50.95; H, 3.63; N, 14.85; found: C, 50.66; H, 3.29; N, 14.49.

Biological evaluation: The NQO1 inducer activity was evaluated using a microtiter plate assay developed by Prochaska and colleagues⁴⁰. Hepa1c1c7 murine hepatoma cells were maintained in a humidified atmosphere at 37 °C, 5 % CO₂. The cell culture medium was α MEM supplemented with 10 % (v/v) heat- and charcoal-inactivated fetal bovine serum. Cells (10⁴ per well) in 96-well plates were grown for 24 h and then exposed in 8 replicates to 8 serial dilutions of compounds for 48 h. Compounds were prepared as stock solutions in DMSO and then diluted in the medium 1:1000. The final concentration of DMSO in the cell culture medium was maintained at 0.1 % (v/v) in all wells. At the end of the incubation period, cells were lysed and the specific activity of NQO1 was evaluated in cell lysates using menadione as a substrate. The concentration which doubles the specific activity of NQO1 (CD value) was used to quantify inducer potency. Protein concentrations were determined in aliquots from the cell lysates by the bicinchoninic acid (BCA) assay (Thermo Scientific). A decrease in protein concentration of the cell lysates was used as an indicator of cytotoxicity. The classical NQO1 inducer sulforaphane served as the positive control.

RESULTS AND DISCUSSION

The aim of the present work was to design, synthesize and evaluate the NQO1 inducer activity of novel thioureido and quinazoline sulfonamide derivatives (**Scheme-I**). The reaction of methyl 2-isothiocyanatobenzoate **2** with 4-amino-*N*-(1-phenyl-1*H*-pyrazol-5-yl) benzenesulfonamide in absolute ethanol at room temperature gave the methyl 2-(3-(4-(*N*-(1-phenyl-1*H*-pyrazol-5-yl)sulfamoyl)phenyl)-thioureido)-

benzoate (**3**) in good yield. The structure of compound **3** was established by elemental analysis, IR, ^1H NMR, ^{13}C NMR spectra and X-ray data. The IR spectrum of **3** revealed the presence of characteristic bands for (NH) at 3448, 3275, (CH aromatic) at 3039 cm^{-1} , (CH aliphatic) at 2977, 2863 cm^{-1} , (C=O) at 1662 cm^{-1} , (C=N) at 1620 cm^{-1} , (SO_2) at 1342, 1168 cm^{-1} and (C=S) at 1261 cm^{-1} . The ^1H NMR spectrum indicated the presence of signals at 4.2 ppm (assigned to OCH_3), 6.6 and 7.6 ppm due to 2CH pyrazole, 7.0–8.1 ppm corresponding to the aromatic protons, 11.4 ppm for SO_2NH and 12.9 ppm for 2NH groups. The ^{13}C NMR spectrum exhibited signals at: 52.4, 95.6, 121.3 (2), 121.8, 127.9 (2), 128.5 (2), 129.0 (2), 131.4, 132.6, 134.3, 137.8, 139.6, 141.2, 141.8, 144.9, 168.1, 181.2.

Scheme-1: Formation of thioureido and quinazoline derivatives **3-5**

The reaction of compound **2** with 4-amino-*N*-(1-phenyl-1*H*-pyrazol-5-yl) benzenesulfonamide and/or 4-amino-*N*-(3,5-dimethoxy-phenyl) benzenesulfonamide in refluxing absolute ethanol containing a catalytic amount of triethylamine afforded the corresponding 4-(4-oxo-2-thioxo-1,2-dihydroquinazolin-3(4*H*)-yl)-*N*-(1-phenyl-1*H*-pyrazol-5-yl) benzenesulfonamide (**4**) and *N*-(2,6-dimethoxypyrimidin-4-yl)-4-(4-oxo-2-thioxo-1,2-dihydroquinazolin-3(4*H*)-yl) benzenesulfonamide (**5**) in good yield. The structure of **4** and **5** was confirmed from its microanalysis, IR, ^1H NMR, ^{13}C NMR. Thus, IR spectrum of **4** exhibited the presence of characteristic bands for (NH) at 3307, 3210 cm^{-1} , (CH aromatic) at 3088 cm^{-1} , (C=O) at 1688 cm^{-1} and (C=N) at 1610 cm^{-1} , (SO_2) at 1365, 1155 cm^{-1} , (C=S) at 1272 cm^{-1} . ^1H NMR spectrum of **4** in ($\text{DMSO}-d_6$) revealed signals at 6.4, 7.4 ppm attributed to 2CH pyrazole, 7.0–8.2 ppm assigned to aromatic protons, 9.3 due to NH and 11.4 ppm corresponding to SO_2NH . The ^{13}C NMR spectrum revealed signals at 91.7, 119.8 (2), 120.6 (2), 122.7, 124.6, 125.1, 126.8 (2), 127.6, 127.9, 128.5 (2), 130.6, 134.1, 134.7, 140.4, 141.3, 141.9, 144.8, 162.7, 187.3. The IR spectrum of **5** had characteristic bands for (NH) at 3216, 3194 cm^{-1} , (CH aromatic) at 3065 cm^{-1} , (C=O) at 1691 cm^{-1} and (C=N) at 1622 cm^{-1} , (SO_2) at 1357, 1163 cm^{-1} , (C=S) at 1265 cm^{-1} . The ^1H NMR spectrum of **5** showed signals at: 3.6, 3.7 ppm assigned to 2OCH_3 , 6.2 ppm due to CH pyrimidine, 7.1–8.2 ppm for

aromatic protons, 8.9 ppm attributed to NH, 10.9 ppm for SO_2NH . The ^{13}C NMR spectrum exhibited signals at: 56.6 (2), 84.3, 120.8 (2), 122.6, 125.7, 126.3 (2), 126.9, 127.8, 129.8, 134.6, 134.9, 138.4, 161.3, 161.7, 163.2, 174.1, 187.8.

Biological evaluation: Compound **3** had a weak NQO1 inducer activity, elevating the specific activity of the enzyme by 1.38-fold at 100 μM , the highest concentration tested (Fig. 1A and Table-1). Compound **4** doubled the NQO1 activity at a concentration of 16 μM . Compound **5** was 3 times more potent than compound **4**, doubling the enzyme activity at a concentration of 5.2 μM . However, it was also more toxic as evident by the significant dose-dependent decrease in the protein content of the lysates of cells that had been exposed to concentrations of compound **4** higher than 25 μM (Fig. 1B). Taken together with our previous findings that the sulfonamide group by itself is not sufficient for NQO1 inducer activity²⁸, these results indicate that introduction of the thioureido moiety confers inducer activity. Furthermore, incorporating the thioureido functionality within a heterocyclic ring system increases the inducer potency substantially.

Fig. 1. Dose response of NQO1 inducer activity of thioureido and quinazoline sulfonamide derivatives. Hepa1c1c7 cells (10^4 per well) were grown in 96-well plates for 24 h. The cell culture medium was then removed and replaced with fresh medium containing serial dilutions of compounds. Sulforaphane was used as a positive control. Cells were grown for a further 48 h, and then lysed with digitonin. The specific activity of NQO1 was determined using menadione as a substrate (A). The protein content was measured in each well by the BCA assay (B). Mean values for 8 replicate wells are shown for each data point. The standard deviation for each was < 5 % of the value

Induction of NQO1 by sulfur heterocyclic compounds such as dithiolethiones (*e.g.* oltipraz) is an effective means for achieving protection against environmental carcinogens⁴¹. Transcriptional control of the expression of NQO1 is coordinately regulated with more than 100 other cytoprotective proteins

TABLE-1
NQO1 INDUCER POTENCY OF THIOUREIDO AND
QUINAZOLINE SULFONAMIDE DERIVATIVES

Compounds	CD (μM)	Induction magnitude (fold)
3	Not reached	1.38
4	16	4.3
5	5.2	5.99
Sulforaphane	0.2	4.5

and is mediated through antioxidant response elements (ARE), specific sequences in the upstream regulatory region of the genes encoding these proteins. The transcription factor Nrf2, which binds to the antioxidant response elements and its major negative regulator, Keap1, which also serves as inducer sensor, are essential for the basal and/or inducible expression of these genes. It can therefore be proposed that these new thioureido compounds induce NQO1 by modifying cysteine residues in the sensor protein Keap1.

In conclusion, we found that the introduction of a thioureido moiety confers NQO1 inducer activity of sulfonamide derivatives and that the incorporation of this moiety within a heterocyclic ring system increases the inducer potency. Importantly, NQO1 deficiency is associated with a reduced survival after metastasis in breast cancer patients⁴², whereas the NQO1 inducer sulforaphane and three synthetic analogues, which had been designed as potent NQO1 inducers, inhibit the formation of mammary tumors in Sprague-Dawley rats treated with the chemical carcinogen 9,10-dimethyl-1,2-benzanthracene⁴³. Moreover, the results from both preclinical and clinical evaluation studies of sulforaphane for chemoprevention in the breast have provided a strong rationale for evaluating the protective effects of this NQO1 inducer in clinical trials of women at risk for breast cancer⁴⁴. In addition, a recent study has reported that combinations of sulforaphane and the aromatase inhibitor exemestane, which is clinically used for prevention and treatment of breast cancers and is also an NQO1 inducer, are synergistic in inhibiting pro-inflammatory responses⁴⁵ which play an important role in the pathogenesis of cancer⁴⁶. Thus, our findings encourage the future development of more potent derivatives bearing a thioureido moiety within a heterocyclic ring system as cytoprotective agents for cancer chemoprevention.

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