Full Paper

3-Nitro-2*H*-chromenes as a New Class of Inhibitors against Thioredoxin Reductase and Proliferation of Cancer Cells

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A series of 3-nitrochromenes were designed and synthesized. These compounds showed good inhibitory activity against thioredoxin reductase (TrxR) and the proliferation of A549 cancer cells. The structure–activity relationship analysis indicates that the 3-nitrochromene scaffold is the crucial pharmacophore for achieving good inhibitory activity. The bromo-substitutions at the 6- and 8-position of 3-nitrochromene significantly increase the inhibitory activity.

Keywords: 3-Nitro-2*H*-chromene / Cancer cell / Inhibitory activity / Synthesis / Thioredoxin reductase

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Introduction

Mammalian thioredoxin reductases (TrxRs) are a family of NADPH-dependent oxidoreductases with a special selenocysteine residue. They catalyze the reduction of the redox protein thioredoxins, as well as other endogenous and exogenous compounds [1]. Biochemical, virological, and clinical evidences suggest that the overactivation/dysfunction of TrxRs play a pathophysiologic role in cancers and chronic diseases such as rheumatoid arthritis, Sjögren's syndrome, and AIDS [2–3]. Inhibiting the activity of TrxR may lead to new treatments for these diseases. Besides, TrxRs from different organisms such as *Escherichia coli*, *Mycobacterium leprae*, *Plasmodium falciparum*, *Drosophila melanogaster*, and human show a surprising diversity in the reduction mechanism. The significant species difference provides the possibility to develop specific TrxR inhibitors for chemotherapeuty [4, 5]. In recent years, extensive efforts have been made to develop TrxR inhibitors. Nitroureas, organochalcogenides (S, Se, and Te), nitroaromatic compounds, polyphenols, and metal complexes were identified as effective TrxR inhibitors. Due to the existence of nucleophilic cysteine and selenocysteine residues in their structure, TrxRs can react with Michael acceptors to form covalent adducts. Several Michael acceptors such as curcumins [6–8], cinnamaldehydes [9], enals [10], and prostaglandins [11] were found to be efficient inhibitors of TrxRs.

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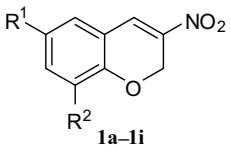
Chromenes and chromans are widespread in plants and are metabolites of microorganisms. They possess a range of interesting biological activities [12]. The 3-nitro-*2H*-choromenes can be readily prepared from salicylaldehydes and 2-nitroethylene [13]. They have been used as Michael acceptors in a couple of reactions [14, 15]. In addition, nitro compounds were reported to exert strong interactions with TrxRs [16]. We speculate that 3-nitro-*2H*-chromenes are efficient inhibitors against TrxRs. In this paper, we report the synthesis of 3-nitro-*2H*-chromenes and their biological evaluations as a new class of inhibitors against TrxRs as well as the proliferation of A549 human cancer cell lines.

Results and discussion

Synthesis

3-Nitro-2*H*-chromenes (**1a–i**) were prepared from substituted salicylaldehydes and 2-nitroethanol according to the reported procedure (Scheme 1) [13]. For a comparison, the structurally related compounds **2–5** were also prepared (Scheme 2). 6-Chloro-2*H*-chromene-3-carbonitrile (**2a**) was obtained from 5-chlorosalicylaldehyde and acrylonitrile in the presence of 1,4-diazabicyclo[2.2.2]octane (DABCO). The basic hydrolysis of **2a** provided 6-chloro-2*H*-chromene-3-carboxylic acid (**2b**). 3-Nitrochromans (**3a–3b**) were prepared by the reduction of **1a–b** with $NaBH_4$. *N*-(6-Chlorochroman-3-yl)acetamide (**4**) was obtained via the reduction of **3b** with zinc powder in the mixed solvent of acetic acid and acetic anhydride. 3-Nitro-2*H*-chromen-2-one (**5**) was prepared from salicylaldehyde and methyl nitroacetate.



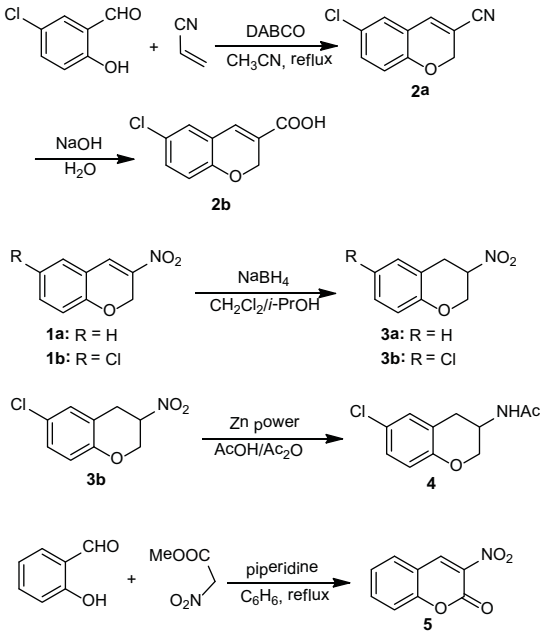
1a–1i

1a: R^1 = H, R^2 = H; **1b:** R^1 = Cl, R^2 = H;
1c: R^1 = F, R^2 = H; **1d:** R^1 = Br, R^2 = H;
1e: R^1 = Cl, R^2 = Cl; **1f:** R^1 = Br, R^2 = Br
1g: R^1 = NO_2, R^2 = H; **1h:** R^1 = MeO, R^2 = H;
1i: R^1 = H, R^2 = MeO

Scheme 1. 3-Nitro-2*H*-chromenes **1a–i**.

Biological activities

The inhibitive activities of compounds **1–5** against TrxR were determined by incubation with DTNB, TrxR, NADPH, and the test compound (25 μM). The initial increase of the absorbance at 405 nm was recorded and IC_{50} was calculated by the standard method. The results are listed in Table 1. The 3-nitro-2*H*-chromenes **1a–i** showed potent inhibitory activities (Table 1, entries 1–9), however the corresponding 3-cyano- and 3-carboxylic acid analogues **2a–b** were inactive (Table 1, entries 10–11). The 3-nitro-chromans **3a–b** showed comparative inhibitory activities with **1a** and **b** (Table 1, entries 12–13). The fact implicates that the 3-nitro group is the crucial function group interacting with TrxR. 6-Bromo-3-nitro-2*H*-chromene **1d** showed better inhibitory activity than 6-chloro-3-nitro-2*H*-chromene **1b**, 6-fluoro-3-nitro-2*H*-chromene **1c** and 3-nitro-2*H*-chromene **1a** (Table 1, entry 4 *vs*. entries 1–3). Further increase of inhibitive activity was observed for 6,8-dibromo-3-nitro-2*H*-chromene **1f** (Table 1, entry 6). These results demonstrate that the bromo substitution at the benzene ring is beneficial for the activity. On the other hand, 3-acetamido-chroman **4** and 3-nitrochromenone **5** were found to be ineffective (Table 1, entries 14–15).



Scheme 2. Synthesis of compounds **2–5**.

Table 1. Inhibitive activities (IC_{50}) of 3-nitro-2*H*-chromenes **1a–i** and their analogs **2–5** against TrxR

Entry	Compound	IC_{50} (μM)
1	**1a**	4.01
2	**1b**	2.18
3	**1c**	1.68
4	**1d**	1.17
5	**1e**	1.79
6	**1f**	1.04
7	**1g**	2.41
8	**1h**	2.82
9	**1i**	4.52
10	**2a**	>50
11	**2b**	>50
12	**3a**	2.58
13	**3b**	3.88
14	**4**	>50
15	**5**	>50

Furthermore, the inhibitive activities of compounds **1–5** against the A549 cancer cell line were studied. Chlormethine was used as the positive control and the results are listed in Table 2. Generally compounds **1a–i** showed good inhibitory activities (Table 2, entries 1–9). The 6-halogen substitutions exerted a beneficial effect on the activity (Table 2, entries 2–4), however 6,8-di-halogen substitutions could not further improve the activity (Table 2, entries 5–6). 6-Methoxyl-3-

Table 2. Inhibition against the proliferation of A549 cancer cells by 3-nitro-2*H*-chromenes **1a–i** and their analogs **2–5**

Entry	Compound	Inhibition of A549
		IC50 (μM)
1	**1a**	5.84
2	**1b**	1.52
3	**1c**	1.42
4	**1d**	1.91
5	**1e**	2.62
6	**1f**	3.19
7	**1g**	1.81
8	**1h**	5.64
9	**1i**	10.46
10	**2a**	>100
11	**2b**	>100
12	**3a**	>100
13	**3b**	>100
14	**4**	>100
15	**5**	19.27
16	Chlormethine	3.80

nitro-*2H*-chromene **1h** and 8-methoxyl-3-nitro-*2H*-chromene **1i** showed significantly lower inhibitory activities than 3,6-dinitro-*2H*-chromene **1g** and halogen substituted compounds **1b–f** (Table 2, entries 8–9 *vs*. entries 2–7). The replacement the 3-nitro group with CN and COOH resulted in the complete loss of the activity (Table 2, entries 10–11). In addition, the reduction of the 3,4-double bond of 3-nitro-*2H*-chromenes also led to inactive compounds **3a** and **b** (Table 2, entries 12–13). 3-Acetoamido-chroman **4** was also found to be ineffective (Table 2, entry 14). On the other hand, 3-nitrochromenone **5** showed much lower inhibitory activity (Table 2, entry 15). These results undoubtedly demonstrate the importance of the 3-nitro-*2H*-chromene scaffold. Since 3-nitrochromenes are efficient Michael acceptors in a couple of organic reactions [14, 15], we reason that their inhibitory activity against the A549 cell line originates from the electrophilic reactivity with DNA. It should be noted that 3-nitro-*2H*-chromenes **1b–g** showed more potent inhibitory activities than chlormethine (Table 2, entries 2–7 *vs*. entry 16). They have the potential as a new class of alkylating reagents for cancer treatment.

Conclusion

In summary, a series of 3-nitro-*2H*-chromenes were found to show good inhibitory activities against TrxR and the proliferation of the A549 cell line. The structure–activity relationship analysis undoubtedly demonstrates that the 3-nitro-*2H*-chromene scaffold is the crucial pharomacophore. Further studies are currently under way to improve the inhibitory activity and to understand the interaction mechanism.

Experimental

General

All solvents were used as commercial anhydrous grade without further purifications. The flash column chromatography was performed over silica gel (230–400 mesh), purchased from Qingdao Haiyang Chemical Co., Ltd. Melting points were recorded on an OptiMelt melting point apparatus. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE 400 MHz spectrometer. Chemical shifts in ^{1}H NMR spectra are reported in parts per million (ppm, δ) downfield from the internal standard Me_4Si (TMS, $\delta = 0.00$ ppm). Chemical shifts in ^{13}C NMR spectra are reported relative to the central line of the chloroform signal ($\delta = 77.0$ ppm). High-resolution mass spectra were obtained with a Shimadzu LCMS-IT-TOF spectrometer. Infrared (IR) spectra were recorded on a Bruker Tensor 37 spectrophotometer. Data are represented as follows: frequency of absorption (cm^{-1}), intensity of absorption (vs, very strong; s, strong; m, medium; w, weak). 3-Nitro-*2H*-chromenes (**1a–i**) were prepared according to the reported procedure [13].

*Synthesis of 6-chloro-2H-chromene-3-carbonitrile (**2a**)*

To a solution of 5-chloro-salicylaldehyde (0.783 g, 5 mmol) in acetonitrile (5 mL) was added acrylonitrile (1.33 g, 25 mmol) and DABCO (0.112 g, 1 mmol). The reaction mixture was refluxed for 20 h and cooled down to room temperature. After aqueous NaOH (2 M, 10 mL) was added, the mixture was extracted with CH_2Cl_2 (25 mL × 3). The combined organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by flash chromatography (petroleum ether/CH_2Cl_2 = 7:1) to give the product **2a** as a white solid. Yield 72%, mp 135.0–136.5°C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.22$ (dd, $J = 8.7$, 2.5 Hz, 1H), 7.12–7.08 (m, 2H), 6.82 (d, $J = 8.7$ Hz, 1H), 4.83 (d, $J = 1.4$ Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 152.76$, 137.56, 132.31, 127.73, 127.40, 121.11, 118.01, 115.93, 104.89, 64.50; IR (KBr) ν/cm^{-1}: 3062 (m), 2925 (w), 2213 (s), 1840 (m), 1451 (s), 1178 (w), 815 (s), 628 (m); HRMS (ESI) calcd for $C_{10}H_5ClNO^-$ $[M-H]^-$: 191.0138, found: 191.0133.

*Synthesis of 6-chloro-2H-chromene-3-carboxylic acid (**2b**)*

A mixture of 6-chloro-2*H*-chromene-3-carbonitrile **1j** (0.575 g, 3 mmol) in aqueous NaOH (10%, 15 mL) was refluxed for 5 h. The reaction mixture was placed in an ice bath and acidified with concentrated HCl to pH 10. The precipitate was filtered and washed with hexane. The crude product was purified by flash chromatography (CH_2Cl_2/MeOH = 9:1) to give **2b** as a yellow solid. Yield 95%, mp 240.1–241.2°C; ^{1}H NMR (400 MHz, DMSO-*d6*): $\delta = 7.46–7.44$ (m, 2H), 7.29 (dd, $J = 8.6$, 2.6 Hz, 1H), 6.88 (d, $J = 8.6$ Hz, 1H), 4.93 (d, $J = 1.5$ Hz, 2H); ^{13}C NMR (100 MHz, DMSO-*d6*): $\delta = 165.19$, 153.07, 130.97, 130.92, 128.13, 125.17, 124.73, 122.44, 117.34, 64.38; IR (KBr) ν/cm^{-1}: 3287 (w), 1700 (s), 1478 (s), 1097 (w), 1018 (w), 820 (s), 758 (s); HRMS (ESI) calcd for $C_{10}H_6ClO_3^-$ $[M-H]^-$: 209.0005, found: 209.0004.

*Synthesis of 3-nitrochromans (**3a**)*

To a solution of 3-nitro-2*H*-chromene **1a** (0.531 g, 3 mmol) in CH_2Cl_2 (40 mL) and *i*-PrOH (16 mL) was added silica gel (2.4 g) at room temperature. Then $NaBH_4$ (0.227 g, 6 mmol) was added slowly over 15 min. The reaction mixture was stirred for 0.5 h and quenched with acetic acid (6 mL). After stirring for another 0.5 h, the reaction mixture was filtered. The insoluble solid was washed thoroughly with CH_2Cl_2 (3 mL × 2). The filtrate was concentrated and the residue was treated with EtOAc (25 mL) and H_2O (25 mL). The organic layer was separated, washed with brine, and dried over anhydrous Na_2SO_4. After the solvent was evaporated under vacuum, 3-nitrochroman (**3a**) was obtained as a white solid. Yield 98%, mp 86.2–87.5°C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.18–7.12$ (m, 2H), 6.95 (td, $J = 7.5$, 1.2 Hz, 1H), 6.88–6.86 (m, 1H), 4.95 (m, 1H), 4.62–4.43 (m, 2H), 3.45 (dd, $J = 16.9$, 5.8 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 153.32$, 129.53, 128.21, 121.90, 117.60, 117.02, 77.62, 65.83, 28.30; IR (KBr) ν/cm^{-1}: 1583 (w), 1549 (s), 1489 (m), 1359 (w), 1259 (s), 1117 (w), 1047 (m), 883 (m), 767 (s); HRMS (ESI) calcd for $C_9H_8NO_3^-$ $[M-H]^-$: 178.0504, found: 178.0506.

*Synthesis of 6-chloro-3-nitrochroman (**3b**)*

3b was prepared by a similar procedure starting from 6-chloro-3-nitro-2*H*-chromene (**1b**). Yellow solid, yield 96%, mp 80.0–81.2°C; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.13–7.11$ (m, 2H), 6.81 (d, $J = 8.9$ Hz, 1H), 4.95 (m, 1H), 4.71–4.39 (m, 2H), 3.43 (dd, $J = 17.2$, 5.2 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 151.93$, 129.05, 128.29, 126.74, 119.18, 118.42, 77.14, 65.96, 27.85; IR (KBr) ν/cm^{-1}: 2997 (w), 2925 (m), 1547 (s), 1482 (s), 1240 (m), 1215 (s), 1021 (w), 925 (w), 818 (s), 638 (m);

HRMS (ESI) calcd for $C_9H_7ClNO_3^-$ $[M-H]^-$: 212.0114, found: 212.0121.

*Synthesis of N-(6-chlorochroman-3-yl)acetamide (**4**)*

To a solution of 6-chloro-3-nitrochroman **3b** (0.214 g, 1 mmol) in acetic anhydride (5 mL) and acetic acid (5 mL) at 0°C was added activated zinc powder (0.523 g, 8 mmol). The reaction mixture was stirred for 8 h at room temperature. The mixture was filtered to remove excess zinc and the filtrate was concentrated under vacuum. The residue was triturated with water to remove zinc acetate. The resulted precipitate was purified by flash chromatography (petroleum ether/EtOAc = 3:1) to give **4** as a white solid. Yield 62%, mp 117.0–118.5°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.08 (dd, J = 8.5, 2.5 Hz, 1H), 7.03 (s, 1H), 6.78 (d, J = 8.7 Hz, 1H), 5.89 (s, 1H), 4.46 (m, 1H), 4.14 (dd, J = 17.1, 6.6 Hz, 2H), 3.11–2.71 (m, 2H), 1.96 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 169.95, 152.47, 129.96, 127.79, 125.96, 121.00, 118.20, 68.21, 41.98, 30.60, 23.28; IR (KBr) ν/cm^{-1}: 3280 (m), 1480 (s), 1214 (m), 1021 (w), 914 (w), 815 (s), 628 (w); HRMS (ESI) calcd for $C_{11}H_{11}ClNO_2^-$ $[M-H]^-$: 224.0478, found: 224.0476.

*Synthesis of 3-nitro-2H-chromen-2-one (**5**)*

To a solution of salicylaldehyde (0.366 g, 3 mmol) in dry benzene (30 mL) was added methyl nitroacetate (0.426 g, 3.6 mmol) and piperidine (0.06 mL, 0.6 mmol). The reaction mixture was refluxed overnight. The water generated in the reaction was removed with a Dean-Stark apparatus. The mixture was then cooled to 0°C and the yellow precipitate was collected with a sintered glass funnel. The precipitate was dissolved in DMF (70 mL) and the solution was cooled to 0°C. After ice-water (60 mL) was added, the bright yellow precipitate was collected, washed with cold water (20 mL × 2) and dried under vacuum. Yield 83%, mp 142.5–144.0°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.75 (s, 1H), 7.80 (ddd, J = 8.6, 7.4, 1.6 Hz, 1H), 7.74 (dd, J = 8.1, 1.6 Hz, 1H), 7.48–7.45 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 154.92, 151.97, 142.40, 136.24, 134.93, 130.74, 125.98, 117.18, 116.22; IR (KBr) ν/cm^{-1}: 3057 (m), 2283 (w), 1756 (s), 1609 (m), 1521 (m), 1348 (s), 770 (s); HRMS (ESI) calcd for $C_9H_4NNaO_4^-$ $[M+Na]^-$: 214.0116, found: 214.0112.

Biological evaluations

TrxR inhibition assay

Commercially available rat liver TrxR (Sigma), DTNB (Sigma), and NADPH (Roche) were used. Compound stocks (10 mM) were prepared in dimethyl sulfoxide (DMSO). All assays were conducted in a total volume of 40 μL in 384-well microplate. In each measurement, the compound stock solution in the indicated volume was added to potassium phosphate buffer (100 mM, pH = 7.0) containing recombinant rat TrxR (2 nM), NADPH (120 μM), and EDTA (1.25 mM). The resulting mixture was incubated for 5 min at 37°C. Then the enzyme reaction was initiated by the addition of DTNB (3.2 μL, 63 mM in ethanol). The first 2 min absorbance increase at 412 nm ($\Delta\varepsilon_{412}$ = 13.6 mM^{-1} cm^{-1}) was recorded by a Tecan Infinite M200 PRO microplate reader. The same volume of DMSO was used instead of the compound solution as the blank control. The inhibitive ratio of TrxR activity was obtained as a percentage of enzyme activity of the control. The IC_{50} values were calculated from the concentration-inhibitory ratio curves by Microsoft Excel.

Growth inhibition assay of A549 cell line

A549 carcinoma human alveolar basal epithelial cell suspensions were prepared and diluted to cell density about 10 000 cells/well. Cells were added by pipette into 96-well microtiter plates. The inoculates were allowed after a pre-incubation period of 24 h at 37°C with 5% CO_2. All tested compounds were used as solution in DMSO. The incubation of the cells with the test compounds lasted for 72 h at 37°C under 5% CO_2 atmosphere and 100% humidity. At the end of the incubation period, MTT stock solution (20 μL, 2.5 mg/mL) was added to each well. The plate was incubated for a further 4 h. The generated formazan was dissolved by the addition of 100 μL/well of DMSO. The optical density (OD) was measured at 490 nm with a BioTek PowerWave XS2 microplate spectrophotometer. The IC_{50} values were calculated from the dose–response curves by Microsoft Excel.

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The authors have declared no conflicts of interest.

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