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EVALUATION OF GENISTEIN ANALOGUES AS ANTI-CANCER AGENTS: AN ANALYSIS

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ABSTRACT
Genistein is a bioactive isoflavone derived from soybeans. The tie-in between the intake of genistein and the decreased incidence of some solid tumors (including prostate cancer) has been demonstrated by epidemiological studies. The potential of genistein in treating prostate cancer has also been displayed by *in vitro* cell-based and *in vivo* animal experiments. Genistein has entered clinical trials for both chemoprevention and potential treatment of prostate cancer. Even though the low oral bioavailability has presented the major challenges to genistein's further clinical development, chemical modulation of genistein holds the promise to generate potential anti-prostate cancer agents with enhanced potency and/or better pharmacokinetic profiles than genistein. As part of our ongoing project to develop natural products-based anti-prostate cancer agents, the current study was undertaken to synthesize eight genistein analogues for cytotoxic evaluation in three prostate cancer cell lines (PC-3, DU-145, LNCaP; both androgen-sensitive and androgen-refractory cell lines), as well as one aggressive cervical cancer cell line (HeLa). Eight genistein analogues have been successfully synthesized with Suzuki-Miyaura coupling reaction as a key step. Their *in vitro* anti-cancer potential was evaluated by trypan blue exclusion assay and WST-1 cell proliferation assay against a panel of four human cancer cell lines. The acquired data suggest i) that the C-5 and C-7 hydroxyl groups in genistein are very important for the cytotoxicity and anti-proliferative activity; and ii) that 1-alkyl-1*H*-pyrazol-4-yl and pyridine-3-yl might act as good bioisosteres for the 4'-hydroxyphenyl moiety in genistein.

Keywords: Cytotoxicity, genistein analogue, prostate cancer, structure-activity relationship, Suzuki-Miyaura coupling reaction

INTRODUCTION
Genistein (1, Fig. 1) is a bioactive isoflavone derived from soybeans. Its potential to prevent and treat prostate cancer has been extensively investigated due to the inverse association between dietary intake of soybeans and a lower risk of prostate cancer, which has been well-observed by epidemiological studies. Genistein is the critical chemical component essential for the preventive effect of soybeans against prostate cancer, which has been confirmed by human clinical studies of genistein. Furthermore, genistein has been established as a prospective cytotoxic agent against various prostate cancer cell lines, including TRAMP-C2 (mouse prostate cancer cell line, IC_{50}, 30 μM), LNCaP (androgen-sensitive human prostate cancer cell line, IC_{50}, 4.3–27 μM), DU-145 (androgen-resistant human prostate cancer cell line, IC_{50}, 4.3–27 μM), and PC3 (androgen-resistant human prostate cancer cell line, IC_{50}, 40 μM). The *in vitro* anti-prostate cancer potential of genistein has been well-mirrored by its *in vivo* anti-tumor efficacy. The development of advanced prostate cancer in castrated TRAMP mice can be suppressed by genistein. The metastasis of human prostate cancer can be suppressed by dietary concentrations of genistein in xenograft mice models]. The animal experiment in Lobund-Wistar rat has attested the prevention of spontaneous development of metastasizing adenocarcinoma by genistein. The blockage of

tumor growth by radiation can be potentiated by genistein in an *in vivo* prostate cancer orthotopic model. Genistein has entered phase 2 clinical trial for potential treatment of metastatic prostate cancer.

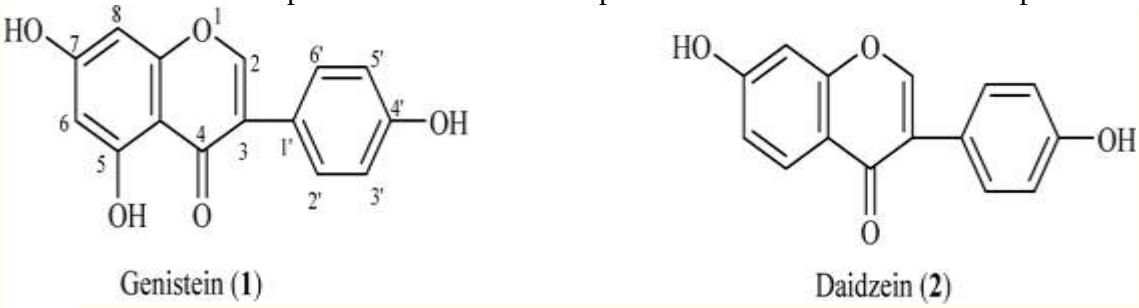


Fig. 1

Structures of genistein and daidzein.

The multi-target mechanism underlying these effects of genistein has been demonstrated by numerous studies supported by experimental data. Genistein has been reported to inhibit cell cycle regulation, tyrosine kinases, DNA topoisomerases, telomerase, apoptosis, and angiogenesis. The action of genistein on multiple cancer-related biological pathways has also been evidenced by the experimental data collected through cDNA microarray and reverse transcription-polymerase chain reaction (RT-PCR) analysis, which revealed that genistein down-regulates several cell-cycle genes (including the mitotic kinesins, cyclins, cyclin-dependent kinases), and decreases various members of the Bcl-2 family of apoptotic proteins, and up-regulates the DefB1 and the HLA membrane receptor genes involved in immunogenicity. Also, genistein was demonstrated to suppress androgen receptor expression and down-regulate androgen-regulated transcript-1 (PART-1) gene expression induced by dihydrotestosterone in human prostate LNCaP cancer cells.

Collectively, genistein with multi-target actions represents a potential agent to treat prostate cancer as suggested by epidemiological, cell-based, animal-based studies, and human clinical trials. However, the low oral bioavailability of genistein has been evidenced by *in vivo* animal pre-clinical and human clinical pharmacokinetic studies, which constituted the major challenge to its further clinical development. Chemical modulation of genistein could be a good way to generate potential anti-prostate cancer agents with improved potency and/or enhanced pharmacokinetic profiles. There are only few reports that have been published so far on the anti-prostate cancer potential of the analogues and derivatives of genistein.

As part of our ongoing research program on the development of natural products-based agents for the potential treatment of aggressive cancer (such as advanced metastatic castration-resistant prostate cancer), we aimed to synthesize genistein analogues for their cytotoxic evaluation towards both androgen-sensitive and androgen-refractory prostate cancer cell lines (PC-3, DU-145, and LNCaP), as well as one aggressive cervical cancer cell line (HeLa). Daidzein (2, Fig. 1), compound 3, and compound 4 were incorporated to investigate the effect of the phenolic hydroxyl groups of genistein on cytotoxicity towards these four cancer cell lines. Nitrogen-containing heteroaromatic rings (compounds 5–10, Fig. 2) were introduced to explore their possibility to act as bioisostere of the 4'-hydroxyphenyl moiety in genistein and to increase water solubility and bioavailability.

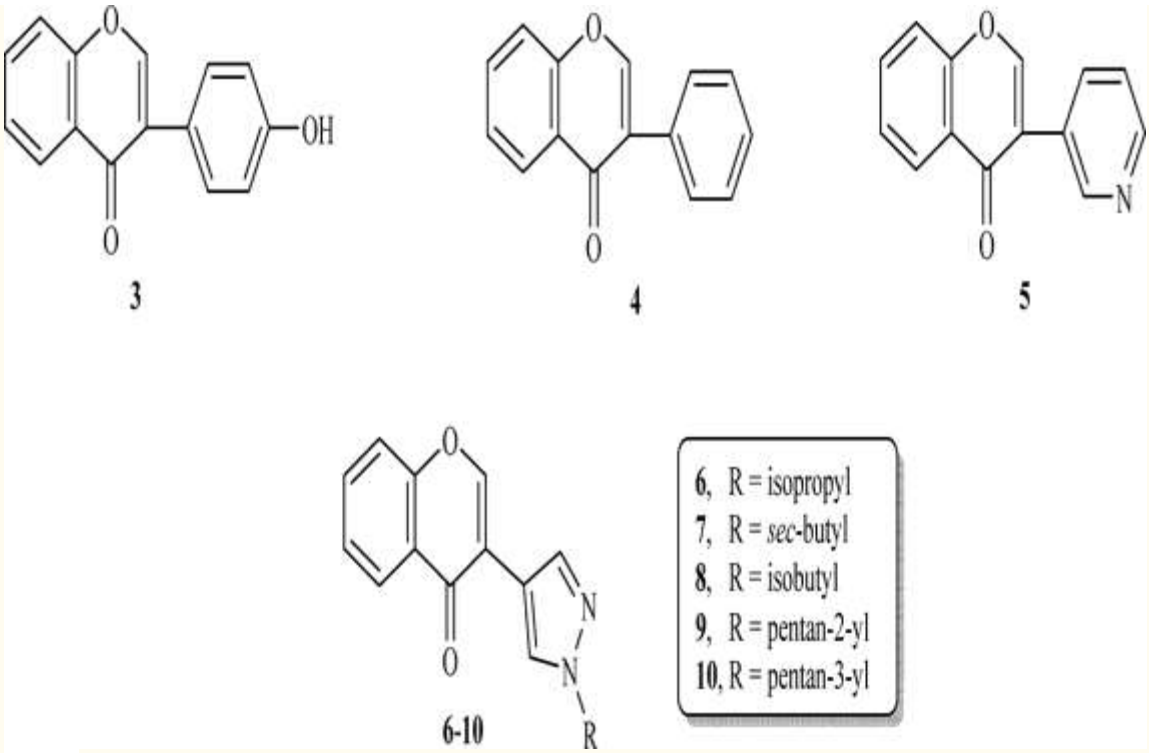


Fig. 2
Synthetic targets.

MATERIALS AND METHODS

Chemical Synthesis-General Methods

HRMS were obtained on an Orbitrap mass spectrometer with electrospray ionization (ESI). NMR spectra were obtained on a Bruker Fourier 300 spectrometer or a Varian NMR 400 MHz Spectrometer (Agilent-Varian) in $CDCl_3$, CD_3OD, or DMSO-d_6. The chemical shifts are given in δppm referenced to the respective solvent peak, and coupling constants are reported in Hz. Anhydrous THF and dichloromethane were purified by PureSolv MD 7 Solvent Purification System from Innovative Technologies (MB-SPS-800). All other reagents and solvents were purchased from commercial sources and were used without further purification. Silica gel column chromatography was performed using silica gel (32–63 µ). Preparative thin-layer chromatography (PTLC) separations were carried out on 1000 µ AnalTech thin layer chromatography plates (Lot No.13401). TLC was carried out on aluminum-backed silica gel GF plates (250 µM thickness), and the compounds were visualized by charring with $KMnO_4$ and/or short wavelength UV light. 3-Iodochromone (11) was synthesized in 88% yield based on the procedure as described in the literature. 1-Alkyl-4-iodopyrazoles (19a–e) were synthesized according to the procedure as illustrated in the literature.

Synthesis of Genistein Analogues (3–10)

General Procedure for the Synthesis of 1-alkyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrazole (15a–e)

A 25mL flask charged with pyrazole (272 mg, 1 mmol), palladium catalyst (245 mg, 0.03 mmol), KOAc (294 mg, 3 mmol), and bis(pinacolato) diboron (381 mg, 1.5 mmol) was flushed with argon. DMSO (6 mL) and the appropriate 1-alkyl-4-iodopyrazole (1 mmol) were added and the reaction mixture was stirred for 12 h at 80 °C prior to being extracted with ethyl ether (100 mL × 3). The combined extracts were rinsed with brine (20 mL × 3), dried over anhydrous $MgSO_4$, filtered, and concentrated under

reduced pressure. The residue obtained was chromatographed over silica gel, eluting with 20% ethyl acetate in hexanes, to furnish the respective 1-alkyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-pyrazole (15a–e). A boron-containing impurity is very harsh to be completely removed from boronates 15a–e even after multiple times of column chromatography purification. We therefore directly used compounds 15a–e contaminating with minor impurity (less than 10%) for the next step reaction.

General Procedure for the Synthesis of Genistein Analogues (3–5)

3-Iodochromone (11, 272 mg, 1 mmol) and the appropriate boronic acid (12, 13, or 14, 1.5 mmol) were dissolved in THF (30 mL), and then aqueous Na_2CO_3 (3.0 mL of 2 M) followed by tetrakis(triphenylphosphine)palladium (35 mg, 0.03 mmol) were added. The reaction was refluxed for 5–6 h prior to being cooled to 25 °C. The reaction mixture was diluted with water (50 mL) and extracted with EtOAc (100 mL × 3). The combined EtOAc extracts were rinsed with water (30 mL × 3), the organic fraction was dried over anhydrous magnesium sulfate, and the solvent was removed *in vacuo* to afford the crude product. Purification of the product by preparative TLC using 30% ethyl acetate in hexanes as eluent furnished the respective title compound (3–5).

3-(4-Hydroxyphenyl)-4H-chromen-4-one (3)

This compound was prepared from the Suzuki coupling of 3-iodochromone (11) and (4-hydroxyphenyl)boronic acid (12) in 21% yield as a brown solid: mp. 221–223 °C; ^{1}H NMR (400 MHz, CD_3COCD_3) δ8.44 (d, *J* = 1.6 Hz, 1H), 8.27 (d, *J* = 2.0 Hz, 1H), 8.22 (dd, *J* = 8.0, 1.6 Hz, 1H), 7.79 (tt, *J* = 7.2, 2.0 Hz, 1H), 7.61-7.58 (m,1H), 7.51-7.48 (m, 3H), 6.93-6.89 (m, 2H); ^{13}C NMR (75 MHz, CD_3COCD_3) δ174.8, 156.8, 155.6, 152.4, 133.1, 129.6, 125.2, 124.5, 124.1, 123.9, 122.7, 117.6, 114.4; IR (KBr) 3296, 1621, 1607, 1594, 1582, 1568, 1509, 1477 cm^{-1}. HRMS (ESI) *m/z*: calcd for $C_{15}H_{11}O_3$ $[M+H]^+$: 239.0708; Found 239.0709.

3-Phenyl-4H-chromen-4-one (4)

This compound was prepared from the Suzuki coupling of 3-iodochromone (11) and phenylboronic acid (13) in 38% yield as a pink needle: mp. 130–131 °C; ^{1}H NMR (400 MHz, CD_3COCD_3) δ8.39 (s, 1H), 8.27 (dd, *J* = 8.0,1.6 Hz, 1H), 7.70-7.69 (m, 1H), 7.68-7.67 (m, 1H), 7.66 (d, *J* = 8.4 Hz, 1H), 7.55 (tt, *J* = 7.2, 1.2 Hz, 1H), 7.50-7.42 (m, 3H); ^{13}C NMR (75 MHz, CD_3COCD_3) δ174.8, 155.8, 153.5, 133.5, 132.0, 128.6, 127.7, 127.5, 125.4, 124.9, 124.4, 124.2, 117.8; IR (KBr) 1639, 1615, 1463 cm^{-1}; HRMS (ESI) *m/z*: calc for $C_{15}H_{11}O_2$ $[M + H]^+$: 223.0759. Found 223.0760.

3-(Pyridin-4-yl)-4H-chromen-4-one (5)

This compound was prepared from the Suzuki coupling of 3-iodochromone (11) and pyridin-3-ylboronic acid (14) in 40% yield as a coral pink solid wax: m.p. 157–158 °C; ^{1}H NMR (300 MHz, CD_3OD) δ8.77 (s, 1H), 8.55 (d, *J* = 4.2 Hz, 1H), 8.47 (s, 1H), 8.23 (d, *J* = 8.1 Hz, 1H), 8.10 (d, *J* = 8.1 Hz, 1 H), 7.82 (t, *J* = 7.5 Hz, 1H), 7.64 (d, *J* = 8.7 Hz, 1H), 7.52 (t, *J* = 6.6 Hz, 2H). ^{13}C NMR (75 MHz, CD_3OD) δ177.6, 157.9, 156.6, 149.9, 149.4, 138.9, 135.7, 130.2, 127.0, 126.8, 125.3, 125.0, 122.9, 119.6; IR (KBr) 3038, 3037, 1634, 1588, 1566, 1470 cm^{-1}; HRMS (ESI): *m/z* calc for $C_{14}H_{10}NO_2$ $[M + H]^+$: 224.0712. Found 224.0714.

General Procedure for the Synthesis of Pyrazole Analogues of Genistein (6–10)

3-Iodochromone (11, 90 mg, 0.3 mmol) and the appropriately substituted 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-pyrazole (15a–e, 0.5 mmol) were dissolved in THF (30 mL), and then aqueous Na_2CO_3 (3.0 mL of 2 M) followed by tetrakis(triphenylphosphine)palladium (12 mg, 0.01 mmol) were added. The reaction was allowed to proceed at reflux overnight, cooled to 25 °C, water was added (50 mL), and the mixture was extracted with EtOAc (100 mL × 3). The combined EtOAc extracts were washed with water (30 mL × 3), the organic fraction was dried over anhydrous magnesium sulfate, and the solvent was removed in vacuo to afford the crude product. Purification of the product by preparative TLC using 30% ethyl acetate in hexanes as eluent furnished the respective title compound (6–10).

3-(1-Isopropyl-1H-pyrazol-4-yl)-4H-chromen-4-one (6)

This compound was prepared from the Suzuki coupling of 3-iodochromone (11) and 1-isopropyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-pyrazole (15a) in 42% yield as a colorless yellow solid: mp. 104–105 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.32 (s, 1H), 8.30 (dd, *J* = 8.2, 1.0 Hz, 1H), 8.25 (s, 1H), 7.79 (s, 1H), 7.67 (dt, *J* = 7.5, 1.8 Hz, 1H), 7.48 (d, *J* = 8.4 Hz, 1H), 7.43 (t, *J* = 7.2 Hz, 1H), 4.57 (hept, *J* = 6.7 Hz, 1H), 1.56 (d, *J* = 6.6 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.8, 155.9, 150.7, 134.9, 133.5, 127.1, 126.1, 125.2, 124.0, 118.1, 117.5, 111.7, 54.2, 22.9; IR (KBr) 3069, 2929, 2978, 1634, 1609, 1540, 1510, 1489, 1469 cm^{-1}; HRMS (ESI) *m/z*: calcd for $C_{15}H_{15}N_2O_2$ $[M + H]^+$: 255.1134. Found 255.1135.

3-(1-(sec-Butyl)-1H-pyrazol-4-yl)-4H-chromen-4-one (7)

This compound was prepared from the Suzuki coupling of 3-iodochromone (11) and 1-*sec*-butyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-pyrazole (15b) in 39% yield as a colorless yellow wax; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.31 (s, 1H), 8.31-8.26 (overlapped, 1H), 8.26 (s, 1H), 7.79 (s, 1H), 7.67 (t, *J* = 7.5 Hz, 1H), 7.48 (d, *J* = 8.4 Hz, 1H), 7.42 (t, *J* = 7.5 Hz, 1H), 4.33-4.22 (m, 1H), 1.98-1.79 (m, 2H), 1.53 (d, *J* = 6.9 Hz, 3H), 0.85 (t, *J* = 7.5 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.6, 153.6, 148.3, 132.8, 131.1, 125.6, 123.8, 122.9, 121.7, 115.8, 115.3, 109.2, 57.9, 27.9, 18.6, 8.4; IR (KBr) 2973, 1644, 1609, 1541 1511, 1490,1465 cm^{-1}; HRMS (ESI) *m/z*: calculated for $C_{16}H_{17}N_2O_2$ $[M + H]^+$: 269.1290. Found 269.1294.

3-(1-Isobutyl-1H-pyrazol-4-yl)-4H-chromen-4-one (8)

This compound was prepared from the Suzuki coupling of 3-iodochromone (11) and 1-isobuyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-pyrazole (15c) in 42% yield as a white solid: m.p. 96–97°C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.29 (d, *J* = 9.3 Hz, 1H), 8.26 (s, 1H), 8.25 (s, 1H), 7.77 (s, 1H), 7.66 (dt, *J* = 7.5 Hz, 1H), 7.47 (d, *J* = 8.4 Hz, 1H), 7.41 (t, *J* = 7.5 Hz, 1H), 3.96 (d, *J* = 7.2 Hz, 2H), 2.31-2.18 (m, 1H), 0.93 (d, *J* = 6.6 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.5, 155.7, 150.4, 135.3, 133.2, 129.7, 125.9, 124.9, 123.8, 117.8, 117.3, 111.5, 59.6, 29.4, 19.7; IR (KBr) 3053, 2961, 2928, 2871, 1640, 1611, 1542, 1465 cm^{-1}; HRMS (ESI) *m/z*: calcd for $C_{16}H_{17}N_2O_2$ $[M + H]^+$: 269.1290. Found 269.1296.

3-(1-(Pentan-2-yl)-1H-pyrazol-4-yl)-4H-chromen-4-one (9)

This compound was prepared from the Suzuki coupling of 3-iodochromone (11) and 1-(pentan-2-yl)-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-pyrazole (15d) in 29% yield as a light yellow wax; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.31 (s, 1H), 8.30-8.26 (overlapped, 1H), 8.26 (s, 1H), 7.78 (s, 1H), 7.67 (t, *J* = 7.5 Hz, 1H), 7.48 (d, *J* = 8.4 Hz, 1H), 7.42 (t, *J* = 7.5 Hz, 1H), 4.43-4.32 (m, 1H), 1.99-1.88 (m, 1H), 1.79-1.67 (m, 1H), 1.53 (d, *J* = 6.6 Hz, 1H), 1.30-1.18 (m, 2H), 0.90 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.9, 154.0, 148.7, 133.2, 131.5, 125.8, 124.2, 123.2, 122.1, 116.2, 115.8, 109.6, 56.5, 37.3, 19.4, 17.5, 11.8; IR (KBr) 3068, 2958, 2930, 2872, 1636, 1609, 1578, 1541, 1463 cm^{-1}; HRMS (ESI) *m/z*: calcd for $C_{17}H_{19}N_2O_2$ $[M + H]^+$: 283.1447. Found 283.1448.

3-(1-(Pentan-3-yl)-1H-pyrazol-4-yl)-4H-chromen-4-one (10)

This compound was prepared from the Suzuki coupling of 3-iodochromone (11) and 1-(pentan-3-yl)-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-pyrazole (15e) in 37% yield as a yellow wax; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.30 (s, 1H), 8.30-8.26 (overlapped, 1H), 8.26 (s, 1H), 7.80 (s, 1H), 7.66 (t, *J* = 7.6 Hz, 1H), 7.47 (d, *J* = 8.4 Hz, 1H), 7.41 (t, *J* = 7.6 Hz, 1H), 3.99-3.94 (m, 1H), 1.95-1.82 (m, 4H), 0.81 (t, *J* = 7.2 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.0, 156.1, 150.8, 135.3, 133.6, 129.1, 126.3, 125.3, 124.2, 118.3, 117.8, 111.5, 67.0, 28.6, 10.9; IR (KBr) 2966, 2931, 2876, 1641, 1610, 1541, 1465 cm^{-1}; HRMS (ESI) *m/z*: calculated for $C_{17}H_{19}N_2O_2$ $[M + H]^+$: 283.1447. Found 283.1452.

Cell Culture

All cell lines were initially purchased from American Type Culture Collection (ATCC™). The PC-3 prostate cancer cells, the LNCaP prostate cancer cells, and the HeLa cervical cancer cells were routinely cultured in RPMI-1640 medium supplemented with 10% FBS and 10% penicillin/streptomycin. Cultures were maintained in 5% carbon dioxide at a temperature of 37°C. The DU-145 prostate cancer cells were routinely cultured in Eagle's Minimum Essential Medium (EMEM) medium supplemented with 10% FBS and 1% penicillin/streptomycin.

Effect of Genistein and Analogues on Cell Viability

PC-3 or DU145 or LNCaP or HeLa cells were plated in 24-well plates at a density of 20,000 each well in the appropriate 10% FBS medium. The cells were then treated with genistein, or synthesized genistein analogues separately at 50 µM and 100 µM (for PC-3, DU-14, and LNCaP cells) or at 1 µM and 10 µM (for HeLa cells) for 3 days, while equal treatment volumes of DMSO were used as vehicle control. Cell numbers were counted with a cell viability analyzer (Beckman Coulter). The ratio of drug treated viable cell numbers to vehicle treated viable cell numbers was defined as percentage viability and variation between replicate experiments is not greater than 5%.

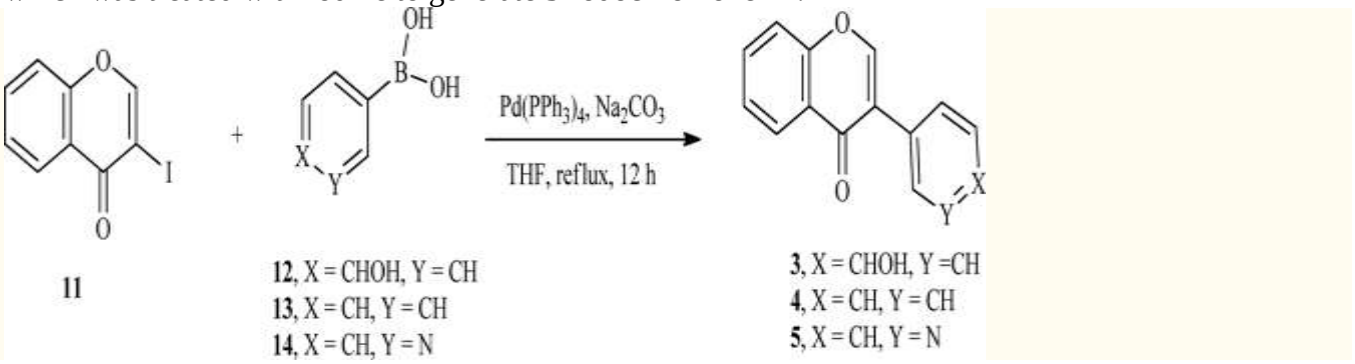
WST-1 Cell Proliferation Assay

PC-3, DU145, LNCaP, or HeLa cells were plated in 96-well plates at a density of 3,200 per well in 200 µL of culture medium. The cells were then treated with genistein or its analogues separately at different doses for 3 days, while equal treatment volumes of DMSO were used as vehicle control. The cells were cultured in a CO_2 incubator at 37 °C for three days. 10 µL of the premixed WST-1 cell proliferation reagent (Clontech) was added to each well. After mixing gently for one minute on an orbital shaker to ensure homogeneous distribution of color, the cells were incubated for additional 3 hours at 37 °C. The absorbance of each well was measured using a microplate-reader (Synergy HT, BioTek) at a wavelength of 430 nm. The IC_{50} value is the concentration of each compound that inhibits cell proliferation by 50% under the experimental conditions and is the average from at least triplicate determinations that were reproducible and statistically significant. For calculating the IC_{50} values, a linear proliferative inhibition was made based on at least five dosages for each compound.

RESULTS AND CONCLUSION

Overview of the Synthesis of Genistein Analogues (3–10)

The genistein analogues (3–10) were synthesized by Suzuki-Miyaura coupling reactions of 3-iodochromone (11) with the appropriate boronic acid (12–14) (Scheme 1) or aryl boronic ester (15a–e) (Scheme 2). The boronic acids (12–14) employed to make genistein analogues 3–5 by Suzuki-Miyaura coupling reaction as described in Scheme 1 are commercially available. The 3-iodochromone (11) was readily synthesized in an excellent yield from commercially available 2-hydroxy-4,5-dimethoxyacetophenone (16) according to the procedure illustrated in the literature (Scheme 3). Specifically, condensation of 16 with *N*,*N*-dimethylformamide dimethyl acetal gave acrylophenone 17, which was treated with iodine to generate 3-iodochromone 11.



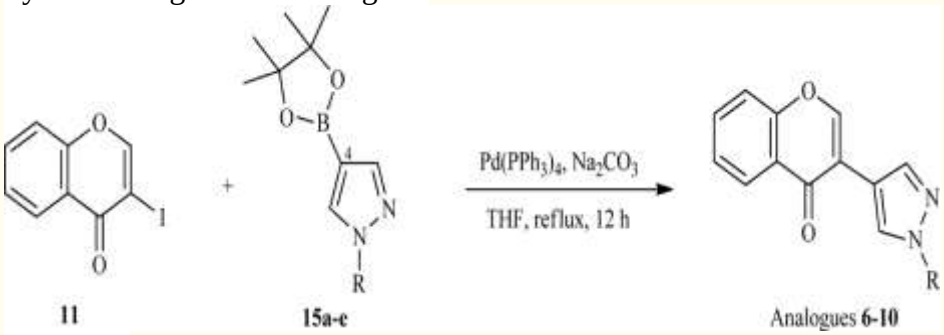
11

12, X = CHOH, Y = CH
13, X = CH, Y = CH
14, X = CH, Y = N

3, X = CHOH, Y =CH
4, X = CH, Y = CH
5, X = CH, Y = N

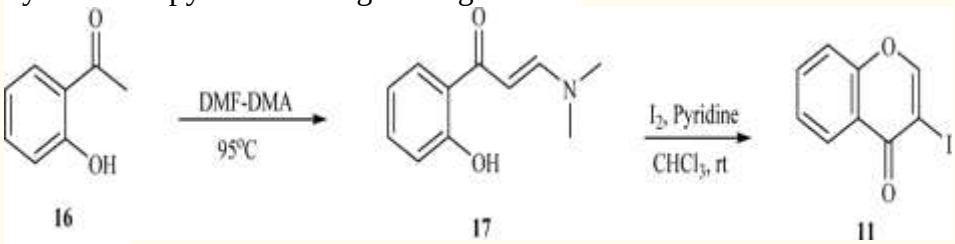
Scheme 1

Synthesis of genistein analogues.



Scheme 2

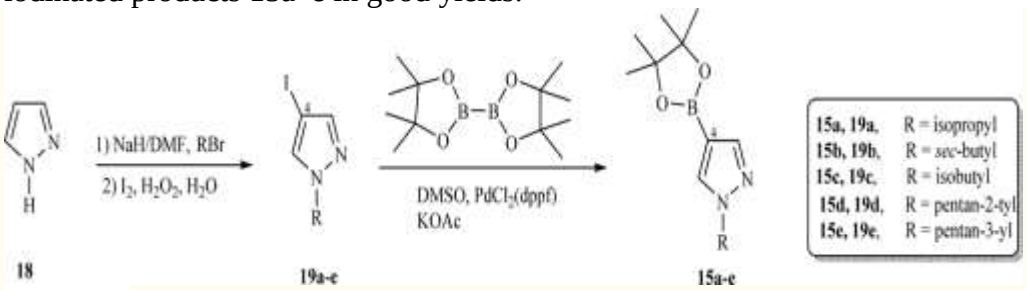
Synthesis of pyrazole analogues of genistein.



Scheme 3

Synthesis of 3-iodochromone (11).

Synthesis of the pyrazole analogues of genistein (6–10) commenced with preparation of appropriate aryl boronic esters (15a–e) from the corresponding aryl iodides (19a–e) and bis(pinacolato)diboron through the palladium-catalyzed Miyaura borylation (Scheme 4). The corresponding aryl iodides (19a–e) were prepared by *N*-alkylation of pyrazole (18) followed by iodination. The *N*-alkylation was achieved by treating pyrazole in DMF with alkyl bromide using sodium hydride as base. The crude products achieved from this step through aqueous work-up are pure enough for the next step reaction. Green iodination of 1-alkylpyrazoles with iodine/hydrogen peroxide in water] provided the corresponding iodinated products 15a–e in good yields.

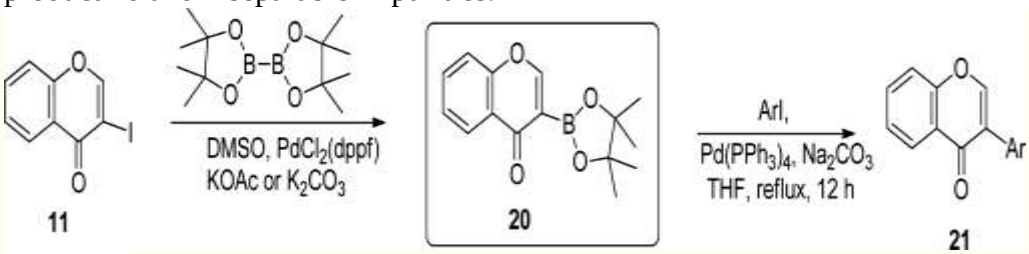


Scheme 4

Synthesis of 1-alkyl-1*H*-pyrazole-4-boronic acid.

In search of a more efficient approach to the synthetic targets with general scaffold 21, we attempted to pursue the synthesis of genistein analogues (Scheme 5) *via* Suzuki-Miyaura coupling reaction of 3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-4*H* chromen-4-one (20) with the appropriate aryl iodide. The advantages of this method are: 1) compound 20 is a more advanced synthetic intermediate, facilitating a more efficient synthetic strategy for our targets, especially for a large number of isoflavones; and 2) most aryl halides are commercially available. Our first attempt of Miyaura borylation of 3-iodochromone using potassium acetate as base failed. The ^{1}H NMR spectrum of the crude product did not exhibit the characteristic signals for desired product 20. We then replaced

potassium acetate with potassium carbonate, a stronger base, to prepare the arylboronic esters from 3-iodochromone according to the procedure described in the literature. The purified product from this reaction was analyzed by the ^{1}H NMR data, implying the existence of the expected borylation product 20 and inseparable impurities.



Scheme 5

Alternative synthetic strategy for genistein analogues.

Cytotoxicity Towards Prostate and Cervical Cancer Cell Lines

The ability of the test compounds to inhibit cell growth was listed in Table 1. The general structure-activity relationship of the genistein analogues can be summarized as below: i) comparison of the cytotoxicity of genistein and that of daidzein indicates the importance of 5-OH for the cytotoxicity in prostate cancer cells, but not in the HeLa cells; ii) removal of both 5- and 7-OH in genistein to give compound 3 was clearly detrimental to the cytotoxicity in both prostate and cervical cancer cell lines. It should be noted that compound 3 has a biphasic action in androgen-sensitive LNCaP prostate cancer cells because the inhibitory activity is better at lower concentration (50 μM). The biphasic action of genistein has been reported in the literature; and iii) Substitution of the 4'-hydroxyphenyl moiety (ring B) in compound 3 with 1-alkyl-1*H* pyrazol-4-yl (compounds 6–10) generally enhances the cytotoxicity against three prostate cancer cell lines at 100 μM concentration, but cannot cause apparent change against the HeLa cell line. This entails that 1-alkyl-1*H*-pyrazol-4-yl can act as a good bioisostere for the 4'-hydroxyphenyl moiety in genistein.

Table 1

Cytotoxicity of genistein analogues towards prostate cancer cells and cervical cancer cells.

Comps	Inhibitory Rate (%)							
	PC-3[a]		DU145[b]		LNCaP[c]		HeLa[d]	
	100 μM	50 μM	100 μM	50 μM	100 μM	50 μM	10 μM	1 μM
Genistein	69.9	61.4	76.5	72.7	72.4	61.9	54.0	9.9
Daidzein	37.6	22.1	32.1	24.6	56.7	48.5	60.5	18.3
3	10.9	9.8	33.7	24.8	11.3	41.2	12.3	6.7
4	37.4	15.6	51.5	24.3	44.3	22.7	13.7	13.1
5	10.5	0	24.6	18.7	52.2	48.5	26.8	9.8
6	46.0	10.5	41.7	5.8	58.2	22.4	14.2	5.4
7	52.9	14.6	27.4	12.5	51.4	29.0	17.0	6.9
8	46.6	9.8	54.8	15.9	77.6	34.6	8.5	7.4
9	67.1	40.0	43.3	16.4	47.7	25.2	11.1	3.4
10	74.7	9.2	44.8	13.0	61.7	36.4	9.8	7.3

Antiproliferative Activity towards Prostate and Cervical Cancer Cell Lines

To further evaluate the anticancer potential of the genistein analogues in cell-based models, we also assessed the cell proliferation after treatment with genistein and its analogues using WST-1 assay according to the manufacturer's instruction. The assay is based on the cleavage of the water-soluble tetrazolium salt WST-1 to formazan catalyzed by the cellular mitochondrial dehydrogenases. The amount of formazan dye yielded directly correlates to the number of live cells in the culture. The detailed procedure for WST-1 cell proliferation assay was described in the section of "MATERIALS AND METHODS." Genistein was used as a positive control for comparison in the parallel experiments and the results were summarized in Table 2. Genistein has potential to attenuate proliferation in three prostate and one cervical cancer cell lines. Compared to androgen-independent PC-3 and DU145 cells, the androgen-dependent LNCaP cells were more sensitive to genistein and its analogues. These findings are in agreement with those reported in the literature. The rank order for antiproliferative activity of genistein and its analogues in different cell lines, as judged by IC_{50} values, was LNCaP and HeLa cells > PC-3 and DU145 cells. Of the genistein analogues that we tested, genistein possessing hydroxyl groups at C-5 and C-7 showed lower IC_{50} values (higher potency) than compound 3, suggesting that these two hydroxyl groups or their bioisosteres on ring A of the isoflavone scaffold are important for cell growth inhibition. This notion is supported by the findings that compound 3, lacking the 5,7-OH substitution pattern, is far less potent than genistein. Replacement of the 4'-hydroxyphenyl moiety (ring B) in compound 3 or the phenyl moiety in compound 4 with a nitrogen-containing heteroaromatic ring (compounds 5–10) significantly enhances the anti-proliferative activity against three prostate cancer and one cervical cancer cell lines. This implies that pyridine-3-yl and 1-alkyl-1*H*-pyrazol-4-yl can serve as good bioisosteres for the 4'-hydroxyphenyl moiety in genistein.

Table 2

Anti-proliferative activity of genistein analogues.

Compd	IC_{50} (μM)[a]			
	PC-3[b]	DU145[c]	LNCaP[d]	HeLa[e]
Genistein	68.6 ± 3.18	96.2.5 ± 3.2	37.4 ± 2.6	10.0 ± 1.5
Daidzein	202.2 ± 21.4	134.5 ± 11.6	63.1 ± 6.1	5.4 ± 0.49
3	> 1000	> 1000	> 1000	> 1000
4	> 1000	> 1000	> 1000	> 1000
5	327.7 ± 34.6	305.2 ± 43.7	218.0 ± 45.9	88.2 ± 15.2
6	277.2 ± 45.0	267.2 ± 15.9	107.9 ± 30.6	51.3 ± 5.0
7	108.3 ± 30.0	137.5 ± 13.3	71.9 ± 2.9	75.9 ± 9.0
8	208.1 ± 45.2	221.3 ± 35.9	116.1 ± 23.7	130.3 ± 24.1
9	132.8 ± 20.4	121.0 ± 12.3	49.3 ± 9.0	73.9 ± 11.0
10	248.1 ± 7.0	118.0 ± 22.4	29.1 ± 5.9	106.8 ± 10.8

In conclusion, we have successfully synthesized eight genistein analogues, including five new pyrazole analogues and one new pyridine analogue, using the Suzuki-Miyaura coupling reaction as the critical transformation. The cytotoxicity and anti-proliferative effect of the synthesized genistein analogues were evaluated towards three prostate cancer cell lines (both androgen-sensitive and androgen-refractory) and one aggressive cervical cancer cell line using trypan blue exclusion method and WST-1 cell proliferation assay, respectively. Both cytotoxicity and antiproliferation of genistein analogues suggest that i) the hydroxyl group located at C-5 and C-7 are very important for the activities; and ii) 1-alkyl-1*H*-pyrazol-

4-yl and pyridine-3-yl can act as suitable bioisosteres for the 4'-hydroxyphenyl moiety in genistein. These results pave the way for further investigation of genistein analogues as potential chemotherapeutics.

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