Synthesis and Structure–Activity Relationships of Tetrahydro- β -carboline Derivatives as Anticancer and Cancer-chemopreventive Agents

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Abstract. *Background/Aim:* There is an unmet clinical need to develop new anticancer and chemopreventive agents. The aim of the present study was to identify β -carboline derivatives with cancer chemopreventive and therapeutic potential. *Materials and Methods:* Forty-eight tetrahydro- β -carboline derivatives were synthesized and evaluated for their anticancer and chemopreventive activities, through induction of quinone reductase 1 (QR1), aromatase inhibition, as well as inhibition of nitric oxide (NO) production. *Results:* 2-((1-Bromonaphthalen-2-yl)methyl)-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole demonstrated the most potent activity in the QR1 induction assay with an induction ratio value of 3.2 (CD=1.3 μ M). The R-isomer of the amide derivative 2-((1-bromonaphthalen-2-yl)methyl)-2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indol-3-yl)(4-methylpiperazin-1-yl)methanone was the most potent inhibitor of NO production with a 50% inhibitory concentration, IC₅₀=6.54 μ M and had a low cytotoxic effect (IC₅₀=17.98 μ M) on RAW 264.7 cells. Subsequent computational docking study revealed that this compound binds to the active site of inducible nitric oxide synthase with favorable interactions. *Conclusion:* our results provided promising β -carboline leads for further optimization and development with therapeutic potential as new chemopreventive and chemotherapy agents.

Cancer is responsible for millions of deaths worldwide and continues to remain one of the leading causes of death each year. According to the World Health Organization, there were an estimated 14.1 million new cancer cases and 8.2 million cancer deaths in 2012, compared to 12.7 million and 7.6 million, respectively, in 2008 (1). It is projected that the number of new cancer cases and cancer deaths worldwide will rise to 21 and 13 million, respectively, by 2030. In the United States, there were an estimated 1.7 million new cancer cases and over half million cancer deaths in 2016 (2). Because of serious side-effects, drug resistance, and relapse associated with cancer chemotherapy, there is a significant unmet medical need for developing new anticancer agents with improved efficacy and low cytotoxicity against healthy normal cells in order to minimize undesirable systemic toxicity.

On the other hand, to circumvent this toxicity issue, cancer chemoprevention represents an alternative and attractive approach to prevent the initiation, promotion, or progression of cancer using chemopreventive agents (3, 4). Mechanistically, the protective effects of chemopreventive agents result from a combination of diverse proposed mechanisms involving antioxidant, anti-inflammatory, immunomodulatory action, apoptosis induction, and chemical intervention against carcinogens, *etc.* (5). In recent years, accumulating evidence supports the notion that inducible nitric oxide synthase (iNOS) is associated with the onset and development of various human diseases, including cancer (6, 7); furthermore, the resulting signal molecule NO can also play a role in drug resistance and cancer metastasis (8, 9). In general, inhibition or down-regulation of carcinogen-metabolizing enzymes, which are capable of modifying potential carcinogens and generating carcinogenic substances, such as aromatase and iNOS, can impede carcinogenesis (10, 11). Specifically, aromatase acts in the late-stage conversion from reproductive hormone androgens to estrogens in peripheral tissues, and high estrogen levels

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can lead to the growth and development of breast tumors. As such, aromatase inhibitors have been clinically used in breast cancer therapy in postmenopausal women (12, 13). Recent clinical studies also supported the use of aromatase inhibitors for breast cancer chemoprevention therapy in high-risk postmenopausal women (14, 15). Furthermore, cancer chemoprevention can also be achieved by induction of anticarcinogenic enzymes, such as quinone reductase 1 (QR1) (16, 17). QR1 plays an important role in cellular defense in preventing the formation of oxidative stress by catalyzing the conversion of quinones to less reactive and less toxic hydroquinones (18).

β -Carboline alkaloids are an important class of anticancer natural products and medicinal molecules (19). Among many naturally occurring β -carboline molecules, callophycin A (**1** in Figure 1), isolated from the red algae *Callophycus oppositifolius*, was found to have antiproliferative effects on various human cancer cell lines at low micromolar concentration (20). Previously, we synthesized and evaluated a series of callophycin A derivatives to explore chemical diversity at the 2 and 3 positions (11). Among them, the callophycin analog **2** with *R*-stereochemistry was discovered to inhibit nitrite production (50% inhibitory concentration, $IC_{50}=2.8 \mu M$) in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Subsequent mechanistic study revealed that **2** inhibits LPS-induced nitrite production by a unique and complex mechanism (21). In this work, an expanded panel of new derivatives around the tetrahydro- β -carboline core scaffold was synthesized and evaluated for anticancer and cancer chemopreventive potential.

Materials and Methods

Reagents and materials. Solvents and reagents were purchased from Sigma-Aldrich, St. Louis, MO, USA or Fisher Scientific, Waltham, MA, USA and were used without further purification. Reactions were monitored either by thin-layer chromatography or by employing a Shimadzu LC-20A series high-performance liquid chromatography (HPLC) system. Compounds were purified by flash column chromatography on silica gel using a Biotage Isolera One system (Biotage AB, Uppsala, Sweden). 1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance III HD-400 spectrometer (Bruker Corp, Billerica, MA, USA) (400 and 100 MHz, respectively). Mass spectroscopy (MS) data were obtained using an Agilent 6120 single quad MS (Agilent Technologies, Santa Clara, CA, USA) *via* direct injection. The purity of compounds was determined by analytical HPLC (Shimadzu LC-20A series) (Shimadzu Scientific Instruments, Inc. Columbia, MD, USA) using a Gemini, 3 μm , C18, 110 Å column (50 mm $\times$ 4.6 mm, Phenomenex, Torrance, CA, USA) and flow rate of 1 ml/min. Gradient conditions: solvent A (0.1% trifluoroacetic acid in water) and solvent B (acetonitrile): 0-2.0 min 100% A, 2.0-7.0 min 0-100% B (linear gradient), 7.0-8.0 min 100% B, UV detection at 254 nm and 220 nm.

Design rationale. From our previous study, the *S*-isomer of callophycin A showed aromatase-inhibitory activity with an IC_{50} value of 10.5 μM (11). To further expand existing structure-activity

relationship (SAR), an extended series of compounds with chemically diversified alkyl substituents at the N^2 position was designed and synthesized (Figure 2). In order to evaluate the importance of the free carboxylic acid or its ester functionality at the 3 position of the tetrahydro- β -carboline scaffold, a set of β -carboline derivatives with carboxylic acid and its ester moieties removed were synthesized (Figure 3). In our previous work, carboline urea and carbamate derivatives at the 2 position exhibited low micromolar activity for the induction of QR1 and nitrite inhibition (11). On the basis of existing SAR, a series of new 2-substituted urea derivatives was designed and synthesized to further expand the SAR. In addition, to explore potential new scaffolds, four tetracyclic intramolecular cyclized hydantoin derivatives were also synthesized for biological evaluation (Figure 4). 3-Substituted amide derivatives and the N^9 -methylated analog were also designed and synthesized (Figure 5).

Synthesis of callophycin A analogs **5-6** is shown in Figure 2. Esterification of chiral carboxylic acid isomer **3a** (*S*) or **3b** (*R*) with corresponding alcohol in the presence of $SOCl_2$ yielded **4a-d**, which were then subjected to reaction with various alkyl bromides in acetonitrile at reflux in the presence of *N,N*-diisopropylethylamine (DIPEA), affording the *N*-alkylated derivatives **5a-l** in 62-82% yields. Finally, **5a-l** were hydrolyzed to give free carboxylic acids **6a-j** in the presence of NaOH in MeOH/H₂O at room temperature in 81-93% yields.

Representative procedure for the preparation of compounds 4a-d. Thionyl chloride (2.1 ml, 24.1 mmol) was added dropwise to a solution of **3a** (1 g, 4.6 mmol) in methanol (50 ml) at 0°C. The reaction was stirred at room temperature and the completion of the reaction was monitored by HPLC. Excess methanol and thionyl chloride were removed by evaporation. The residue was dissolved in dichloromethane and washed successively with saturated Na₂CO₃ and NaCl solutions. The dried dichloromethane layer was evaporated and purified by flash column chromatography on silica gel to provide **4a**.

General procedure for the preparation of compounds 5a-l. Substituted bromide (0.12 mmol) and DIPEA (0.15 mmol) were added to the solution of **4** (0.1 mmol) in acetonitrile (10 ml). The reaction was heated under reflux for 6 h. After the completion of the reaction (monitored by HPLC), solvent was removed by evaporation. The residue was dissolved in cold EtOAc (10 ml) and the mixture was then filtered to remove the precipitate. The filtrate was concentrated *in vacuo* to yield a light-yellow crude residue, which was further purified by flash column chromatography on silica gel to give products **5a-l**.

General procedure for the preparation of compounds 6a-j. A solution of an appropriate ester **5a-l** (0.1 mmol) and NaOH (0.5 mmol) in MeOH/H₂O (10:1, 5 ml) was stirred at room temperature until the completion of the reaction (monitored by HPLC). Most MeOH was removed *in vacuo* and the residue was carefully neutralized with glacial acetic acid, whereupon a yellow precipitate was formed, the product was collected by filtration, washed with cold water, and dried.

General procedure for the preparation of compounds 8a-h. As shown in Figure 3, the commercially available secondary amine **7a** or **7b** was reacted with an appropriate alkyl bromide/chloride in

Figure 1. Callophycin A (**1**) and its synthetic analog **2**. Figure 2. Synthesis of callophycin A analogs **5-6**. Reagents and conditions: a: SOCl_2 , $R^1\text{OH}$, 0°C to rt; b: $R^2\text{Br}$, DIPEA, CH_3CN , reflux; c: NaOH , $\text{MeOH}/\text{H}_2\text{O}$ (10:1). Figure 3. Synthesis of **8a-h**. Reagents and conditions: a: $R^2\text{-Br}$ or $R^2\text{-Cl}$, *N,N*-diisopropylethylamine (DIPEA), CH_3CN , reflux.

acetonitrile under reflux in the presence of DIPEA, affording **8a-h** in 77-94% yields. Substituted bromide or chloride (0.12 mmol) and DIPEA (0.15 mmol) were added to the solution of **7a** or **7b** (0.1 mmol) in acetonitrile (10 ml). This reaction was heated under reflux for 2 h. After the completion of the reaction (monitored by HPLC), the solvent was removed by evaporation. The residue was dissolved in cold EtOAc (10 ml) and the mixture was then filtered

to remove the precipitate. The filtrate was concentrated *in vacuo* to yield a light-yellow crude residue, which was further purified by flash column chromatography on silica gel to give products **8a-h**.

General procedure for the preparation of compounds 9a-f and 10a-c. The urea derivatives **9a-f** and **10a-c** were synthesized from **4b-d** or **7a** and corresponding isocyanates in 66-84% yields in

Figure 4. Synthesis of β -carboline derivatives **9-11**. Reagents and conditions: a: $R^2\text{-NCO}$, CH_2Cl_2 , rt; b: NaOH , $\text{THF}:\text{H}_2\text{O}$ (1:1), rt.

Figure 5. Synthesis of β -carboline amide derivatives **12a-g** and N^9 -methylated **13**. Reagents and conditions: a: $R^1R^2\text{NH}$, EDC , HOBT , CH_2Cl_2 , 0°C to rt., overnight; b: MeI , NaH , DMF , 0°C to rt., 12 h.

dichloromethane at room temperature (Figure 4). Subsequently, under the basic reaction condition, **9a-d** were transformed into their corresponding intramolecular cyclized hydantoin products **11a-d** in 58-68% yields.

The reaction of **4b-d** or **7a** (0.5 mmol) and an appropriate isocyanate in dichloromethane (5 ml) was stirred at room temperature overnight. After the completion of the reaction (monitored by HPLC), the reaction mixture was concentrated. The

residue was purified by flash column chromatography on silica gel to give products **9a-f** and **10a-c**.

General procedure for the preparation of compounds 11a-d. A solution of an appropriate methyl ester **9a-d** (0.1 mmol) and NaOH (1 mmol) in THF/H₂O (1:1, 5 ml) was stirred at room temperature until the completion of the reaction (monitored by HPLC). THF was removed *in vacuo* and the residue was dissolved in EtOAc (10 ml) and washed with H₂O. The organic phase was collected, dried, and concentrated. The residue was purified by flash column chromatography on silica gel to give products **11a-d**.

Finally, given the promising activity of **6e** and **6j** in our preliminary screen, the free carboxylic acid group at the 3 position of **6e** and **6j** was converted to the amide functionality in **12a-g** by reacting with different amines using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole monohydrate (HOBt) in dichloromethane (Figure 5). To investigate the importance of the N⁹ hydrogen, the N⁹ methylated derivative **13** was also synthesized from **5j**.

General procedure for the preparation of compounds 12a-g. At 0°C, to a solution of an appropriate amine (0.345 mmol) in dry CH₂Cl₂ (5 ml), HOBt (0.69 mmol) and EDC (0.69 mmol) were added and stirred for 30 min. Then compound **6e** or **6j** (0.23 mmol) was added and the reaction was allowed to return to room temperature and stirred overnight. The reaction mixture was washed by water and extracted with CH₂Cl₂. The organic layer was washed with saturated aqueous NaHCO₃ solution, brine, dried over anhydrous Na₂SO₄, and evaporated under reduced pressure. The residue was purified by flash column chromatography on silica gel to give products **12a-g** in 59-73% yields.

Procedure for the preparation of compound 13. To a stirred solution of **5j** (100 mg, 0.22 mmol) in dry dimethylformamide (DMF) (25 ml), NaH (11 mg, 60% suspension in mineral oil, 0.26 mmol) was added in portions under N₂ atmosphere at 0°C. The reaction mixture was then warmed to room temperature and stirred for 30 min. After cooling to 0°C again, MeI (17 μ l, 0.27 mmol) was added dropwise. The reaction mixture was stirred at room temperature overnight, quenched with water and the aqueous layer was extracted with ether. The combined organic layer was washed with brine, dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The residue was purified by flash column chromatography to give compound **13** in 79% yield.

Determination of QR1 activity in cell culture. QR1 activity was determined using Hepa 1c1c7 murine hepatoma cells as previously described (22). Briefly, cells were incubated in a 96-well plate with test compounds at a maximum concentration of 50 μ M for 48 h prior to permeabilization with digitonin. Enzyme activity was then determined as a function of the NADPH-dependent menadiol-mediated reduction of 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue formazan. Production was measured by absorption at 595 nm. Total protein, quantified using crystal violet staining, was assessed in parallel. The induction ratio (IR) of QR activity represents the specific enzyme activity of agent-treated cells compared with a dimethyl sulfoxide (DMSO)-treated control. The concentration required for double activity (CD) was determined through a dose-response assay for active substances (IR >2). Data presented are the result of three independent experiments run in duplicate. 4'-Bromoflavone (CD=0.01 μ M) was used as a positive control.

Figure 6. Interaction of **12f** in the active site of inducible nitric oxide synthase (PDB ID: 2Y37).

Aromatase assay. Aromatase activity was assessed by measuring the fluorescent intensity from the final product of a substrate, dibenzylfluorescein (DBF). The test substance was preincubated with a NADPH-regenerating system followed by further incubation with aromatase enzyme and DBF mixture for 30 min. Fluorescence was measured at 485 nm (excitation) and 530 nm (emission). Compounds with inhibition value of 50% or higher at the concentration of 50 μ M were further assessed to determine IC₅₀ values using TableCurve software 2D (version 4) (Systat Software Inc., Chicago, IL, USA) (22).

Measurement of the production of NO in LPS-stimulated RAW 264.7 murine macrophage cells (nitrite assay). The level of NO in the cultured media was estimated by measuring the level of nitrite due to the instability of NO and its subsequent conversion to nitrite. The nitrite assay was performed as previously described (23). Briefly, RAW 264.7 cells (ATCC, Manassas, VA, USA) were incubated in 96-well culture plates at 37°C, with 5% CO₂ in a humidified air incubator for 24 h. Then cells were treated with serially diluted compounds for 15 min, followed by treatment with or without LPS (1 μ g/ml) for an additional 20 h. After incubation, the nitrite released in the culture media was measured using Griess reagent [1:1 mixture (v/v) of 1% sulfanilamide in 5% H₃PO₄ and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride solution], and absorbance was measured at 540 nm. The concentration of nitrite was calculated using a standard curve created with known concentrations of sodium nitrite. To evaluate the cytotoxic effects of compounds on RAW 264.7 cells under the same experimental condition, sulforhodamine B (SRB) assays were performed (24).

SRB assay. After incubation of RAW 264.7 cells with test compounds, cells were fixed with 10% trichloroacetic acid solution for 30 min and stained with 0.4% SRB in 1% acetic acid solution for 30 min. Protein-bound SRB was dissolved in 10 mM Tris buffer (pH 10.0) and the absorbance was measured at 515 nm. The effect of compounds on cell survival was demonstrated as percentage survival in comparison with vehicle (DMSO)-treated control cells.

Molecular docking. Molecular docking was performed using a protein-ligand docking program GLIDE (XP mode) (Schrödinger, LLC, Portland, OR, USA) (25, 26). In brief, this was achieved by simulation of docking of compounds into the active binding site of iNOS protein for which the 3D structure was obtained from Protein Data Bank (<https://www.rcsb.org/>, PDB ID: 2Y37). Compounds were built with ChemBio3D (CambridgeSoft Corp, Waltham, MA, USA) and optimized at molecular mechanical level using Schrödinger software (Schrödinger, LLC, Portland, OR, USA). They were then docked into the binding site of iNOS protein and the binding position with the most favorable score was examined.

Results

Chemistry. All the compounds were successfully synthesized in moderate to good yields and their structures were characterized by ^1H and ^{13}C NMR and MS. The purity of all compounds was determined to be >95% (monitored at 254 and 220 nm) by reverse phase HPLC.

Biological assays. Forty-eight β -carboline derivatives (Figures 2-5) were evaluated in cancer chemopreventive and anticancer assays including the induction of QR1 and inhibition against aromatase and nitrite production. Briefly, testing for activity was initially performed at 50 μM . For compounds exhibiting >50% inhibition or with IR value >2.0 in the QR1 assay at this concentration, further testing was performed to determine an IC_{50} (or CD for QR1) value. The antiproliferative effects of these compounds were also tested using a SRB assay; compounds leading to <50% of cell survival at 50 μM were considered cytotoxic, and IC_{50} values were determined. Results are summarized in Table I.

QR1 induction. For QR1 induction, among the *N*-alkylation derivatives of **5**, the *R*-isomer **5f** demonstrated higher activity (IR=4.0, CD=10.9 μM) than its *S*-isomer **5a** (IR=2.9, CD=42.4 μM). There were no major differences observed between the two chiral isomers with other *N*²-substituents. In contrast, most compounds derived from **6** with a free carboxylic acid group showed increased QR1 induction activity. Among the series of compounds without the carboxylic acid group (**8** and derivatives), only **8e** with 1-bromo-2-naphthylmethyl group at the *N*² position demonstrated potent activity, with an IR value of 3.2 (CD=1.3 μM). For urea derivatives with an alkyl side chain (**9** and **11**), only weak QR1 inductive activity was observed. However, higher activity was observed with the cyclized hydantoin compound **11c** (IR=2.2, CD=41.4 μM). In general, the introduction of an amide functionality at the 3 position instead of the carboxylic acid group led to increased QR1 induction (**12a**, **12e**, **12f** vs. **6e**, **6j**). In contrast, the *S*-isomer ethylamino-substituted amide derivative **12a** (IR=2.6, CD=32.3 μM) was much more active than its corresponding *R*-isomer **12b** (IR=1.4). Among the *R*-isomer amide derivatives **12b-g**, isobutyl **12e** (IR=2.2, CD=39.9 μM) and 4-

methylpiperazinyl **12f** (IR=2.4, CD=30.4 μM) showed higher activity than other substituents. In addition, the *N*¹-methylated compound **13** (IR=3.0, CD=26.0 μM) showed higher activity relative to its free NH variant **5j**.

Aromatase inhibitory activity. Among all the tested compounds, only tetracyclic derivatives **11a-c** with an extended hydantoin ring system led to over 34% inhibition when tested at 50 μM , and **11a** exhibited the most potent inhibition (59.9% inhibition at 50 μM).

Nitrite inhibition. As shown in Table I, among the *N*²-alkylated 3-substituted ester derivatives **5a-l**, the *R*-isomers (**5f**, IC_{50} =30.97 μM ; **5j**, IC_{50} =16.63 μM) generally exhibited activity comparable to their corresponding *S*-isomers (**5a**, IC_{50} =33.10 μM ; **5e**, IC_{50} =17.69 μM) except that the *R*-isomer **5g** (IC_{50} =33.62 μM) was more active than its *S*-isomer **5b**. Moreover, the bulky ethyl and isopropyl ester compounds **5k** and **5l** were inactive compared to their methyl ester counterparts **5e** (the *S*-isomer, IC_{50} =17.69 μM) and **5j** (the *R*-isomer, IC_{50} =16.63 μM). In contrast, in the free carboxylic acid series **6a-j**, the stereoisomers of alkylation derivatives **5-6** had a stereospecific effect on nitrite inhibitory activity. For instance, the *R*-isomers (**6h**, IC_{50} =38.43 μM ; **6i**, IC_{50} =28.43 μM ; **6j**, IC_{50} =22.22 μM) were more active than their corresponding *S*-isomers. In addition, compounds with *N*² substituted 1-bromo-2-naphthylmethyl group (**5e**, **5j**, **6e**, **6j**) demonstrated more potent activity, with IC_{50} values ranging from 16.63 to 27.13 μM , lower than all the other *N*² alkyl substituents tested. Among the series of compounds **8a-h** without a carboxylic acid group, **8c** with *N*² substituted 2-naphthoxypropyl group and **8e** with 1-bromo-2-naphthylmethyl exhibited inhibitory activity with IC_{50} values of 31.06 and 28.91 μM , respectively. For the urea compounds (**9c**, **9e**, **9f** and **10a-c**) with an alkyl side chain, none of these compounds showed notable NO inhibition activity. Interestingly, tetracyclic hydantoin derivatives **11c** and **11d** inhibited nitrite production, with IC_{50} values of 27.80 and 35.11 μM , respectively. In general, the introduction of the amide functionality at the 3 position (*e.g.*, **12a**, IC_{50} =13.39 μM ; **12b**, IC_{50} =14.49 μM ; **12e**, IC_{50} =42.88 μM ; and **12f**, IC_{50} =6.54 μM) led to enhanced inhibitory activity compared to **6e** (IC_{50} =27.13 μM) and **6j** (IC_{50} =22.22 μM) with a 3-substituted carboxylic acid group. It should be noted for the ethyl-substituted amide derivatives, *S*-isomer **12a** (IC_{50} =13.39 μM) had almost the same activity as its corresponding *R*-isomer **12b** (IC_{50} =14.49 μM). Overall, the *R*-isomer amide derivative **12f** with 4-methylpiperazinyl moiety had the most potent activity (IC_{50} =6.54 μM) among all the compounds tested.

Cytotoxic effects on RAW 264.7 cells. None of the alkylated derivatives **5-6** had any cytotoxic effect on RAW cells. Among the series of alkylated compounds of **8** without a

Table I. *Biological activities (data mean value $\pm$ standard deviation, μ M) of compounds 5-13 evaluated in cancer-chemopreventive and anticancer assays.*

Compound	Configuration	QR1 assay		Aromatase assay		Nitrite assay		
		IR at 50 μ M	CD (μ M)	Inhibition at 50 μ M (%)*	Inhibition at 50 μ M (%)*	IC ₅₀ (μ M)	Survival at 50 μ M (%)*	Cytotoxic IC ₅₀ (μ M)
5a	S	2.9 $\pm$ 0.2	42.4 $\pm$ 3.3	0.0 $\pm$ 7.1	61.45 $\pm$ 0.9	33.10 $\pm$ 2.50	68.83 $\pm$ 1.32	
5b	S	1.2 $\pm$ 0.2		0.0 $\pm$ 5.6	49.36 $\pm$ 3.78		77.39 $\pm$ 2.66	
5c	S	1.0 $\pm$ 0.1		0.0 $\pm$ 7.2	5.59 $\pm$ 2.76		86.31 $\pm$ 1.07	
5d	S	1.6 $\pm$ 0.3		2.3 $\pm$ 8.5	36.51 $\pm$ 2.87		86.29 $\pm$ 0.93	
5e	S	1.3 $\pm$ 0.2		0.0 $\pm$ 4.4	63.48 $\pm$ 2.07	17.69 $\pm$ 2.02	81.16 $\pm$ 0.98	
5f	R	4.0 $\pm$ 0.6	10.9 + 1.2	0.6 $\pm$ 7.9	65.84 $\pm$ 1.66	30.97 $\pm$ 1.42	60.19 $\pm$ 1.74	
5g	R	1.1 $\pm$ 0.3		0.5 $\pm$ 6.1	64.77 $\pm$ 2.32	33.62 $\pm$ 1.75	69.65 $\pm$ 0.73	
5h	R	1.0 $\pm$ 0.1		0.0 $\pm$ 9.5	12.72 $\pm$ 2.47		84.48 $\pm$ 0.52	
5i	R	1.3 $\pm$ 0.2		5.6 $\pm$ 9.5	43.97 $\pm$ 2.66		69.24 $\pm$ 1.54	
5j	R	1.0 $\pm$ 0.1		2.9 $\pm$ 3.7	72.44 $\pm$ 0.77	16.63 $\pm$ 0.69	78.34 $\pm$ 5.40	
5k	R	1.0 $\pm$ 0.1		3.7 $\pm$ 5.5	34.71 $\pm$ 1.23		82.33 $\pm$ 1.40	
5l	R	1.9 $\pm$ 0.3		9.1 $\pm$ 4.1	35.10 $\pm$ 1.56		80.55 $\pm$ 0.85	
6a	S	3.2 $\pm$ 0.4	23.3 $\pm$ 0.9	0.0 $\pm$ 2.7	4.52 $\pm$ 1.67		96.19 $\pm$ 2.15	
6b	S	4.8 $\pm$ 0.1	18.8 $\pm$ 2.6	19.5 $\pm$ 5.1	35.10 $\pm$ 2.59		94.29 $\pm$ 0.43	
6c	S	1.8 $\pm$ 0.3		15.8 $\pm$ 7.4	43.73 $\pm$ 1.38		80.36 $\pm$ 0.45	
6d	S	2.4 $\pm$ 0.1	36.3 $\pm$ 4.1	15.1 $\pm$ 6.7	29.98 $\pm$ 3.41		81.29 $\pm$ 3.24	
6e	S	1.8 $\pm$ 0.1		0.0 $\pm$ 4.6	85.65 $\pm$ 0.99	27.13 $\pm$ 1.41	65.14 $\pm$ 2.12	
6f	R	3.3 $\pm$ 0.1	16.4 $\pm$ 0.6	3.4 $\pm$ 3.9	6.71 $\pm$ 2.45		96.64 $\pm$ 1.89	
6g	R	0.6 $\pm$ 0.2		0.7 $\pm$ 8.4	44.76 $\pm$ 1.45		83.08 $\pm$ 1.99	
6h	R	3.4 $\pm$ 0.1	21.9 $\pm$ 3.1	6.4 $\pm$ 2.2	68.25 $\pm$ 3.72	38.43 $\pm$ 1.97	84.56 $\pm$ 3.29	
6i	R	0.4 $\pm$ 0.1		15.8 $\pm$ 8.5	91.79 $\pm$ 0.74	28.34 $\pm$ 2.64	56.25 $\pm$ 1.38	
6j	R	1.4 $\pm$ 0.0		0.0 $\pm$ 2.6	84.14 $\pm$ 0.85	22.22 $\pm$ 1.04	78.39 $\pm$ 1.55	
8a		1.2 $\pm$ 0.1		21.6 $\pm$ 6.2	17.60 $\pm$ 2.49		107.74 $\pm$ 1.60	
8b		1.8 $\pm$ 0.3		5.5 $\pm$ 4.4	7.26 $\pm$ 0.15		108.90 $\pm$ 3.22	
8c		1.2 $\pm$ 0.1		6.3 $\pm$ 1.8	99.13 $\pm$ 0.14	31.06 $\pm$ 2.03	15.57 $\pm$ 0.19	39.90 $\pm$ 2.35
8d		1.2 $\pm$ 0.4		2.1 $\pm$ 4.1	13.08 $\pm$ 0.79		101.34 $\pm$ 1.58	
8e		3.2 $\pm$ 0.1	1.3 $\pm$ 0.4	7.0 $\pm$ 9.8	80.91 $\pm$ 2.91	28.91 $\pm$ 0.63	50.59 $\pm$ 3.81	
8f		1.3 $\pm$ 0.2		0.0 $\pm$ 11.0	8.72 $\pm$ 0.29		90.23 $\pm$ 1.30	
8g		1.5 $\pm$ 0.1		9.0 $\pm$ 2.8	31.66 $\pm$ 2.15		81.81 $\pm$ 0.74	
8h		1.0 $\pm$ 0.1		9.2 $\pm$ 6.0	38.91 $\pm$ 0.69		102.19 $\pm$ 1.59	
9c	R	1.0 $\pm$ 0.1		6.3 $\pm$ 2.3	35.46 $\pm$ 1.03		91.11 $\pm$ 2.70	
9e	R	1.0 $\pm$ 0.0		8.9 $\pm$ 3.7	21.13 $\pm$ 0.83		97.12 $\pm$ 1.52	
9f	R	1.0 $\pm$ 0.1		1.7 $\pm$ 2.9	12.28 $\pm$ 1.18		97.76 $\pm$ 1.63	
10a		1.1 $\pm$ 0.2		24.0 $\pm$ 6.8	-2.21 $\pm$ 3.01		102.30 $\pm$ 0.77	
10b		1.4 $\pm$ 0.2		13.8 $\pm$ 4.3	18.84 $\pm$ 0.71		88.45 $\pm$ 0.80	
10c		1.0 $\pm$ 0.0		0.0 $\pm$ 4.4	15.98 $\pm$ 1.54		98.98 $\pm$ 2.34	
11a	R	1.6 $\pm$ 0.1		59.9 $\pm$ 7.0	4.39 $\pm$ 4.15		106.22 $\pm$ 2.36	
11b	R	1.2 $\pm$ 0.1		34.5 $\pm$ 2.0	18.31 $\pm$ 2.09		101.45 $\pm$ 1.42	
11c	R	2.2 $\pm$ 0.0	41.4 $\pm$ 4.0	35.8 $\pm$ 5.2	91.38 $\pm$ 0.92	27.80 $\pm$ 1.89	46.88 $\pm$ 1.05	49.95 $\pm$ 0.02
11d	R	1.3 $\pm$ 0.3		26.4 $\pm$ 2.3	66.30 $\pm$ 2.72	35.11 $\pm$ 0.73	104.83 $\pm$ 3.52	
12a	S	2.6 $\pm$ 1.0	32.3 $\pm$ 2.1	11.5 $\pm$ 4.3	73.41 $\pm$ 0.47	13.39 $\pm$ 1.83	92.42 $\pm$ 3.09	
12b	R	1.4 $\pm$ 0.1		12.6 $\pm$ 4.6	76.08 $\pm$ 1.15	14.49 $\pm$ 0.83	78.43 $\pm$ 1.05	
12c	R	1.2 $\pm$ 0.1		5.8 $\pm$ 1.3	43.61 $\pm$ 1.29		103.97 $\pm$ 3.22	
12d	R	1.7 $\pm$ 0.3		10.8 $\pm$ 3.0	50.86 $\pm$ 2.06		107.74 $\pm$ 0.77	
12e	R	2.2 $\pm$ 0.0	39.9 $\pm$ 3.3	8.6 $\pm$ 3.2	51.91 $\pm$ 0.74	42.88 $\pm$ 2.38	89.86 $\pm$ 0.57	
12f	R	2.4 $\pm$ 0.2	30.4 $\pm$ 3.3	9.0 $\pm$ 1.4	99.38 $\pm$ 0.14	6.54 $\pm$ 0.77	28.63 $\pm$ 0.39	17.98 $\pm$ 1.46
12g	R	1.2 $\pm$ 0.3		8.8 $\pm$ 2.2	32.95 $\pm$ 2.07		108.66 $\pm$ 2.86	
13	R	3.0 $\pm$ 0.0	26.0 $\pm$ 2.8	0.0 $\pm$ 5.9	41.44 $\pm$ 4.63		70.23 $\pm$ 1.85	

CD: Concentration that doubles activity, determined for compounds with induction ratio (IR)>2; QR1: quinone reductase 1. *In comparison with vehicle-treated controls. 50% inhibitory concentration (IC₅₀) values for control inhibitors in these assays: for QR1: 4'-bromoflavone, 0.01 μ M; for aromatase: letrozole, 7.0 $\pm$ 2.0 nM; and for the nitrite assay: L-NG-monomethyl arginine, 22.1 $\pm$ 0.2 μ M.

carboxylic acid group, **8c** with a *N*²-substituted 2-naphthoxypropyl moiety was cytotoxic toward RAW cells with an IC₅₀ value of 39.90 μM. From the urea and tetracyclic hydantoin series (**9-11**), only **11c** with a hexyl group exhibited a cytotoxic effect on RAW cells, with an IC₅₀ value of 49.95 μM. Among 3-substituted amide derivatives **12a-g**, only **12f** (containing a 4-methylpiperazinyl group; IC₅₀=17.98 μM) had cellular cytotoxic effect on RAW cells.

Molecular docking with iNOS. In an effort to provide structural insight and gain further understanding of the potency of our tested compounds, we proceeded to examine the potential interactions of **12f** with iNOS protein using the 3D structure obtained from Protein Data Bank (PDB ID: 2Y37). As shown in Figure 6, the carboline compound **12f** was found to bind in the same pocket as the co-crystallized ligand. The tetrahydro-β-carboline moiety of **12f** was buried in a pocket woven between TYR367, VAL346, PRO344, and iron protoporphyrin IX (HEME). One hydrogen bond was formed between NH on the carboline moiety with a carboxylic acid group on HEME. The naphthalene ring moiety was accommodated in another side pocket defined by GLU488, GLN486, and TYR485, forming hydrophobic interactions. Moreover, two additional hydrogen bonds were formed between the acylpiperazine moiety and amino acid GLN381 and ARG260.

Discussion

A series of callophycin A analogs bearing a tetrahydro-β-carboline motif was synthesized and evaluated. This work revealed several compounds to have low micromolar activity in our assays. Specifically, **8e** with *N*²-substituted 1-bromo-2-naphthylmethyl group demonstrated excellent activity in QR1 induction with CD of 1.3 μM. Consistent with our previous findings (11), this series of tetrahydro-β-carboline derivatives were largely inactive in the aromatase inhibition assay. Among all the tested compounds, **11a** exhibited the most potent inhibition (59.9% inhibition at 50 μM) against aromatase. With regard to the nitrite assay, the *R*-isomer of the amide derivative **12f** bearing a 4-methylpiperazinyl group exhibited the most potent inhibitory activity (IC₅₀=6.54 μM) against NO production, with a cytotoxic effect (IC₅₀=17.98 μM) on RAW cells. The cytotoxicity of **12f** is likely due to the extra amine functionality and increased cellular membrane penetration. In general, only three compounds (**8c**, **11c**, and **12f**) developed cellular cytotoxic effects on RAW cells simultaneously with iNOS inhibition under the same experimental condition. Since the cytotoxic IC₅₀ values of these compounds were higher than their corresponding IC₅₀ values against NO production, it can be concluded that iNOS inhibition was not caused by a false-positive effect due to general cellular cytotoxicity.

There are several factors that can affect NO synthesis: i) the cellular level of L-arginine as a substrate of iNOS and iNOS-

specific cofactors, ii) enzymatic activity of iNOS, iii) protein and mRNA expression levels of iNOS, and iv) existence of endogenous antagonists (27). Active compounds found in the current study may affect enzymatic activity of iNOS by interacting with the active site or allosteric site (*e.g.* interaction with the dimerization site of iNOS). It is known that NOS is active only as a homodimer (28). Thus, inhibitors of iNOS dimerization have therapeutic potential in effectively controlling iNOS activity and reducing the high levels of NO produced under pathological conditions (29). Recently, pyrimidine imidazole derivatives were reported to be versatile iNOS inhibitors by interacting with both dimer and monomer entities of the iNOS enzyme (30). On the other hand, active compounds from nitrite assay may affect enzymatic activity by directly binding to the active enzyme site (substrate-binding site). For example, L-*N*^G-monomethyl arginine, a derivative of arginine (iNOS substrate), is known to act as a competitive inhibitor of NOS (31).

In order to predict the binding mode of **12f**, found to be the most active compound in the nitrite assay, molecular docking simulation was performed. The docking study revealed that **12f** interacts favorably with and binds with iNOS in the active site, including three hydrogen bonds, as well as hydrophobic interactions. There are two main domains in iNOS, the oxygenase domain and the reductase domain. Compound **12f** is predicted to bind with the oxygenase domain that includes regions involved in dimerization and the L-arginine binding site. None of the residues (TYR367, VAL346, PRO344, GLU488, GLN486, and TYR485) that interact with **12f** exist outside of dimerization regions (32); the inhibition may occur not by allosteric inhibition, but by active-site inhibition. Nevertheless, further experimental studies remain to be performed to determine the exact mechanism of inhibition by **12f**, including directly inhibiting the enzyme or another alternative mechanism such as induction by LPS. Together, these results provide promising β-carboline leads for further optimization and development with therapeutic potential as new chemopreventive and chemotherapy agents.

Conflicts of Interest

The Authors declare that there are no conflicts of interest in regard to this study.

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