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Brief Article

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Inhibition of Acyl-CoA: Cholesterol Acyltransferase (ACAT), Overexpression of Cholesterol Transporter Gene, and Protection of Amyloid β (Aβ) Oligomers-Induced Neuronal Cell Death by Tricyclic Pyrone Molecules

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Supporting Information

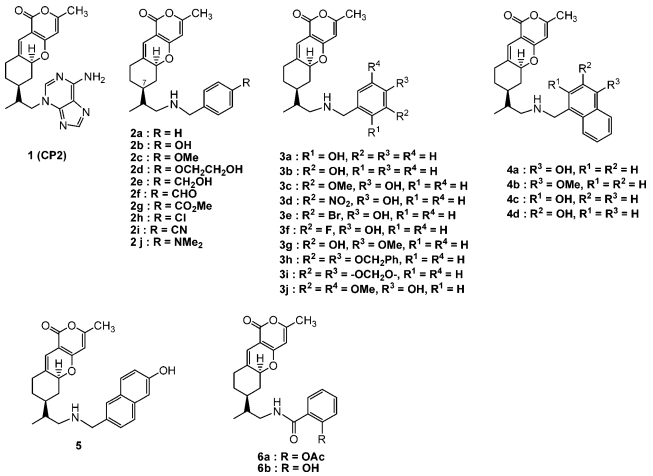
ABSTRACT: A major effort in Alzheimer's disease therapeutic development has targeted Aβ and downstream events. We have synthesized a small library of tricyclic pyrone compounds. Their protective action in MC65 cells and inhibition of ACAT along with the upregulation of cholesterol transporter gene were investigated. Five active compounds exhibited potencies in the nanomolar ranges. The multiple effects of the compounds on Aβ and cellular cholesterol pathways could be potential mechanisms underlying the protective effects in vivo.

INTRODUCTION

Over 35 million people worldwide suffer from Alzheimer's disease (AD), and current available treatments for AD, donepezil, rivastigmine, galantamine, and memantine, temporarily ameliorate some symptoms but do not modify the underlying disease. New drug discovery is urgently needed.[1] In search of small molecules against intracellular Aβ oligomers (AβO) toxicity, we have used MC65 cells protection assay as our primary screen for bioactive compounds.[2−4] MC65 cells are neuroblastoma cells that degenerate after induction of intraneuronal accumulation of AβO. This line has been used in a high throughput assay that reliably selects compounds that penetrate the membranes, bind, neutralize, and reduce intraneuronal levels of AβO.[4,5] This assay generates few false positive results and gives a high likelihood of identifying leads that penetrate cells and ameliorate AβO-induced toxicity.[5] Previously, we identified a tricyclic pyrone (TP) molecule, **1** (code name CP2; Chart 1),[3,6] from MC65 cells assay that prevented cell death associated with intracellular AβO and inhibits Aβ aggregation in vitro and reduced amyloid plaques and soluble AβO in vivo.[3,6] In search of other TP molecules that possess greater cell protective action, we synthesized a library of TPs and evaluated their MC65 cells protective potencies and additional beneficial effects such as lipid modulation activities including the inhibition of acyl-CoA:cholesterol acyltransferase (ACAT) and upregulation of cholesterol transporter gene, ATP-binding cassette subfamily A, member 1 (ABCA1).

Recent results from genetic, cell-culture, mouse model, and epidemiologic data suggest that cellular cholesterol (lipid) metabolism is important in the control of the production and/or accumulation of Aβ.[7] For example, natural and synthetic liver X receptor (LXR) agonists including oxysterols, retinoic acids, T0901317 and GW3965 have been shown to induce cholesterol efflux,[8] which associates with the reduction of Aβ formation and secretion of Aβ in vitro and in vivo.[9,10] The induction of ABCA1 is important for cholesterol efflux and is also shown to mediate the secretion of Aβ from the cells.[9] Moreover, a well-established AD biomarker, ε4 allele[11] of

Chart 1. Synthesized and Bioevaluated Tricyclic Pyrone Compounds 1−6 Using MC65 Cells



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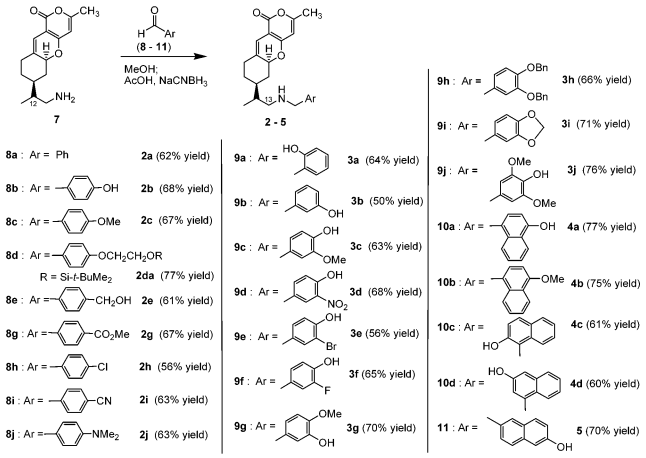
apolipoprotein E (APOE), is involved in the cholesterol homeostasis. ACAT plays an important role in the cholesterol homeostasis by converting free cholesterol to neutral cholesteryl ester for storage, and ACAT inhibitors have been implicated in antiatherosclerosis and reduction of amyloid pathology by regulating cholesterol homeostasis.[12−14] Also ACAT inhibitors were shown to induce cholesterol efflux.[15]

RESULTS AND DISCUSSION

In mimicking CP2,[16] various TP compounds (Chart 1) containing aryl substituted alkylamino functions attached at the C7 isopropyl side chain were synthesized from a facile reductive amination reaction starting from amine **7** (Scheme 1).

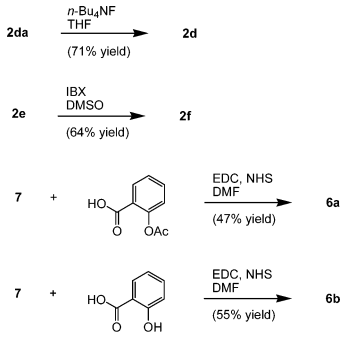
Scheme 1. Synthesis of TP Compounds 2−5



Two TP amides, compounds **6a** and **6b**, were also prepared for comparison of their MC65 cells protective activities with that of TP amines **2−5**. Hence, treatment of TP amine **7** (a pair of diastereomers possessing *R* and *S* configurations at C12)[17] with aldehydes **8−11** in methanol followed by sodium cyanoborohydride and acetic acid afforded compounds **2−5** in good yields. Various functionalities including hydroxyl, methyl ester, aromatic halides, cyanide, amine, and nitro remain intact under the reaction conditions.

Compound TP **2d** was obtained from removal of the silyl ether protecting group of compound **2da**, derived from the above reductive amination of **7** and **8d**, in 71% yield, and TP aldehyde **2f** from oxidation of alcohol **2e** with 2-iodoxybenzoic acid (IBX) and DMSO[18] in 64% yield (Scheme 2). TP amides **6a** and **6b** were prepared from coupling reactions of amine **7** with acetylsalicylic acid and salicylic acid, respectively, using 3-

Scheme 2. Syntheses of TP Compounds 2d, 2f, 6a, and 6b



(3-dimethylaminopropyl)-1-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) as the activating reagents.

Aryl aldehydes **8a−8c**, **8h−8j**, **9a−9d**, **9g**, **9i**, **9j**, **10a**, and **10c** were obtained from commercial sources. Aryl aldehydes **8d**,[19] **8e**,[20] and **8g**[21] were prepared by following the literature methods. Bromination of **8b** with bromine in chloroform and 1,2-dimethoxyethane (4:1) afforded aldehyde **9e**.[22] Reduction of 4-cyano-2-fluorophenol with platinum oxide in formic acid[23] gave compound **9f**. Dibenzylation of 3,4-dihydroxybenzaldehyde with potassium carbonate and benzyl bromide[24] produced aldehyde **9h**, and similarly, methylation of 4-hydroxynaphthalenecarboxaldehyde with potassium carbonate and methyl iodide furnished aldehyde **10b**.[25] The metalation/Vilsmeier−Haack reaction of 6-bromo-2-naphthol with sodium hydride, *n*-BuLi, and DMF[26] gave aldehyde **11**.

3-Hydroxynaphthalenecarboxaldehyde (**10d**) was similarly made from the metalation/Vilsmeier−Haack reaction of 4-bromo-2-naphthol,[27] which was derived regioselectively from a sequence of bromination, oxidiazotization, and reduction of 1-aminonaphthalene.[28]

As described previously, we used MC65 cell line to screen bioactive compounds.[4] The cells are readily propagated, and cell death occurs after three days and is measured quantitatively by a simple 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[4] Tetracycline (TC) is used to suppress the induction of the SβC gene which produces the C99 fragment of amyloid precursor protein (APP). Hence, in the presence of TC, MC65 cells survive, and absence of TC leads to cell death. In the absence of TC, compounds that protect neuron cell death could be used for screening of new leads in anti-Aβ. In the presence of TC and the compound, toxicity of the compound to MC65 cells is revealed. The EC_{50} (median effective concentration), TD_{50} (median toxic concentration), and TI (therapeutic index; equals to TD_{50}/EC_{50}) values of various TP compounds are listed in Table 1. Compounds **2b**, **2e**, **3f**, and **4a** showed the greatest potencies in protecting MC65 cells death having EC_{50} values of 70, 101, 67, and 145 nM, respectively. Compounds **2b**, **2e**, and **3f** are more active than our initial lead compound, **1**, EC_{50} value of 120 nM. It appears that a hydroxyl (**2b** and **4a**) or hydroxymethyl (**2e**) substituent at the para-position of the C13 phenyl- and naphthyl-methylamino moieties (for numbering, see structures **2−5** of Scheme 1) enhances the bioactivity, and additional fluorine atom at the meta-position provides similar activity as that of **2b**. Other substituents such as hydrogen, methoxy, 2-hydroxyethyloxy, aldehyde, ester, chlorine, and dimethylamino at the para-position of the phenylmethylamino group decrease the activity. The *para*-cyano group of compound **2i** abolishes the activity. The *ortho*- or *meta*-hydroxyl group lowers the activity. It is encouraging that additional functionality to the phenyl ring of compound **2b** only lowers the activity moderately, implying that further modification of compound **2b** is possible. Other regioisomers such as **4c**, **4d**, and **5** of 4-hydroxynaphthyl analogue **4a** also possess weaker activities. Amide **6b** showed poor activity, and its acetyl ester analogue, **6a**, is inactive in the protection of MC65 cell death, hence, other amide derivatives were not investigated. Notably, data presented in our previous report suggests that TP compounds achieve MC65 protection neither by inhibiting γ-secretase-catalyzed Aβ production nor by a general antioxidation effect.[6]

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Table 1. EC_{50}, TD_{50}, and TI Values of 1−6 from MC65 Cell Protection Assays[29]

compd	EC_{50} (μM)	TD_{50} (μM)	TI
1	0.120 ± 0.015	39.0 ± 1.12	325
2a	3.24 ± 0.160	37.3 ± 1.77	11.5
2b	0.070 ± 0.002	49.3 ± 0.127	704
2c	2.77 ± 0.191	>50	>18.1
2d	24.5 ± 1.31	>50	>2.04
2e	0.101 ± 0.004	>50	>495
2f	6.41 ± 0.961	39.4 ± 1.87	6.15
2g	0.769 ± 0.033	>50	>65.0
2h	2.79 ± 0.107	48.0 ± 0.080	17.2
2i	>50	>50	
2j	4.36 ± 0.055	38.2 ± 1.02	8.76
3a	2.44 ± 0.111	30.7 ± 0.429	12.6
3b	4.66 ± 0.339	>50	>10.7
3c	0.242 ± 0.014	26.3 ± 1.34	109
3d	3.85 ± 0.416	>50	>13.0
3e	0.639 ± 0.001	>50	>78.2
3f	0.067 ± 0.002	>50	>746
3g	1.56 ± 0.025	>50	>32.1
3h	0.662 ± 0.070	9.38 ± 0.217	14.2
3i	1.18 ± 0.010	>50	>42.4
3j	1.26 ± 0.182	>50	>39.7
4a	0.145 ± 0.002	13.7 ± 0.704	94
4b	0.621 ± 0.051	15.3 ± 0.183	24.6
4c	0.198 ± 0.013	26.4 ± 0.264	133
4d	0.586 ± 0.045	18.2 ± 1.84	31.1
5	0.459 ± 0.026	24.1 ± 1.48	52.5
6a	>50	8.35 ± 0.319	
6b	6.25 ± 0.171	6.69 ± 0.105	1.07

In light of the finding of alleviation of cholesterol accumulation in Huntington's disease neurons by **1**[30] and to explore other proteins that TPs may affect, inhibitions of ACAT in MC65 cells by five most active compounds selected from MC65 assay, **1**, **2b**, **2e**, **3f**, and **4a**, were carried out. Cells were incubated with mock-medium, TP compounds, and CI-976 (an ACAT inhibitor)[31] for 24 h. ACAT activities were examined by staining with NBD-cholesterol, which is a fluorescent probe for cholesteryl ester (CE)-rich lipid droplets.[32] The intensity of fluorescence were measured on a fluorescent plate reader equipped with 485 nm excitation and 535 nm emission filters. The ACAT activity in the presence of each compound was assessed by the comparison of fluorescence intensity with mock-treated cells. Like CI-976, the incubation with TP compounds significantly reduced the fluorescence intensity in MC65 cells, and results are summarized in Table 2. Compound **2b**, with IC_{50} value of 0.3 μM, possesses similar inhibitory activity as that of CI-976 and more potent than that of **2e**, **3f**, and **4a**. Compounds **1**, **2e**, **3f**, and **4a** are slightly less active, with IC_{50} values in the range of 0.8−1.8 μM. It appears that TPs' ACAT inhibitory activities correlate with MC65 cells' protective activities. Anti-ACAT effects of each compound were also confirmed in human hepatoma cells (Huh-7 cells), and similar results were obtained (data not shown). TD_{50} values of **1**, **2b**, **2e**, **3f**, and **4a** are 39, 37, >50, >50, and 14 μM, respectively, and their respective TI values are 33, 123, >36, >28, and 18.

Table 2. ACAT Inhibitory Activity and Increase of ABCA1 Gene Expression of the Most Active TP Compounds and CI-976 in MC65 Cells

compd	IC_{50} value of ACAT inhibition (μM)	EC_{50} value of ABCA1 gene expression (μM)
1	1.2 ± 0.2	0.9 ± 0.1
2b	0.3 ± 0.08	1.1 ± 0.1
2e	1.4 ± 0.2	2.5 ± 0.4
3f	1.8 ± 0.3	2.2 ± 0.2
4a	0.8 ± 0.06	1.3 ± 0.1
CI-976	0.2 ± 0.1	0.6 ± 0.07

The inhibition of ACAT may increase the level of free cholesterol, subsequently induce oxidation of cholesterol (oxycholesterol), and activate LXR pathway. We examined whether these compounds induced the expression of cholesterol efflux-related protein gene, ABCA1, in MC65 cells, and Huh-7 cells. Cells were incubated with mock-medium, TP compounds, and CI-976 for 24 h, and expression of the gene was assessed with the gene expression assay.[33] The treatment with compounds **1**, **2b**, **4a**, and CI-976 in both MC65 (Table 2) and Huh-7 cells significantly increased the expression of ABCA1 with EC_{50} values in the range of 0.6−1.3 μM compared to mock-treated cells (Table 2). Compounds **2e** and **3f** are less active, having EC_{50} values of 2.5 and 2.2 μM, respectively. Because TP compounds and CI-976 inhibit ACAT activity in the cells and the role of ACAT in cholesterol homeostasis, we speculate that the induction of expression is due to the inhibition of ACAT activity. Notably, CI-976 is inactive in protection of MC65 cell death up to 50 μM.

CONCLUSION

Newly synthesized TP compounds **2b**, **2e**, **3f**, and **4a**, containing 4-hydroxyphenyl-, 4-(hydroxymethyl)phenyl-, 3-fluoro-4-hydroxyphenyl-, and 4-hydroxynaphthyl-methylamino moiety at C13 of the tricyclic pyrone skeleton, respectively, possess strong cell protective properties against intracellularly induced Aβ toxicity, inhibitory activities against ACAT, and enhancing properties of ABCA1 cholesterol transporter gene in nanomolar to low micromolar ranges. Additional fluorine atom at C3 on the 4-hydroxyphenyl ring of compound **2b** retains cell protective activity. The therapeutic index values of the TP compounds in MC65 cells are high (>100), and they may serve as lead compounds for the discovery of AD drugs.

EXPERIMENTAL SECTION

Chemistry. A representative synthesis of compound **2b** is described below. The general bioassays, experimental information, and synthesis of all other compounds are supplied in the Supporting Information. Purity of all final compounds determined by HPLC analysis is >95%.

(5a*S*,7*S*)-3-Methyl-7-[(1*R*) and (1*S*)1-(4-hydroxybenzylamino)propan-2-yl]-1*H*,7*H*-5a,6,8,9-tetrahydro-1-oxopyrano[4,3-*b*][1]benzopyran (2b). A solution of 85 mg (0.31 mmol) of amine **7** and 38 mg (0.31 mmol) of aldehyde **8b** in 5 mL of dry MeOH was stirred under argon at 25 °C for 12 h. To it were added acetic acid (5 drops) and a solution of 68 mg (1.1 mmol) of $NaBH_3CN$ in MeOH. After stirring for 1 h, the reaction solution was diluted with 40 mL of 5% aqueous ammonium hydroxide and extracted three times with dichloromethane. The combined organic layer was washed with brine, dried ($MgSO_4$), concentrated, and column chromatographed on silica gel using a mixture of CH_2Cl_2 and MeOH (9:1) as eluant to give 80 mg (68% yield) of light-yellow solid **2b**; mp 75−78 °C. FTIR (solid) ν 3276 (bw), 1682, 1637, 1565, 1514, 1446, 1231, 1146, 879, 762 cm^{-1}. ^1H NMR δ 7.16 (d, J = 8.0 Hz, 2 H), 6.76 (d, J = 8.4 Hz, 2 H), 6.06 (s, 1 H), 5.71 (s, 1 H), 5.09−4.99 (m, 1

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H), 3.70 (s, 2 H), 2.66−2.57 (m, 1 H), 2.51−2.39 (m, 2 H), 2.19 (s, 3 H), 2.07−1.90 (m, 2 H), 1.73−1.47 (m, 4 H), 1.29−1.08 (m, 1 H), 0.90 and 0.89 (2 d, J = 6.4 Hz, 3 H, CH_3 of two diastereomers). ^{13}C NMR δ 163.7, 163.6, 163.0, 161.8, 156.5, 133.0, 129.9, 129.7, 115.9, 109.1, 100.1, 97.5, 79.7, 79.65, 53.5, 52.8, 52.7, 39.2, 38.8, 38.7, 37.2, 37.1, 36.8, 32.4, 32.3, 31.0, 28.4, 20.2, 14.7, 14.6. MS (electrospray ionization) m/z 382.4 ($M + H^+$), 276.5. HRMS calcd for $C_{23}H_{28}NO_4^+$ ($M + H^+$), 382.2018; found, 382.2013.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures and spectroscopic data for compounds **2**−**6**, detailed protocols for MC65 cells assay, inhibition of ACAT, and upregulation of ABCA1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

Professors Hua, Jin, and Chang directed and designed the chemistry and biological chemistry. L. Pokhrel and T.D.T. Nguyen carried out the chemical synthesis, Dr. Maezawa conducted the MC65 cell assays, and Dr. Chang performed the inhibition of ACAT and expression of ABCA1 transporter gene.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AβO, amyloid beta oligomers; ACAT, acyl-CoA:cholesterol acyltransferase; ABCA1, ATP-binding cassette subfamily A member 1; AD, Alzheimer's disease; APP, amyloid precursor protein; APOE, apolipoprotein E; CE, cholesteryl ester; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; EDC, 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide hydrochloride; IBX, 2-iodoxybenzoic acid; IC_{50}, inhibition concentration at 50%; LXR, liver X receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NBD, 25-[*N*-[(7-nitro-2-1,3-benzoxadiazol-4-yl)methyl]amino]-27-norcholesterol; NHS, *N*-hydroxysuccinimide; TC, tetracycline; TP, tricyclic pyrone

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