Note

Structure–Activity Relationships of Thiophene Carboxamide Annonaceous Acetogenin Analogs: Shortening the Alkyl Chain in the Tail Part Significantly Affects Their Growth Inhibitory Activity against Human Cancer Cell Lines

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In a previous study, we found that the thiophene carboxamide solamin analog, which is a mono-tetrahydrofuran annonaceous acetogenin, showed potent antitumor activity through the inhibition of mitochondrial complex I. In this study, we synthesized analogs with short alkyl chains instead of the n-dodecyl group in the tail part. We evaluated their growth inhibitory activities against human cancer cell lines. We found that the alkyl chain in the tail part plays an essential role in their activity.

Key words annonaceous acetogenin; antitumor agent; structure–activity relationship; organic synthesis

Introduction

The development of novel antitumor agents with unique modes of action is one of the most urgent and vital research challenges, as cancer is the first or second most common cause of death in people under 70 years of age in over half of the countries of the world according to the WHO’s 2015 report.1) To contribute to the fight against cancer, we studied the structure–activity relationships (SARs) of annonaceous acetogenins. These polyketides (annonaceous acetogenins)2–8) were isolated from plants of the Annonaceae family, which grow in tropical and subtropical regions. Three components characterize the structure of acetogenins: i) a long hydrocarbon chain, ii) one to three adjacent or non-adjacent 2,5-tetrahydrofuran (THF) moieties at the center of the molecule, and iii) an α,β-unsaturated-γ-lactone ring moiety at the end of the molecule. They exhibit potent growth inhibitory effects against human cancer cell lines by inhibiting mitochondrial complex I (reduced nicotinamide-adenine dinucleotide-ubiquinone oxidoreductase).9–11)

Our group has reported on the synthesis of various analogs12–16) of solamin,17–19) a natural mono-THF acetogenin with a simple structure containing all the components that characterize acetogenins (Fig. 1). Recently, we found that analog 1 (JCI-20679), which has a thiophene-3-carboxamide moiety in place of the γ-lactone ring unit, exhibited in vivo antitumor activity in xenograft mice, without any significant toxicity, due to its inhibitory effects on mitochondrial complex I.20–23)

Our lead compound 1 showed promising results, but its low water solubility prevented further biological evaluation. Compound 1 was found to have a high CLogP value (9.82)24) due to 2 lipophilic hydrocarbon chains, the linker part between the THF and thiophene moieties, and the tail part, linked to the THF ring. Miyoshi and colleagues reported that the length and flexibility of the linker parts in acetogenins affected their inhibitory activities against complex I.25) It was shown that a bullatacin (a bis-THF acetogenin) analog with a methyl group in the place of the n-decanyl group as the tail part, exhibited almost similar activity.26) Recently, we reported that an acetogenin thiophen-3-carboxamide analog bearing an ethylene glycol unit in the tail showed potent growth inhibitory activity against human cancer cell lines, with similar activity to that of compound 1.27) Based on this, we synthesized novel analogs with short tail parts to enhance their water solubility. Two analogs, 2a and 2b, were designed to investigate the effects of the tail length on the activity of the compound (Fig. 1). The CLogP values of 2a and 2b (6.65 and 5.06, respectively24)) were lower than that of compound 1 (i.e., 9.82); these 2 compounds are expected to be new drug candidates.28–32) In this study, we describe the synthesis and cancer cell growth inhibi-

![Fig. 1. Structure of Solamin and Its Analogs](image-url)
tory effects of the short-tail analogs, 2a and 2b.

Results and Discussion

As shown in Fig. 2, analogs 2a and 2b can be synthesized by the ring-opening reaction of a known epoxide, 3, using the corresponding Grignard reagent. We adopted this divergent route because it allows for access to many analogs with various tail lengths.

The starting epoxide 3 was stereoselectively synthesized through a 12-step process from o-mannitol following a previously reported route.27 Treatment of 3 with n-pentylmagnesium bromide in the presence of copper (I) iodide regioslectively led to the formation of the diol 4a with a 77% yield (Table 1, Entry 1). Then, hydrogenation of the alkyne moiety of 4a was carried out through Pearlman’s catalysis in MeOH, forming the desired compound 2a with a 70% yield. Finally, another analog 2b with a shorter alkyl chain was also synthesized from 3 following the same procedure as that used for 2a using ethyl magnesium bromide (Entry 2).

With analogs 2a and 2b already obtained, their growth inhibitory activities against 39 human cancer cell lines (JFCR39) were evaluated. The results are shown in Fig. 3, and the activities of lead compound 1 are also presented for comparison. In this figure, the x-axis represents the 50% growth inhibitory concentration logarithm (GL50) relative to the control values for the 39 human cancer cell lines. Thus, we found that the MG-MID values (i.e., the mean GL50 value for all tested cell lines) for both new analogs (2a and 2b) were about ten times higher (i.e., −4.54 and −4.64, respectively) than that for compound 1 (i.e., −5.56). However, their water solubility would be expected to increase because of their lower CLogP values (i.e., 6.65 and 5.06, respectively) than that of the lead compound (i.e., 9.82). From these results, it is expected that the shorter the carbon chain, the weaker is the activity because the tail portion is a simple alkyl chain without a distinct functional group, thus making it unlikely to interact with the target site. According to Lipinski’s rule, the value of CLogP should be less than 5. However, in our analog, and probably in natural acetogenin, the presence of some lipophilic moiety would be necessary for the inhibitory activity against human cancer cell lines. This result is contrary to Miyoshi and colleagues, who found that an analog with a shortened tail part showed almost similar activity to that of the natural product with a long hydrocarbon chain.26 However, direct comparisons were not possible as the methods used to evaluate activity in the two studies are different (i.e., growth inhibitory activity against human cancer cell lines and inhibitory activity against mitochondrial complex I). In contrast, analogs 2a and 2b showed significant activity against DMS-114 (a human small cell lung cancer cell line), MKN-B (a human stomach cancer cell line), and MKN-A (a human stomach cancer cell line).

Similarities between the antitumor mechanisms of the compounds were predicted by COMPARE analysis and Pearson correlation coefficients were calculated to determine similarities between various combinations of compounds (Table 2). In a previous study, we found that lead compound 1 exhibited similar fingerprints to those of known complex I inhibitors, such as deguelin, buformin, and phenformin. We demonstrated that analog 1 elicits potent antitumor activity by inhibiting mitochondrial complex I.21 The short-chain analogs, 2a and 2b (especially 2a), also showed significant correlations with complex I inhibitors, suggesting that analogs 2a and 2b inhibit cancer cell growth through mitochondrial complex I inhibition. However, they showed lower MG-MID values than compound 1. However, the tested compounds dose–response curves revealed an interesting difference between lead compound 1 and analogs 2a and 2b (Supplementary Materials, Figs. S1–6). Although compound 1 elicits significant cytotoxic effects against some cancer cell lines (DMS273, NCI-H522, HBC5, MCF7, HCC2998, HCT116, LOX-IMVI, OVCAR4, OVCAR3, St-4, MKN1, MKN74, and PC-3), the short-tail analogs, 2a and 2b, and known complex I inhibitors exhibit cytostatic effects against cancer cell lines, but not cytotoxic effects. The role of the tail alkyl chain in these analogs and in natural acetogenins is unclear. The decrease in activity may be due to lower membrane permeability brought about by the shortening of the lipophilic alkyl chain, as analogs 2a and 2b may have the same mode of action as lead compound 1, though their activities were lower.

Conclusion

This study reported the synthesis of thiophene carboxamide annonaceous acetogenin analogs with short alkyl moieties.

![Fig. 2. Retrosynthetic Analysis of the Short-Alkyl Chain Analogs, 2a, and 2b](image)

Table 1. Syntheses of 2a and 2b

| Entry | R       | Elongation Yield (%) | Hydrogenation Yield (%) |
|-------|---------|----------------------|-------------------------|
| 1     | n-C₅H₁₁ | 77 (4a)              | 70 (2a)                 |
| 2     | C₅H₁₀   | 84 (4b)              | 67 (2b)                 |
We found that shortening the alkyl chain in the tail significantly decreases their growth inhibitory activities against 39 human cancer cell lines. However, COMPARE analysis results suggested that, as with the lead compound, these analogs also inhibit mitochondrial complex I; this report is the first to show that the length of the tail alkyl chain on acetogenin analogs plays a vital role in their activity. Additional structural optimization of acetogenin analogs in terms of the alkyl chain length in the tail would improve their antitumor activity. Further structure–activity relationship studies are ongoing in our group, and the results will be presented in due course.

**Experimental**

**Chemistry** Melting points were uncorrected. Optical rotations were measured using a JASCO DIP-360 digital polarimeter or a JASCO P-1020 digital polarimeter. 1H-NMR spectra were recorded in the specified solvent using a JEOL JNM-GX-500 spectrometer (500 MHz) or a JEOL JNM-EX-270 spectrometer (270 MHz). 13C-NMR spectra were recorded in the specified solvent using a JEOL JNM-AL300 spectrometer (75 MHz), a JEOL JNM-ECS-400 spectrometer (100 MHz), or a JEOL JNM-GX-500 spectrometer (125 MHz). Chemical shifts were recorded in ppm relative to the internal solvent signal [CDCl3: 7.26 ppm (1H-NMR), 77.0 ppm (13C-NMR)] or to that of tetramethylsilane [0 ppm] as the internal standard. The following abbreviations were used: broad singlet = brs, singlet = s, doublet = d, triplet = t, quartet = q, quintet = qn, sextet = sext, septet = sept, and multiplet = m. IR absorption spectra (FT = diffuse reflectance spectroscopy) were recorded in KBr powder using a Horiba FT-210 1R spectrophotometer or as neat films on NaCl plates using a Shimadzu FTIR-8400S, and only noteworthy absorptions (in cm−1) were listed. Mass spectra were obtained using JEOL JMS-600H and JEOL JMS-700 mass spectrometers. Column chromatography was carried out using a Kanto Chemical Silica Gel 60N (spherical,
neutral, 63–210 µm) column, and flash column chromatography was carried out using a Merck Silica Gel 60 (40–63 µm) column. All air- or moisture-sensitive reactions were carried out in flame-dried glassware under an Ar or N₂ atmosphere. All solvents were dried and distilled according to standard procedures, if necessary. All organic extracts were dried over anhydrous MgSO₄, filtered, and concentrated under reduced pressure with a rotary evaporator.

**N-((R)-(R)-11-Hydroxy-11-((2R,5R)-5-((R)-1-hydroxybutylyltetrahydrofuran-2-yl)undec-9-ynyl)thiophene-3-carboxamide (4a)**

Cul (13.0 mg, 0.0683 mmol) was added to a solution of (25.0 mg, 0.0616 mmol) in THF (2.1 mL) with stirring at room temperature (r.t.). After stirring for 20 min at the same temperature, pentylmagnesium bromide (2.0 M in THF, 0.154 µL, 0.308 mmol) was added dropwise slowly to the mixture at −40°C. After warming to r.t. over 2 h with stirring, saturated NH₄Cl was added to the reaction mixture. The mixture was extracted with Et₂O prior to drying and solvent evaporation. Purification by flash column chromatography over silica gel with n-hexane/EtOAc (5:7) as eluent yielded 4a (22.8 mg, 77%) as a colorless oil. [α]D²⁴ +12.2 (c 0.92, CHCl₃); [H]-NMR (400 MHz, CDCl₃) δ: 0.81 (t, 3H, J = 6.9 Hz), 1.17–1.66 (m, 23H), 1.71–1.80 (m, 1H), 1.88–2.07 (m, 2H), 2.12 (td, 2H, J = 6.9, 1.8 Hz), 2.29 (br, 2H), 3.31–3.37 (m, 3H), 3.78 (dt, 1H, J = 7.8, 6.4 Hz), 3.97 (q, 1H, J = 6.9 Hz), 4.14 (dt, 1H, J = 6.9, 1.8 Hz), 6.09 (br, 1H), 7.26 (dd, 1H, J = 5.0, 2.8 Hz), 7.31 (dd, 1H, J = 5.0, 1.4 Hz); [13C]-NMR (100 MHz, CDCl₃) δ: 14.1, 18.6, 22.6, 25.2, 25.53, 26.8, 28.2, 28.3, 28.5, 28.8, 29.0, 29.3, 29.6, 31.8, 33.4, 39.8, 39.6, 76.0, 74.8, 82.5, 83.2, 86.5, 126.0, 126.4, 127.9, 137.7, 163.1; IR (NaCl) cm⁻¹: 3312, 1633; MS (FAB) m/z: 478 [M + H]⁺; high resolution (HRMS) (FAB) m/z: Calcd for C₂₇H₄₄NO₄S: 478.2991. Found: 478.2997 [M⁺]

**N-((R)-(R)-11-Hydroxy-11-((2R,5R)-5-((R)-1-hydroxybutylyltetrahydrofuran-2-yl)undec-9-ynyl)thiophene-3-carboxamide (4b)**

The procedure was the same as that used for preparation of 2a by use of 4b instead of 4a, giving 2b (Yield: 67%) as a white powder. mp 69.0–71.5°C; [α]D²⁴ +13.6 (c 0.68, CHCl₃); [H]-NMR (400 MHz, CDCl₃) δ: 0.86 (t, 3H, J = 7.1 Hz), 1.18–1.66 (m, 24H), 1.86–1.96 (m, 2H), 2.22 (br, 2H), 3.31–3.38 (m, 4H), 3.71–3.76 (m, 2H), 6.02 (br, 1H), 7.26 (dd, 1H, J = 5.0, 2.8 Hz), 7.30 (dd, 1H, J = 5.0, 1.4 Hz), 7.78 (dd, 1H, J = 2.8, 1.4 Hz); [13C]-NMR (100 MHz, CDCl₃) δ: 14.1, 18.8, 25.5, 26.9, 28.71, 28.72, 29.2, 29.4 (2C), 29.5, 29.6, 29.7, 33.4, 35.5, 39.8, 73.7, 74.0, 82.6, 82.7, 125.9, 126.4, 127.9, 137.7, 163.1; IR (NaCl) cm⁻¹: 3327, 1631; MS (FAB) m/z: 440 [M + H]⁺; HRMS (FAB) m/z: Calcd for C₂₆H₄₂NO₄S: 440.2835. Found: 440.2837 [M⁺]

**Biology**

**Determination of Cell Growth Inhibition Profiles (Fingerprint)**

This experiment was carried out at the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research. The screening panel consisted of the following 39 human cancer cell lines (JFCR39): breast cancer HBC-4, BSY-1, HBC-5, MCF-7, and MDA-MB-231; brain cancer U251, SF-268, SF-295, SF-539, SNB-75, and SNB-78; colon cancer HCC2998, KM-12, HT-29, HCT-15, and HCT-116; lung cancer NCI-H232, NCI-H522, NCI-H460, A549, DMS273, and DMS114; melanoma LOX-IMVI; ovarian cancer OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, and SK-OV-3; renal cancer RXF-631 L and ACHN; stomach cancer St-4, MKN1, MKN-B, MKN-A, MKN45, and MKN74; and prostate cancer DU-145 and PC-3. Inhibition of cell growth was assessed by measuring changes in total cellular protein levels following 48 h treatment with a given test compound, using the sulforhodamine B colorimetric assay. The molar concentration of a test compound, using the sulforhodamine B colorimetric assay. The molar concentration of a test compound, using the sulforhodamine B colorimetric assay.

**COMPARE Analysis of the Analog Antitumor Fingerprints across the JFCR39 Panel**

COMPARE analysis was performed by calculating the Pearson correlation coefficient (r) between the GI₅₀ mean graphs of two compounds (depicted with X and Y) using the following formula: \( r = \frac{(\bar{X}_i - \bar{Y}_i)(\bar{X}_j - \bar{Y}_j)}{\sqrt{\sum(X_i - \bar{X})(X_j - \bar{Y})\sqrt{\sum(Y_i - \bar{Y})(Y_j - \bar{Y})}} \), where \( \alpha \) and \( \beta \) are the Log GI₅₀ values of X and Y, respectively, for each cell line, and \( \alpha_m \) and \( \beta_m \) are used to determine the degree of the mean values of \( \alpha \) and \( \beta \), respectively (n = 39). The Pearson correlation coefficients are used to determine the degree of similarity, with a larger coefficient indicating a higher similarity between X and Y.

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