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Synthesis and biological evaluation of quinic acid derivatives as anti-inflammatory agents

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ABSTRACT

Quinic acid (QA) esters found in hot water extracts of *Uncaria tomentosa* (a.k.a. cat's claw) exert anti-inflammatory activity through mechanisms involving inhibition of the pro-inflammatory transcription factor nuclear factor kappa B (NF-κB). Herein, we describe the synthesis and biological testing of novel QA derivatives. Inhibition of NF-κB was assessed using A549 (Type II alveolar epithelial-like) cells that stably express a secreted alkaline phosphatase (SEAP) reporter driven by an NF-κB response element. A549-NF-κB cells were stimulated with TNF-α (10 ng/mL) in the presence or absence of QA derivative for 18 hours followed by measurement of SEAP activity. Amide substitution at the carboxylic acid position yielded potent inhibitors of NF-κB. A variety of modifications to the amide substitution were tolerated with the N-propyl amide derivative being the most potent. Further examination of the SAR demonstrated that acetylation of the hydroxyl groups reduced NF-κB inhibitory activity. QA amide derivatives lacked anti-oxidant activity and were found to be neither anti-proliferative nor cytotoxic at concentrations up to 100 μM. In conclusion, we have discovered a novel series of non-toxic QA amides that potently inhibit NF-κB, despite their lack of anti-oxidant activity. Mechanistic studies and pre-clinical efficacy studies in various inflammatory animal models are on-going.

The woody plant *Uncaria* is widely dispersed in tropical regions, including Southeast Asia, Africa, and South America. *Uncaria* genus plants have provided numerous structurally diverse medicinal natural products (e.g., alkaloids, terpenes, quinovic acid glycosides, flavonoids, and coumarins).[1] The species with the most compounds identified (≈50; 15 of which are reported as novel) is the Peruvian *Uncaria tomentosa* commonly known as Uno de Gato or cat's claw. Most commercial cat's claw preparations are based on oxindole alkaloid content as alkaloids represent the most abundant class of compounds found in *Uncaria*.[2]

Hot water cat's claw extracts (e.g., C-Med 100) have very low alkaloid content (<0.5%) and yet retain significant immune enhancing activity. For example, the extract significantly accelerated recovery from doxorubicin-induced leukopenia in rats.[3] Moreover, elevated leukocyte numbers were noted in humans, mice, and rats receiving repeat doses of the extract.[4,5] Enhanced leukocyte counts correlated with prolonged lymphocyte survival, thus providing a potential mechanistic basis for the immune enhancing properties of the extract.[5] Prolonged cell survival has been linked to enhanced DNA repair.[4,6,7] Recently, quinic acid (QA) esters have been identified as biologically active components in the extract.[8]

The mechanism by which QA and its esters exert their anti-inflammatory activity is unclear, but appears to be related to inhibition of the pro-inflammatory transcription factor nuclear factor kappa B (NF-κB). For example, QA inhibited phorbol myristate acetate (PMA) and ionomycin-induced NF-κB activation in Jurkat T cells at concentrations that neither induced cell death nor inhibited proliferation.[9] Moreover, QA prevented degradation of the NF-κB inhibitor protein IκB-α without affecting levels of the phosphorylated IκB-α protein.[9] Base hydrolysis of the extract dramatically reduces biological activity. For example, the lactone ester of QA (QAL) inhibits proliferation of mitogen-stimulated mouse lymphocytes, whereas QA does not affect proliferation.[9] Further, base hydrolysis of the extract dramatically reduces its anti-proliferative effect against HL-60 and human mononuclear cells.[8] Together, these data suggest the ester derivatives of QA found in the extract comprise a significant fraction of the biological activity.

QA is utilized by gastrointestinal bacteria as a carbon source for aromatic acid synthesis. Consequently, only a small fraction of QA is absorbed after oral administration of either QA or chlorogenic acid (CGA), which is a caffeic acid (CA)-containing ester of QA.[10] Our group has focused on the discovery and development of stable QA derivatives. Toward this end, we have identified water soluble amide analogs of QA (1,3,4,5-tetrahydroxy-1-cyclohexanecarboxylic acid) which possess potent anti-inflammatory activity.

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Synthesis of the QA analogs was carried out using chemistry previously described.[11–13] The synthesis of the lactone **2** was carried out using PTSA in refluxing benzene and DMF according to the procedure of Neelu Kaila et al. with minor revision.[13,14] The lactone was then allowed to react with *N*-propyl amine with acetic acid at 85 °C and the resulting amide **3** was purified using flash chromatography.[11,15] The amides **4–11**, and **13** were formed by using different amines with lactone **2** in a manner similar to what was described for compound **3**. QA, **1**, was treated with acetic anhydride /pyridine to give compound **12**.[13] Each compound was characterized with Mass Spectroscopy, NMR, and elemental analysis.

The general synthesis of QA analogs is shown in Scheme 1.

As previously described, human alveolar Type II-like epithelial cells (A549 cells) stably transfected with the secreted alkaline phosphatase (SEAP) gene containing the response element for NF-κB, were used to screen for anti-inflammatory activity.[16] Briefly, cells (6×10^4 cells/well) were plated overnight followed by treatment with 10 ng/mL human recombinant TNF-α (Biosource, Camarillo, CA) and QA analogs (1 μM). SEAP activity was measured 18 h later in supernatant samples (50 μL) using the Great EscAPe™ chemiluminescence kit (Clontech, Mountain View, CA) and a microplate luminometer (Packard αHT microplate reader). Cells were lysed for protein quantification (Pierce BCA Protein Assay Kit, Microplate Procedure) and SEAP activity was normalized to the total protein content. Inhibitory potency (IC_{50}) was determined from dose–response curves ($n = 3$ separate experiments).

The results of QA derivative anti-inflammatory high throughput screening are presented in Figure 1. The *N*-propyl amide derivative (**3**) was found to have the greatest extent of NF-κB inhibition following derivative screening. We initially focused on substitution at the carboxylic acid position, as previous work has shown that QA ester derivatives have greater biological activity compared to QA.[8,9] The carboxylic acid substituent appears critical to the interaction of QA derivatives with E- and P-selectin, key mediators of leukocyte endothelial cell interactions during inflammation.[13] Interestingly, our most potent QA analog **3** demonstrates anti-inflammatory activity despite the fact that it lacks a carboxylic acid functional group. Further examination of the SAR demonstrated that acetylation of the hydroxyl groups, which yields compound **12**, leads to reduced NF-κB inhibitory activity. A variety of modifications to the amide substitution of **3** were tolerated, for example, **9**, **10** and **13**, a *N*-isopropyl amide, but no substitution was better than the *N*-propyl amide. The NF-κB inhibitory potency (IC_{50}) of our most active analog **3** was determined as 2.83 ± 1.76 μM.

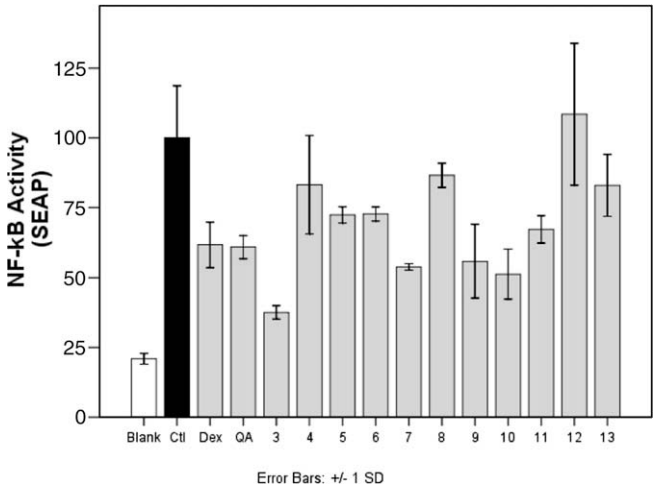
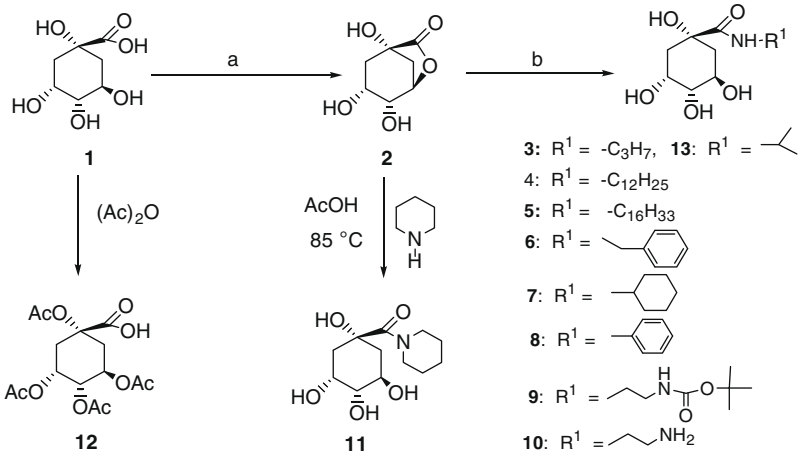


Figure 1. NF-κB inhibition by QA and analogs using A549 cells stably transfected with a secreted alkaline phosphatase (SEAP) reporter. NF-κB activity was measured 18 hours after addition of TNF-α (10 ng/mL) and either QA or synthesized derivatives (**3–13**; 1 μM). Dexamethasone (Dex; 1 μM) was used as a positive control. Data are presented as percent (%) inhibition relative to control (ctl; TNF-α alone) and represent mean % inhibition ± standard deviation ($n = 3$). Blank: media only.

Next, we determined the anti-oxidant potential of QA derivatives using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method.[17] The dietary polyphenol CGA, an ester of QA and CA, possesses potent anti-oxidant activity, which is attributed to the presence of CA. We thus compared the anti-oxidant potential of CGA, CA, QA and **3** to determine if the anti-inflammatory activity of QA and **3** was attributable to anti-oxidant activity. Ascorbic acid 6-palmitate was included as a structurally-distinct molecule with described anti-oxidant activity. As expected, Figure 2 demonstrates the anti-oxidant activity of CGA, CA, and ascorbic acid. However, QA and **3** have no such anti-oxidant activity. The fact that QA does not possess anti-oxidant activity is consistent with a previous report.[18] Therefore, the NF-κB inhibitory activity of QA derivatives is attributed to mechanisms unrelated to anti-oxidant activity.

QA esters potently inhibit proliferation of mitogen-stimulated mouse lymphocytes without increasing cytotoxicity.[9] We thus determined the anti-proliferative potential of QA and **3** against A549 cells using the MTS assay.[19] Neither QA nor **3** exhibited cytotoxic activity toward A549 cells at concentrations up to 100 μM.



Scheme 1. Reagents and conditions: (a) PTSA, DMF, C_6H_6, Dean-Stark, reflux, 78%; (b) R^1–NH_2, AcOH, oil bath 85 °C.

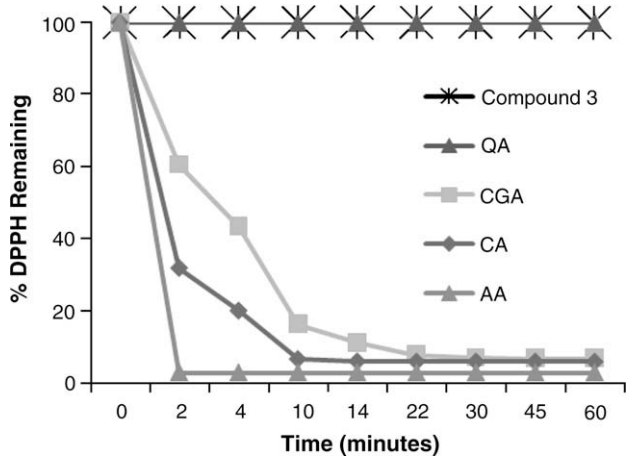


Figure 2. Reaction kinetics of DPPH for determining antioxidant activity. Caffeic acid (CA), chlorogenic acid (CGA), ascorbic acid (AA), QA and Compound **3** were prepared in 50% acetone. The conventional colorimetric DPPH·scavenging capacity was determined by UV absorption measured at 515 nm. CA, CGA and AA showed strong anti-oxidant activity, while QA and **3** had no anti-oxidant activity.

Thus, it appears that inhibition of NF-κB by QA and **3** is related not to anti-proliferative or cytotoxic activity, but rather to a yet to be determined mechanism.

In conclusion, we have synthesized novel QA analogs that potently inhibit NF-κB activity in TNF-α-stimulated human alveolar Type II-like epithelial cells (A549). We have demonstrated that the QA analogs presented in this work do not exert their activity via anti-oxidant or cytotoxic mechanisms. Mechanistic studies and pre-clinical efficacy studies of our lead molecule **3** in various inflammatory animal models are on-going.

Acknowledgements

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14. Lactone. 1,3,4-Trihydroxy-6-oxa-bicyclo[3.2.1]octan-7-one **(2)**. In a 200 mL round-bottom flask fitted with a stirring bar, reflux condenser, Dean-Stark trap, and argon inlet, 5.0 g of QA (26.0 mmol) was placed and 10 mL of dry DMF was added via syringe and the slurry stirred at room temperature. Next, benzene 60 mL and PTSA 0.5 g were added, and the slurry was heated to reflux for 26 h. TLC was used to confirm reaction completion. A 1:1 mixture of EtOAc and heptane (100 mL) was added to the cooled reaction mixture. The mixture was stirred for 1 h at room temperature and filtered. The collected solid was again stirred with a 1:1 mixture of EtOAc and heptane (100 mL) for 1 h at room temperature and filtered. Tituration was repeated once more with a 1:1 mixture of EtOAc and heptane (100 mL) and the precipitate was purified by flash column chromatography with EtOAc to give 3.5 g of Lactone **2** (78% yield).
15. Amide. **3**. In a 50 mL round-bottom flask fitted with a stirring bar, reflux condenser, 0.2 g lactone **2** (1.1 mmol), and propylamine (0.8 mL, 0.6 g, 10.1 mmol) were combined, then glacial acetic acid (0.2 mL, 0.20 g, 3.4 mmol) was added. The solution was warmed to 85 °C in an oil bath for 30 min, at which time TLC (EtOAc) indicated complete consumption of the starting lactone. The reaction mixture was purified by flash column with $CHCl_3$:MeOH:NH_4OH (100:10:1). The amide **3** was isolated as a white solid 206.0 mg (mp: 132–133 °C, 80% yield).
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17. DPPH. DPPH· and all standard antioxidant compounds were dissolved in 50% acetone. The DPPH· stock solution at a concentration of 0.625 mM was prepared monthly and kept at 4 °C in dark. The 0.208 mM fresh DPPH· working solution was made daily by further diluting the stock solution in 50% acetone for each test. Stock solutions of CA, CGA, AA and compound **3** were prepared in 50% acetone at concentrations of 10 mM, respectively, and stored at 4 °C. A series of working solutions were made by appropriate dilutions of the above standard phenolic acid stock solutions with 50% acetone.Conventional colorimetric analysis. The conventional colorimetric DPPH·scavenging capacity assay was performed according to a previously described laboratory protocol. Briefly, an aliquot of 500 μL of different concentrations of sample in 50% acetone was added to 500 μL of 0.208 mM DPPH· solution. The initial concentration was 0.104 mM for DPPH· in all reaction mixtures. Each mixture was vortexed for a few seconds and test immediately. The absorbance (*A*) of each reaction mixture at 515 nm was measured against a blank of 50% acetone using a UV–isible spectrometer. The level of DPPH· remaining for each reaction time was calculated as:

$$\%\text{DPPH}\cdot \text{ remaining} = (A_{\text{sample-t}}/A_{\text{control}}) \times 100.$$

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19. MTS. Cytotoxicity Studies in Cultured human lung adenocarcinoma A549SN cells. A549SN cells were suspended in culture medium at a density of 10×10^4 cells/mL. Then 1×10^4 cells in 200 μL were plated into 96-well flat-bottom plates. Following incubation for 24 h at 37 °C, drugs, vehicles and controls consisting only of medium and cells were dispensed in 200 μL volumes in duplicate into the appropriate wells, and incubated for 18 h. QA derivatives were tested at concentrations ranging from 0.01 to 100 μM.Cell viability was assessed by the MTS-CellTiter 96® aqueous non-radioactive cell proliferation assay (Promega, Madison, WI, USA) according to the manufacturer's protocol after 2 h of culture. The number of living cells in the culture is directly proportional to the absorbance at 490 nm by a formazan product bioreduced from MTS by living cells. Absorbance was measured using a DTX 880 multimode detector (Beckman Coulter, Fullerton, CA). The absorbance of media-only well was subtracted from reading of control or treated wells.