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Synthesis, in vitro, and in vivo evaluation of novel functionalized quaternary ammonium curcuminoids as potential anti-cancer agents

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ABSTRACT

Novel functionalized quaternary ammonium curcuminoids have been synthesized from piperazinyl curcuminoids and Baylis–Hillman reaction derived allyl bromides. These molecules are found to be highly water soluble with increased cytotoxicity compared to native curcumin against three cancer cell lines MIAPaCa-2, MDA-MB-231, and 4T1. Preliminary in vivo toxicity evaluation of a representative curcuminoid **5a** in healthy mice indicates that this molecule is well tolerated based on normal body weight gains compared to control group. Furthermore, the efficacy of **5a** has been tested in a pancreatic cancer xenograft model of MIAPaCa-2 and has been found to exhibit good tumor growth inhibition as a single agent and also in combination with clinical pancreatic cancer drug gemcitabine.

Curcumin is a yellow organic compound isolated from rhizome of the herbaceous perennial plant *Curcuma longa* L. Curcumin has been shown to possess a multitude of pharmacological properties including cancer chemoprevention and anticancer properties.[1–16] Although curcumin is readily available, inexpensive and non-toxic, it is besieged with numerous problems for clinical use. Some of the problems include insolubility in water, poor absorption, fast metabolism, and also rapid glucuronidic elimination.[17] To circumvent these problems, there have been several attempts to improve its water solubility and potency.[1–3,9,10,12,14] However, many of these studies were met with limited clinical success and development of novel curcuminoids with high water solubility and good in vivo activity is important.

The Baylis–Hillman (BH) reaction has gained a lot of attention as an important C–C bond forming reaction because of its simple experimental conditions, high atom efficiency, and easy assembly of densely functionalized allyl alcohols and amines.[18–29] The allyl alcohol product can be further functionalized in numerous ways to produce important synthons for organic synthesis. Allyl bromides derived from BH alcohols can be readily isomerized with various nucleophiles in S_N^2 or $S_N^{2'}$ fashion (Scheme 1). Recently, BH reaction products have also received attention as pharmacologically important structural motifs for various types of diseases.[18,22,26]

Our interest in developing novel small molecule therapeutics has prompted us to explore molecules derived from BH allyl bromides as anti-cancer agents.[30–32] BH derived allyl bromides are highly reactive electrophiles with the ability to react with various types of N, S, O, C nucleophiles.[18–29] We envisioned that these functionalized allyl bromides would interact with intracellular N and/or S molecules such as DNA, glutathione, or cysteine causing cellular death.[33–36] As representative examples, we synthesized BH allyl alcohols and bromides **1a**–**1e** by the reaction of formaldehyde and benzaldehyde with corresponding acrylates or acrylamide. Initially, we evaluated **1a**, **1b** and their parent alcohols for cytotoxicity against triple negative breast cancer cell line MDA-MB-231. It was found that BH alcohols did not show any significant activity up to 50 μM but their bromides **1a** and **1b** exhibited cytotoxicity at lower concentrations of ~20–50 μM. However, these bromides do not have the required chemical stability and water solubility to develop them as anti-cancer agents.

Owing to the above mentioned problems associated with curcumin and BH bromides, we hypothesized that hybridization of these two structural units on a single molecular framework would lead to novel molecules with desirable pharmacological and pharmaceutical properties. We also envisioned that quaternary ammonium curcuminoids derived from BH allyl bromides would retain some of their electrophilic character and would have the required

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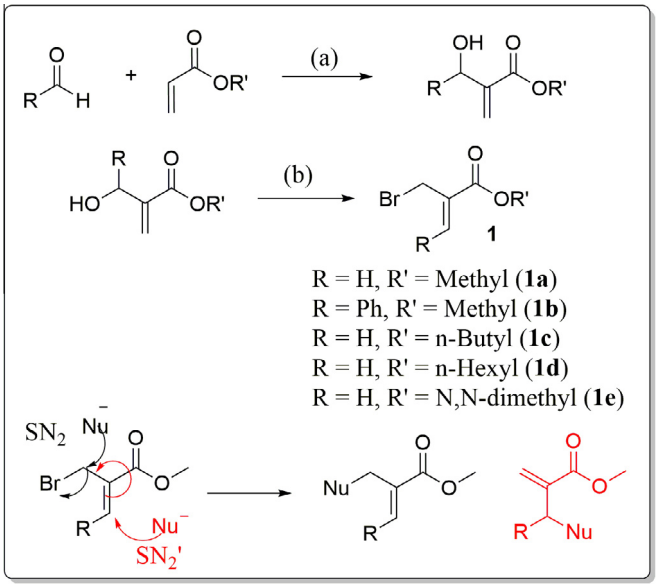


Scheme 1. Baylis–Hillman reaction and nucleophilic rearrangements of Baylis–Hillman Bromides: (a) DABCO, rt, 7 days; (b) HBr, H_2SO_4, 0 °C, 15 min.

chemical stability and water solubility to develop them as exploratory anti-cancer agents (Fig. 1). Herein, we report the synthesis of novel Baylis–Hillman (BH) reaction based quaternary ammonium curcuminoids, their in vitro cytotoxicity in breast and pancreatic cancer cell lines, preliminary in vivo systemic toxicity in healthy mice, and in vivo tumor growth inhibition properties of a lead derivative in a pancreatic tumor xenograft model.

The required piperazinyl benzaldehydes **3** and **4** were prepared from mono-*N*-substituted piperazine via a base mediated *ipso* substitution of 4-fluorobenzaldehyde.[37] Aldehydes **3** and **4** were condensed with 2,4-pentanedione to synthesize piperazinyl curcuminoids **5** and **6** using established literature protocols (Scheme 2).[38–41] Compounds **5** and **6** were evaluated for their in vitro cytotoxic properties utilizing the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay.[42] Human pancreatic cancer (MIAPaCa-2), human triple negative breast cancer (MDA-MB-231), and highly metastatic murine breast cancer (4T1) cell lines were chosen for

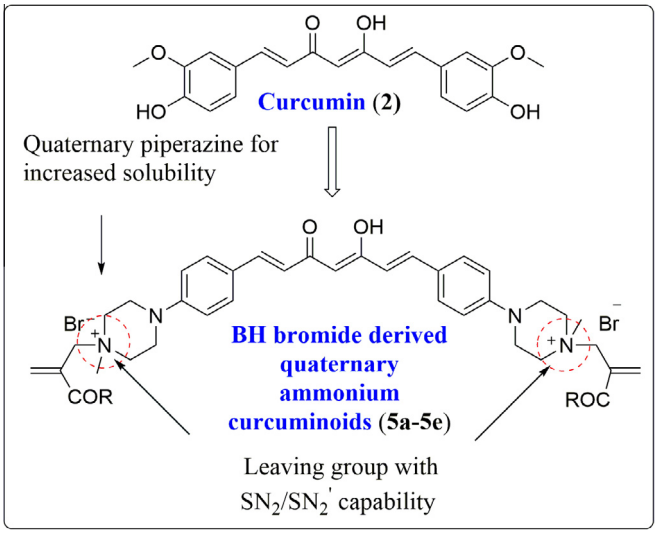
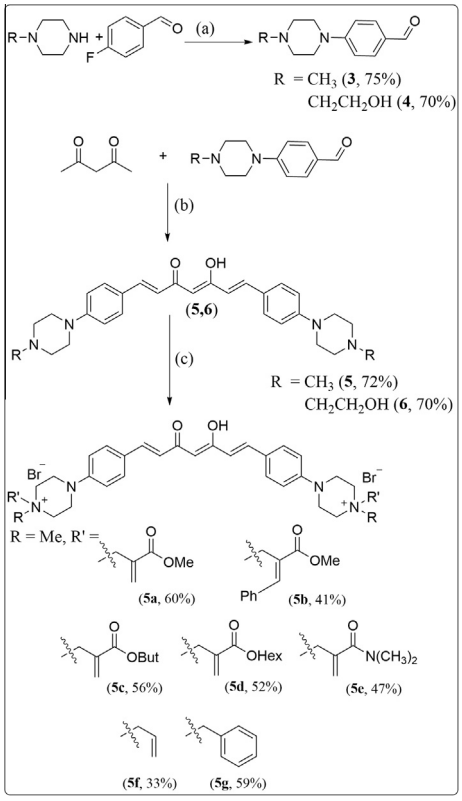


Figure 1. Quaternary piperazinyl curcuminoids.



Scheme 2. Baylis–Hillman bromide derived quaternary ammonium curcuminoids: (a) K_2CO_3, DMSO, 100 °C, 12 h; (b) (i) B_2O_3, DMF, 170 °C, 10 min, (ii) tributylborate, piperidine, 120 °C, 1 h; (c) allylic bromide, DMF, rt, 1 h.

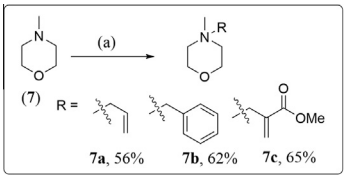
cytotoxic evaluation as these cancers have limited treatment options and high patient mortality. Curcuminoids **5** and **6** did not show significant cytotoxicity even at 100 μM concentration and hence were considered to be non-toxic.

Utilizing the piperazinyl curcuminoid template **5**, we then synthesized their corresponding quaternary ammonium salts **5a**–**5e**.[43] Reaction of **5** with BH bromides **1a**–**1e** in DMF provided curcuminoid salts **5a**–**5e** in moderate to good yields (Scheme 2). These novel derivatives have been found to exhibit excellent water solubility in the range of ~1 mg/mL to >100 mg/mL. Gratifyingly, **5a**–**5e** exhibited increased cytotoxicity compared to the native curcumin **2** in all three cell lines (Table 1). Quaternary ammonium salt **5a** derived from formaldehyde BH bromide **1a** has good activity of 3.6 μM against MDA-MB-231 cell line when compared to parent curcuminoid **5** (>100 μM) and also native curcumin **2** (~15 μM). Similarly, **5a** exhibited cytotoxicity against MIAPaCa-2 and 4T1 in

Table 1
IC_{50} values of curcuminoids in MDA-MB-231, MIAPaCa-2 and 4T1 cell lines (in μM)*

Sl. No.	MDA-MB-231	MIAPaCa-2	4T1
2	~15	~20	~20
5	>100	>100	>100
6	>100	>100	>100
5a	3.6 ± 0.7	2.7 ± 0.5	4.1 ± 1.1
5b	6.2 ± 1.4	4.1 ± 0.3	5.0 ± 1.6
5c	3.7 ± 0.8	4.4 ± 0.4	4.1 ± 1.6
5d	4.2 ± 0.5	4.2 ± 0.7	3.4 ± 0.8
5e	15.1 ± 1.8	14.2 ± 0.4	17.2 ± 2.8
5f	>100	>100	>100
5g	>100	>100	>100

* Values are reported as an average of a minimum of three individual experiments ±SEM.



Scheme 3. Synthesis of allylic quaternary ammonium salts of *N*-methylmorpholine: (a) allylic bromide, Et_2O, rt, 1 h.

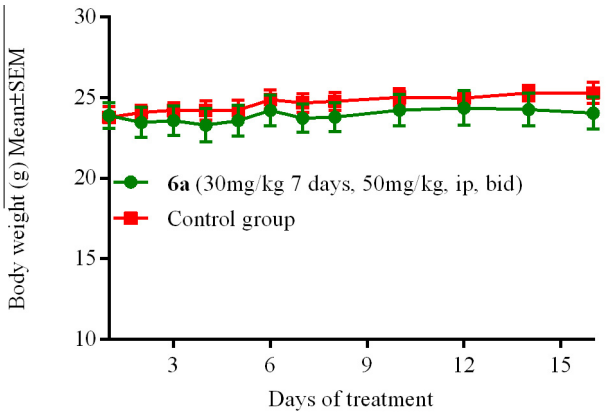


Figure 2. In vivo toxicity study of **5a** in CD-1 mice ($n = 6$).

low micro molar concentrations (Table 1). Compound **5b** derived from benzaldehyde BH bromide **1b** also exhibited a similar type of cytotoxicity against these three cell lines. To further understand the structure activity relationship (SAR), we modified the methyl ester to butyl **5c** and hexyl **5d** esters and also replaced the ester moiety with *N*,*N*-dimethyl amide **5e**. However, these modifications did not result in enhanced activity compared to the methyl ester.

We then synthesized the quaternary ammonium curcuminoids **5f** and **5g** from simple allyl and benzyl bromides to understand the role and importance of BH derived allyl bromides. These molecules **5f** and **5g** have good water solubility (~15 mg/mL) but did not exhibit in vitro cytotoxicity against three cancer cell lines even at higher concentrations of 100 μM. Quaternary ammonium curcuminoid salt derived from piperazinyl ethanol curcuminoid **6** and BH bromide **1a** was found to be highly deliquescent and did not show any enhanced activity compared to **5a**.

We also investigated the role of the curcuminoid template in relation to the cytotoxic properties. In this regard, we have synthesized quaternary ammonium salts **7a**–**7c** derived from *N*-methylmorpholine **7** in place of curcuminoid unit (Scheme 3). Although these salts **7a**–**7c** were found to be highly water soluble (>100 mg/mL), they did not exhibit any appreciable cytotoxic properties (IC_{50} > 100 μM, 4T1 cells). These results clearly illustrate the importance of allylic rearrangement with S_N^2, $S_N^{2'}$, or 1,4-addition mechanism in combination with curcuminoid template in providing the pharmacological activity.

To explore the translational potential of these quaternary ammonium curcuminoid salts as anticancer agents, we carried out systemic toxicity and in vivo tumor growth inhibition studies in mice.[44–47] As a representative example, compound **5a** was chosen for all the in vivo studies due to its higher water solubility (>100 mg/mL) and slightly better potency than the other derivatives. For systemic toxicity studies, we utilized healthy CD-1 mice. Group 1 and 2 mice ($n = 6$) were administered twice daily (bid), intraperitoneally (ip) with compound **5a** (30 mg/kg) and vehicle (saline), respectively. The dosage for group 1 was increased to 50 mg/kg after 7 days. In both cases, body weight gains were found to be similar in treated group and control group (Fig. 2). All the

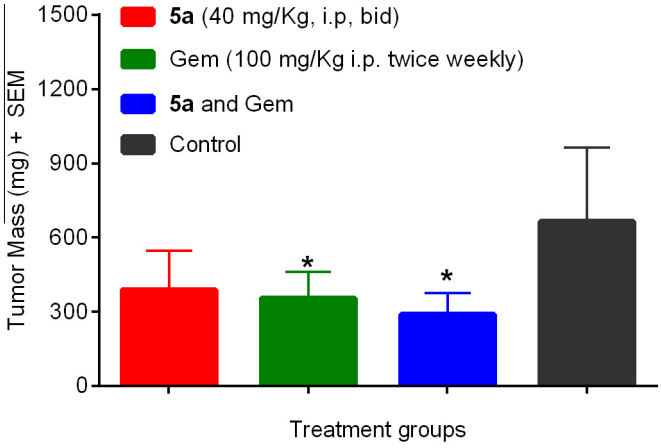


Figure 3. In vivo tumor growth inhibition of **5a** based on isolated tumor mass in MIAPaCa-2 xenograft model ($n = 6$, $^*P \leq 0.05$).

treated animals survived and **5a** was found to be well tolerated based on observed normal body weights compared to the control group. None of the animals exhibited any signs of stress during the treatment.

We next carried out an in vivo tumor growth inhibition study using flank based tumor xenografts in female athymic nude mice. For this study, we utilized human pancreatic cancer cell line MIAPaCa-2. We chose this tumor model due to the lack of effective therapeutics for pancreatic cancer treatment. MIAPaCa-2 cells (5×10^6) in 1:1 PBS–matrigel were inoculated onto the right flank of mice. When the tumor volume reached ~250 mm^3, mice were assigned into four groups ($n = 6$). Mice in group 1 were administered with compound **5a** (40 mg/Kg, ip, bid) whereas group 2 mice were treated with gemcitabine (100 mg/Kg, ip, twice weekly). Group 3 mice were given a combination of **5a** (40 mg/Kg, ip, bid) and gemcitabine (100 mg/Kg, ip, twice weekly) and group 4 was designated as a control group (saline administration). At the end of the study, mice were euthanized and tumors were resected and weighed. Based on these studies, 42%, 47%, and 57% tumor growth inhibition was observed in groups 1, 2 and 3, respectively, compared to the control group (Fig. 3).

In conclusion, we have developed several BH bromide based highly water soluble quaternary ammonium curcuminoids as potential anti-cancer agents. Many of these novel derivatives have been found to exhibit several fold higher activity than native curcumin against three solid tumor cell lines. We have evaluated the systemic toxicity of one of the derivatives (**5a**) in healthy CD-1 mice. Compound **5a** has been found to be well tolerated based on normal body weight changes compared to control group. Furthermore, we have carried out tumor growth inhibition studies with **5a** in a pancreatic cancer xenograft model. **5a** has good tumor growth inhibition as a single agent and also in combination with gemcitabine. Future studies include determining the mechanism of action, pharmacokinetics, pharmacodynamics, metabolic stability and off-target effects of these novel derivatives.

Acknowledgments

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38. *Synthesis of (1E,4Z,6E)-5-hydroxy-1,7-bis(4-(4-methylpiperazin-1-yl)phenyl) hepta-1,4,6-trien-3-one (Compound **5**):* To a solution of acetylacetone (10 mmol) in DMF (5 mL) was added boric anhydride (10 mmol) and the reaction mixture was heated at 170 °C for 0.5 h. The reaction was then cooled to 120 °C and 4-(4-methylpiperazin-1-yl)benzaldehyde (20 mmol) was added. A 1:1 mixture of piperidine (10 mmol) in DMF was then added drop wise and the reaction was stirred for 2 h. Upon the completion of the reaction (TLC), cold water (20 mL) was added and the solid was filtered and washed with water (3 × 20 mL). **5** was obtained as the pure compound upon recrystallization in ethyl acetate–methanol mixture. Similarly compound **6** was also synthesized. ^{1}H NMR (500 MHz, $CDCl_3$): δ ppm 7.59 (d, J = 15 Hz, 2H), 7.46 (d, J = 10 Hz, 4H), 6.88 (d, J = 10 Hz, 4H), 6.46 (d, J = 15 Hz, 2H), 5.75 (s, 1H), 3.32 (t, J = 5 Hz, 8H), 2.58 (t, J = 5 Hz, 8H), 2.36 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ ppm 183.27, 152.12, 140.19, 129.61, 125.82, 120.68, 115.03, 101.16, 54.73, 47.71, 45.97. HRMS (ESI) m/z: calcd for $C_{29}H_{36}N_4O_2$ $[M]^+$: 472.2838, found $[M+H]^+$: 473.2763.
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42. *Cell culture conditions and cytotoxicity:* Pancreatic cancer cells MIAPaCa-2 were purchased from ATCC. Culture medium includes DMEM supplemented with 10% FBS, 2.5% horse serum, and 1% penicillin–streptomycin. Triple negative breast cancer cells MDA-MB-231 were purchased from ATCC and were cultured in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin. Stage IV murine breast cancer cells 4T1 were purchased from ATCC. Culture medium consists of RPMI-1640 supplemented with 10% FBS and 1% penicillin–streptomycin. Standard MTT assay was used to determine IC_{50} values (average ± SEM, n = 3) in MDA-MB-231, MIAPaCa-2 and 4T1 cell lines.
43. *Synthesis of (1E,4Z,6E)-5-hydroxy-1,7-bis(4-(4-methylpiperazin-1-yl)phenyl) hepta-1,4,6-trien-3-one dibromide salts (Compound **5a**):* To a solution of **5** (10 mmol) in DMF (10 mL) was added methyl 2-(bromomethyl)acrylate bromide (30 mmol) and the reaction mixture was stirred at rt for 3 h. Upon the completion of the reaction, acetone (20 mL) was added and the resulting dibromide salt, **5a** was filtered and washed repeatedly with acetone to obtain the pure product. ^{1}H NMR (500 MHz, CDCl3-DMSO-d_6) δ ppm 8.33 (s, 1H), 7.94 (s, 1H), 7.63 (d, J = 8.79 Hz, 4H), 7.55 (d, J = 15.62 Hz, 2H), 7.06 (d, J = 8.79 Hz, 4H), 6.85 (s, 2H), 6.75 (d, J = 15.62 Hz, 2H), 6.54 (s, 2H), 6.06 (s, 1H), 4.47 (s, 4H), 3.85 (d, J = 13.67 Hz, 7H), 3.78 (s, 6H), 3.49–3.63 (m, 14H), 3.10 (s, 6H); ^{13}C NMR (125 MHz, DMSO-d_6) δ ppm 183.54, 166.08, 151.08, 141.66, 140.51, 130.28, 128.81, 126.12, 121.32, 115.39, 62.63, 59.19, 53.16, 45.85, 41.39; HRMS (ESI) m/z: calcd for $C_{39}H_{50}Br_2N_4O_6$ $[M]^+$: 830.2077, found $[M-2Br]^{2+}$: 335.1487.
44. *In vivo studies ethical consideration:* Animal studies conducted at the University of Minnesota Duluth were in compliance with the U.S. National Institutes of Health Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (IACUC protocols 1311-31063A for systemic toxicity study and 1312-31124A for MIAPaCa-2 xenograft study).
45. *Systemic toxicity study:* Compound **5a** was evaluated for systemic toxicity in healthy CD-1 (ICR) mice. **5a** was dissolved in saline and administered to mice via ip injection (30 mg/kg, bid and 50 mg/kg, bid) (n = 6). Control animals received saline administration. All treatments were carried out for 16 days. Body weights were recorded every 2–3 days.
46. *Tumor growth inhibition study of compound **5a** in MIAPaCa-2 tumor xenograft model* (Fig. 3): At the end of day 20, mice were euthanized with CO_2 and tumor masses were isolated and weighed. The tumor growth inhibition was determined using the formula % inhibition = $[(C-T)/C) * 100]$ where C is average tumor weight of the control group and T is the average tumor weight of the test group. The treatment for two mice in group-3 was suspended from day-13 onwards due to the body weight loss. One mouse each from group 1 and group 3 were euthanized due to weight loss and lethargy.
47. *Statistical analysis:* Mann–Whitney test was used to compare the treated and untreated groups in in vivo studies. A P-value of $\leqslant$0.05 was considered significant. GraphPad Prism version 6.0 was used for statistical analysis and generation of graphs for in vivo studies.