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Synthesis, anti-proliferative activity, SAR study, and preliminary in vivo toxicity study of substituted *N,N'*-bis(arylmethyl)benzimidazolium salts against a panel of non-small cell lung cancer cell lines

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Abstract

A series of *N,N'*-bis(arylmethyl)benzimidazolium salts have been synthesized and evaluated for their in vitro anti-cancer activity against select non-small cell lung cancer cell lines to create a structure activity relationship profile. The results indicate that hydrophobic substituents on the salts increase the overall anti-proliferative activity. Our data confirms that naphthylmethyl substituents at the nitrogen atoms ($N^1(N^3)$) and highly lipophilic substituents at the carbon atoms (C^2 and $C^5(C^6)$) can generate benzimidazolium salts with anti-proliferative activity that is comparable to that of cisplatin. The National Cancer Institute's Developmental Therapeutics Program tested **1**, **3–5**, **10**, **11**, **13–18**, **20–25**, and **28–30** in their 60 human tumor cell line screen. Results were supportive of data observed in our lab. Compounds with hydrophobic substituents have higher anti-cancer activity than compounds with hydrophilic substituents.

GRAPHICAL ABSTRACT

Synthesis, anti-proliferative activity, SAR study, and preliminary in vivo toxicity study of substituted *N,N'*-bis(arylmethyl)benzimidazolium salts against a panel of non-small cell lung cancer cell lines

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Supporting Information

The ¹H and ¹³C NMR for **2**, **4**, **13**, and **20**; the thermal ellipsoid plot of **8-Cl/Br**; solubility information for **1–25** and **28–31**; and results from the NCI-60 human tumor cell line screen five-dose assays for compounds **1**, **4**, **11**, **14**, **15**, **18**, **23**, **25**, and **29**. Crystallographic information files (CIF) for **11-Cl**, **12**, **13**, **15**, **17**, **18**, **19**, **20**, **21**, **24**, **30** (CCDC #s 1481965 – 1481970 and 1481972 – 1481976) can be found on the Cambridge Crystallographic Data Center website. This material is available free of charge via the Internet at <http://www.ccdc.cam.ac.uk/pages/Home.aspx>.

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Keywords

Lung cancer; Benzimidazolium Salt; Anti-tumor; Anti-cancer; Anti-proliferative

1. Introduction

Lung cancer is expected to kill over 150,000 people in the United States this year alone, accounting for one in every four cancer deaths.[1] The treatment of lung cancer is based upon the type and stage the cancer is in. There are two types of lung cancer including small cell and non-small cell lung cancer (NSCLC) the latter of which makes up 83% of the diagnosed cases. Treatments include, surgery, radiation, targeted therapies, and chemotherapy depending on the stage. Although platinum-based treatments can be effective for certain cancers, the five-year survival rate for NSCLC is only 21% and platinum agents can cause severe side effects such as nephrotoxicity.[2] Therefore, there is a need for new treatments for this terrible disease to increase survival rates and improve quality of life for patients.

Imidazolium salts are a class of compounds that have received attention for their in vitro anti-cancer activity against a variety of cancers, particularly NSCLC, beginning with the naturally occurring Lepidiline A and Lepidiline B (Figure 1).[3] Also, imidazolium salts with one naphthylmethyl and one tetrahydrobenzodifuran substituent have shown high anti-cancer activity against the A549 cell line.[4] Also of interest concerning this class of compounds, the benzimidazolium and 4,5-dimethyl benzimidazolium salts have higher anti-cancer activity than the less lipophilic imidazolium salt derivative. This suggests the lipophilicity of the benzimidazole ring is desirable when fine-tuning the compounds anti-cancer activity against NSCLC.

The Youngs' group has concentrated on enhancing the anti-proliferative properties of *N,N'*-bis(naphthylmethyl)imidazolium salts as anti-tumor agents against NSCLC cell lines.[5,6] The highly lipophilic *N,N'*-bis(naphthylmethyl)imidazolium salt **1** exhibited high anti-proliferative activity against several NSCLC cell lines (Figure 2). It was shown that the in vitro anti-tumor activity of **1** was strongly correlated to the two naphthylmethyl substituents at the N^1 and N^3 positions of the imidazole ring. However, the limited water solubility of **1** prohibits the clinical use of the compound due to anticipated problems with systemic administration. Herein, a series of *N,N'*-bis(arylmethyl)benzimidazolium salts with hydrophilic and hydrophobic substituents at all positions of the benzimidazole ring are presented. Derivatives in this series were evaluated against a panel of NSCLC cell lines by us and the National Cancer Institute's (NCI) Developmental Therapeutics Program (DTP) to

determine the structure activity relationship of the substituents and the resultant anti-proliferative activity.

2. Experimental

2.1. General Considerations

All reactions were carried out under aerobic conditions unless otherwise specified. 5,6-Dimethylbenzimidazole and 5-carboxylic acid-benzimidazole were purchased from Tokyo Chemical Industries (TCI), Co. Ltd. Benzimidazole, benzimidazole-5,6-dicarboxylic acid and 5-methoxybenzimidazole, methyl 2-(benzimidazol-2-yl)acetate, 3-(benzimidazol-2-yl)propanoic acid, 2-(methylthio)benzimidazole, 2-methylbenzimidazole, o-phenylenediamine and 4,5-dimethylbenzene-1,2-diamine were purchased from Alfa Aesar. γ -Butyrolactone, 2-hydroxybenzimidazole and 2-methoxypropionic acid were purchased from Sigma Aldrich. 2-(2-(2-Methoxyethoxy)ethoxy)acetic acid (synthesis grade, 95%) was purchased from Merck. 2-(Bromomethyl)naphthalene and 2-(chloromethyl)quinoline hydrochloride were purchased from Waterstone Technologies. All solvents were purchased from Fisher Scientific. All reagents were used as received without purification except for the following materials listed below. 2-(Chloromethyl)quinoline was obtained by deprotonating 2-(chloromethyl)quinoline hydrochloride with sodium bicarbonate. Methyl benzimidazole-5-carboxylate was synthesized according to literature procedure.[7] Methyl 3-(benzimidazol-2-yl)propanoate was synthesized by esterification of 3-(benzimidazol-2-yl)propanoic acid. 3-(Benzimidazol-2-yl)propan-1-ol was synthesized according to literature procedure.[8] 2-((2-(2-Methoxyethoxy)ethoxy)methyl)benzimidazole (**26**) was synthesized according to literature procedure.[9] Column chromatography utilized silica gel (60 Å, ICN Medicals) embedded with fluorescent indicator green 254 nm (Fluka Analytical). Melting points were obtained on a MelTemp apparatus. ^1H and $^{13}\text{C}\{^1\text{H}\}$ NMR spectra were collected on a Varian 300, 400 or 500 MHz instrument with spectra referenced to the residual protons in DMSO- d_6 (Cambridge Isotope Laboratories, 2.50 ppm, 39.51 ppm, for ^1H or ^{13}C NMR respectively) or CD $_3$ OD- d_4 (Cambridge Isotope Laboratories, 3.31 ppm, 49.00 ppm, for ^1H or ^{13}C NMR respectively). Infrared spectroscopy was performed using a Thermo Scientific ATR-IR Nicolet iS5 FT-IR spectrometer equipped with an iD5 ATR attachment. Mass spectrometry (ESI-MS) was performed by the University of Akron mass spectrometry laboratory (Akron, OH). Elemental analysis was performed by the microanalysis laboratory at Atlantic Microlabs, Inc. (Norcross, GA) or by the University of Akron Department Of Geology (Akron, OH).

2.2. X-ray Structure Determination Details

Crystals of the compounds were coated in paratone oil, mounted on a CryoLoop and placed on a goniometer under a stream of nitrogen. Crystal structure data sets were collected on either a Bruker SMART APEX I CCD diffractometer with graphite-monochromated Mo K α radiation ($\lambda = 0.71073$ Å) or a Bruker Kappa APEX II Duo CCD system equipped with a Mo ImuS source and a Cu ImuS micro-focus source equipped with QUAZAR optics ($\lambda = 1.54178$ Å). The unit cells were determined by using reflections from three different orientations. Data sets were collected using SMART or APEX II software packages. All data sets were processed using the APEX II software suite.[10,11] The data sets were integrated

using SAINT.[12] An empirical absorption correction and other corrections were applied to the data sets using multi-scan SADABS.[13] Structure solution, refinement, and modelling were accomplished by using the Bruker SHELXTL package.[14] The structures were determined by full-matrix least-squares refinement of F^2 and the selection of the appropriate atoms from the generated difference map. Hydrogen atom positions were calculated and $U_{iso}(H)$ values were fixed according to a riding model.

2.3. Cell Viability Protocol

The human non-small cell lung cancer cell lines NCI-H1975, HCC827 and A549 were donated by Dr. Daniel Lindner from the Cleveland Clinic. The human non-small cell lung cancer cell line NCI-H460 was purchased from ATCC (Manassas, VA, USA). All cell lines were grown in RPMI 1640 media supplemented with 10% fetal bovine serum. All cell lines were grown under physiological conditions, specifically at 37 °C with 5% CO₂ and passed every 2–3 days.

Cells were grown to confluency and plated in 96-well plates at 5,000–7,000 cells per well. Cells were incubated for 24 h prior to adding the compounds. All compounds were dissolved in a 1% DMSO solution and diluted in fresh media to the desired concentrations of 1, 4, 16, and 32 μ M. Compounds were added in sextuplet and cells were incubated for 72 h at which time the optional MTT assay protocol was followed. MTT reagent (10 μ L) was added to each well and cells were incubated for 3–4 h. Media was removed by vacuum and DMSO (100 μ L) was added to each well. Plates were incubated for 15 min at 37 °C. The optical density was read at 540 nm on a BioTek Epoch plate reader.

2.4 In Vivo Toxicity Study

All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Akron. Eight week old male C57BL/6 mice were obtained from Charles River laboratories. Animals were housed in a 12h light/dark cycle and food and water were provided ad libitum (n = 3 animals per cage). Prior to the toxicity testing, animals were allowed to acclimate for 5 days. Vehicle control mice received injections on days 0, 7, 14, 21, and 25 that consisted of a 100 μ L solution of a 20% 2-HP β CD sterile PBS solution by IP injection. Experimental mice received 100 μ L of a 4 mg/mL 20% 2-HP β CD sterile PBS solution (0.4 mg/100 μ L or ~ 20 mg/kg assuming 20 g mice) of **29** by IP injection on day 0. Animals were closely monitored and weighed on a daily basis. On day 2, two of the experimental mice were sacrificed. On day 16, the lone surviving experimental mouse was sacrificed. On day 29, the vehicle control animals were sacrificed.

2.4. General Synthesis

2.4.1. Synthesis of 5-methoxy-1,3-bis(naphthalen-2-ylmethyl)benzimidazolium bromide (2)—5-Methoxybenzimidazole (0.50 g, 3.37 mmol) was dissolved in acetonitrile (5 mL), potassium hydroxide (0.39 g, 6.95 mmol) was added, and the mixture was refluxed for 30 min. 2-(Bromomethyl)naphthalene (0.76 g, 3.44 mmol) was added, and the mixture was refluxed overnight. The reaction was filtered hot via vacuum filtration to remove a white solid, presumed to be potassium bromide. The filtrate was stirred with 2-

(bromomethyl)naphthalene (0.76 g, 3.44 mmol) and refluxed overnight. The resulting precipitate was isolated via vacuum filtration of the hot reaction mixture. The solid was purified by column chromatography on silica gel using a mobile phase of methanol/chloroform (10:90) to yield a white solid **2** (1.26 g, 73%). Mp: 217 – 220 °C. HRMS (ESI⁺) calcd for C₃₀H₂₅N₂O⁺ [M-Br]⁺ m/z = 429.1967, found at m/z = 429.1926. ¹H NMR (500 MHz, DMSO-*d*₆) δ = 10.02 (s, 1H), 8.16 (s, 1H), 8.11 (s, 1H), 8.01 – 7.90 (m, 7H), 7.67 (m, 2H), 7.61 (m, 1H), 7.58 – 7.53 (m, 4H), 7.24 – 7.21 (m, 1H), 5.98 (s, 2H), 5.96 (s, 2H), 3.83 (s, 3H). ¹³C{¹H} NMR (125 MHz, DMSO-*d*₆) δ = 158.7, 142.0, 132.68, 132.67, 132.66, 132.6, 132.5, 131.5, 131.4, 128.8, 128.7, 127.81, 127.79, 127.62, 127.61, 127.58, 127.4, 126.7, 125.7, 125.5, 125.3, 116.5, 114.9, 96.7, 56.3, 50.2, 49.9.

2.4.2. Synthesis of 5-(methoxycarbonyl)-1,3-bis(naphthalen-2-

ylmethyl)benzimidazolium bromide (**3**)—Methyl benzimidazole-6-carboxylate (0.38 g, 2.16 mmol) was stirred with sodium carbonate (0.34 g, 3.21 mmol) in acetonitrile (3 mL) for 30 min. 2-(Bromomethyl)-naphthalene (1.13 g, 5.11 mmol) was added and the mixture was refluxed overnight, during which a white precipitate formed. The volatile components of the reaction mixture were removed under reduced pressure. The white residue was dissolved in methanol and gravity filtered to remove any inorganic salts. The filtrate was concentrated under reduced pressure to produce a cream solid. The solid was purified by column chromatography on silica gel using a mobile phase of methanol/chloroform (5:95) to yield a white solid **3** (0.23 g, 19%). Mp: 133 – 136 °C. HRMS (ESI⁺) calcd for C₃₁H₂₅N₂O₂⁺ [M-Br]⁺ m/z = 457.1916, found m/z = 457.1882. ¹H NMR (300 MHz, DMSO-*d*₆) δ = 10.24 (s, 1H), 8.62 (m, 1H), 8.17 (m, 2H), 8.13 – 8.10 (m, 2H), 8.02 – 7.91 (m, 6H), 7.66 – 7.63 (m, 2H), 7.59 – 7.56 (m, 4H), 6.09 (s, 2H), 6.01 (s, 2H), 3.87 (s, 3H). ¹³C{¹H} NMR (125 MHz, DMSO-*d*₆) δ = 165.1, 145.4, 134.1, 132.72, 132.69, 132.66, 131.3, 131.2, 131.1, 128.79, 128.76, 128.0, 127.83, 127.81, 127.7, 127.64, 127.60, 127.4, 127.2, 126.74, 126.72, 126.69, 125.64, 125.62, 115.6, 114.6, 52.7, 50.5, 50.4. ATR-IR (cm⁻¹): 1720 (C=O).

2.4.3. Synthesis of 5,6-dimethyl-1,3-bis(naphthalen-2-

ylmethyl)benzimidazolium bromide (**4**)—5,6-Dimethylbenzimidazole (1.11 g, 7.59 mmol) was dissolved in acetonitrile (8 mL), potassium hydroxide (0.61 g, 10.87 mmol) was added, and the mixture was refluxed for 30 min. 2-(Bromomethyl)naphthalene (1.66 g, 7.51 mmol) was added, and the mixture was refluxed overnight, during which a white precipitate formed. The reaction mixture was filtered hot to remove a white solid, presumed to be potassium bromide, and the volatile components of the filtrate were removed under reduced pressure. The residue was combined with 2-(bromomethyl)-naphthalene (1.68 g, 7.60 mmol) in chloroform (17 mL) and refluxed overnight. The volatile components were removed under reduced pressure to form a solid. The solid was stirred at RT with diethyl ether (15 mL). The product was isolated via vacuum filtration, washed with diethyl ether and air dried to yield a cream solid **4** (3.14 g, 81%). Mp: 167 – 170 °C. HRMS (ESI⁺) calcd for C₃₁H₂₇N₂⁺ [M-Br]⁺ of m/z = 427.2174, found m/z = 427.2138. ¹H NMR (500 MHz, DMSO-*d*₆) δ = 10.12 (s, 1H), 8.10 (s, 2H), 7.98 (m, 2H), 7.93 (m, 4H), 7.85 (m, 2H), 7.63 (m, 2H), 7.56 (m, 4H), 5.94 (s, 4H), 2.31 (s, 6H). ¹³C{¹H} NMR (125 MHz, DMSO-*d*₆) δ = 141.7, 136.6, 132.69, 132.68, 131.6, 129.7, 128.8, 127.8, 127.7, 127.3, 126.7, 125.5, 113.4, 50.0, 20.0.

2.4.4. Synthesis of 5,6-bis(methoxycarbonyl)-1,3-bis(naphthalen-2-ylmethyl)benzimidazolium bromide (5)—Dimethyl benzimidazole-5,6-dicarboxylate (0.25 g, 1.07 mmol) was dissolved in acetonitrile (3 mL), sodium carbonate (0.18 g, 1.70 mmol) was added, and the mixture was refluxed for 30 min. 2-(Bromomethyl)naphthalene (0.38 g, 1.72 mmol) was added and the mixture was refluxed overnight. The reaction mixture was filtered hot to remove a white solid, presumed to be sodium bromide. The filtrate was refluxed overnight with 2-(bromomethyl)-naphthalene (0.38 g, 1.72 mmol). The volatile components of the reaction mixture were removed under reduced pressure, and the residue was dissolved in hot methanol and gravity filtered. The volatile components of the filtrate were removed under reduced pressure to yield a white solid **5** (0.40 g, 62%). Mp: 169 – 172 °C. Anal. Calcd. for $C_{33}H_{27}BrN_2O_4$: C, 66.56; H, 4.57; N, 4.70%. Found: C, 66.08; H, 4.41; N, 4.54%. 1H NMR (300 MHz, DMSO- d_6) δ = 10.30 (s, 1H), 8.53 (s, 2H), 8.08 (m, 2H), 8.02 – 7.90 (m, 6H), 7.63 (m, 2H), 7.57 (m, 4H), 6.05 (s, 4H), 3.84 (s, 6H). $^{13}C\{^1H\}$ NMR (125 MHz, DMSO- d_6) δ = 166.2, 146.6, 132.73, 132.68, 132.5, 131.0, 129.8, 128.8, 127.8, 127.7, 127.5, 126.8, 126.7, 125.6, 115.4, 53.1, 50.5. MS (ESI $^+$) calcd for $C_{33}H_{27}N_2O_4^+$ [M-Br] $^+$ of m/z = 515.5, found m/z = 515.1. ATR-IR (cm $^{-1}$): 1733 (C=O).

2.4.5. Synthesis of 5-((naphthalen-2-ylmethoxy)carbonyl)-1,3-bis(naphthalen-2-ylmethyl)benzimidazolium bromide (6)—Benzimidazole-6-carboxylic acid (0.50 g, 3.08 mmol) was dissolved in acetonitrile (150 mL), sodium bicarbonate (1.11 g, 13.2 mmol) was added, and the mixture was refluxed for 1 h. 2-(Bromomethyl)naphthalene (3.86 g, 17.5 mmol) was added, and the mixture was refluxed for 3 d. The volatile components were removed under reduced pressure. The slurry was dissolved in dichloromethane (200 mL) and filtered to remove a white solid, presumed to be sodium bromide. The filtrate was concentrated under reduced pressure to generate a white solid. The solid was purified by column chromatography on silica gel using a mobile phase of methanol/chloroform (10:90) to yield a cream solid **6** (1.68 g, 82%). Mp: 139 – 142 °C. Anal. Calcd. for $C_{41}H_{31}BrN_2O_2$: C, 74.21; H, 4.71; N, 4.22%. Found: C, 74.04; H, 4.70; N, 4.20%. 1H NMR (300 MHz, DMSO- d_6) δ = 10.22 (s, 1H), 8.65 (s, 1H), 8.23 (m, 1H), 8.17 – 8.11 (m, 3H), 8.01 – 7.83 (m, 10H), 7.65 – 7.50 (m, 8H), 6.08 (s, 2H), 6.00 (s, 2H), 5.53 (s, 2H). $^{13}C\{^1H\}$ NMR (125 MHz, DMSO- d_6) δ = 164.5, 145.5, 134.3, 133.3, 132.8, 132.73, 132.69, 132.67, 132.65, 132.6, 131.3, 131.2, 131.1, 128.8, 128.2, 128.0, 127.9, 127.81, 126.81, 127.80, 127.69, 127.66, 127.6, 127.3, 126.9, 126.8, 126.7, 126.41, 126.36, 125.8, 125.74, 125.70, 115.9, 114.7, 67.0, 50.6, 50.5. MS (ESI $^+$) calcd for $C_{41}H_{31}N_2O_2^+$ [M-Br] $^+$ of m/z = 583.2, found m/z = 583.1. ATR-IR (cm $^{-1}$): 1719 (C=O).

2.4.6. Synthesis of 5,6-dicarboxy-1,3-bis(naphthalen-2-ylmethyl)benzimidazolium chloride (7)—Compound **5** (0.38 g, 0.64 mmol) in 11 M aqueous hydrochloric acid (10 mL) was heated at reflux for 3 d. The hydrochloride salt was isolated via vacuum filtration to yield a white solid **7** (0.20 g, 60%). Mp: 210 – 213 °C. Anal. Calcd. for $C_{31}H_{23}ClN_2O_4$: C, 70.72; H, 4.43; N, 5.26%. Found: C, 71.20; H, 4.43; N, 5.36%. 1H NMR (300 MHz, DMSO- d_6) δ = 13.63 (s, 2H), 10.28 (s, 1H), 8.40 (s, 2H), 8.08 (s, 2H), 8.02 – 7.90 (m, 6H), 7.63 – 7.55 (m, 6H), 6.03 (s, 4H). $^{13}C\{^1H\}$ NMR (125 MHz, DMSO- d_6) δ = 167.4, 146.0, 132.73, 132.68, 132.1, 131.7, 131.1, 128.8, 127.8, 127.7,

127.4, 126.76, 126.74, 125.6, 114.8, 50.4. MS (ESI⁺) calcd for C₃₁H₂₃N₂O₄⁺ [M-Cl]⁺ of m/z = 487.2, found m/z = 486.9. ATR-IR (cm⁻¹): 1690 (C=O).

2.4.7. Synthesis of 5-carboxy-1,3-bis(naphthalen-2-ylmethyl)benzimidazolium chloride/bromide (8)—Compound **6** (2.01 g, 3.03 mmol) in 6 M aqueous HCl (46 mL) was refluxed for 3 d. The product was filtered, and the salt was stirred with a mixture of de-ionized water and acetone (1:1, v/v, 30 mL). The blue-grey solid was filtered and purified by column chromatography on silica gel using a mobile phase of methanol/chloroform (10:90) to yield a white solid **8** (0.98 g, 66%). Mp: 217 – 220 °C. Anal. Calcd. for C₃₀H₂₃Cl_{0.68}Br_{0.32}N₂O₂: C, 73.06; H, 4.70; N, 5.68%. Found: C, 72.60; H, 5.24; N, 5.34 %. HRMS (ESI⁺) calcd for C₃₀H₂₃N₂O₂⁺ [M-Cl_{0.68}Br_{0.32}]⁺ = 443.1760, found m/z = 443.1573. ¹H NMR (300 MHz, CD₃OD-*d*₄) δ = 9.84 (s, 1H), 8.26 (s, 1H), 8.03 (m, 2H), 7.93 – 7.87 (m, 7H), 7.55 – 7.51 (m, 7H), 5.95 (2H, s), 5.93 (2H, s). ¹³C{¹H} NMR (125 MHz, DMSO-*d*₆) δ = 166.0, 145.4, 133.9, 132.74, 132.72, 132.70, 131.4, 131.3, 129.4, 128.81, 128.79, 127.89, 127.85, 127.71, 127.68, 127.6, 127.5, 127.4, 126.8, 126.7, 125.72, 125.67, 115.6, 114.4, 50.5, 50.3. ATR-IR (cm⁻¹): 1703 (C=O).

2.4.8. Synthesis of 2-(2-methoxy-2-oxoethyl)-1,3-bis(naphthalen-2-ylmethyl)benzimidazolium bromide (9)—Methyl 2-(benzimidazol-2-yl)acetate (1.00 g, 5.26 mmol) and sodium carbonate (0.86 g, 8.11 mmol) was dissolved in acetonitrile (7.5 mL), and the mixture was refluxed for 30 min. 2-(Bromomethyl)naphthalene (1.29 g, 5.83 mmol) was added, and the mixture was refluxed overnight. The reaction mixture was filtered hot to remove a white solid, presumed to be sodium bromide. 2-(Bromomethyl)naphthalene (1.29 g, 5.83 mmol) was added to the filtrate and the mixture was refluxed overnight. The volatile components were removed under reduced pressure to yield a residue. The residue was stirred in diethyl ether (10 mL) at RT. The solid was isolated via vacuum filtration, washed with diethyl ether and air-dried to yield a white solid **9** (0.78 g, 26%). Mp: 197 – 200 °C. Anal. Calcd for C₃₂H₂₇BrN₂O₂: C, 69.69; H, 4.94; N, 5.08%. Found: C, 70.09; H, 5.17; N, 4.95%. ¹H NMR (300 MHz, DMSO-*d*₆) δ = 8.05 (m, 2H), 7.95 – 7.86 (m, 8H), 7.67 (m, 2H), 7.55 (m, 4H), 7.42 (m, 2H), 6.11 (s, 4H), 5.03 (s, 2H), 2.98 (s, 3H). ¹³C{¹H} NMR (125 MHz, DMSO-*d*₆) δ = 165.2, 148.2, 132.54, 132.48, 131.29, 131.28, 128.6, 127.7, 127.6, 127.1, 126.7, 126.6, 126.5, 125.0, 114.0, 52.4, 49.0, 30.7. MS (ESI⁺) calcd for C₃₂H₂₇N₂O₂⁺ [M-Br]⁺ of m/z = 471.2, found m/z = 471.1. ATR-IR (cm⁻¹): 1748 (C=O).

2.4.9. Synthesis of 2-(3-methoxy-3-oxopropyl)-1,3-bis(naphthalen-2-ylmethyl)benzimidazolium bromide (10)—Methyl 3-(benzimidazol-2-yl)propanoate (0.31 g, 1.52 mmol) and sodium carbonate (0.27 g, 2.55 mmol) were stirred in acetonitrile (4 mL) for 30 min. 2-(Bromomethyl)naphthalene (0.41 g, 1.84 mmol) was added, and the mixture was refluxed overnight. The reaction mixture was filtered hot to remove a white solid, presumed to be sodium bromide. 2-(Bromomethyl)naphthalene (0.41 g, 1.84 mmol) was added to the filtrate and the mixture was refluxed overnight. The volatile components were removed under reduced pressure to yield a residue. The residue was washed in diethyl ether (10 mL) at RT. The solid was isolated via vacuum filtration, washed with diethyl ether and air-dried to yield a colorless solid **10** (0.57 g, 66%). Mp: 179 – 181 °C. Anal. Calcd for C₃₃H₂₉BrN₂O₂: C, 70.09; H, 5.17; N, 4.95%. Found: C, 69.18; H, 5.29; N, 5.01%. ¹H NMR

(500 MHz, DMSO- d_6) δ = 7.99–7.94 (m, 6H), 7.87–7.84 (m, 4H), 7.62 (m, 2H), 7.54 (m, 6H), 7.47 (m, 2H), 6.11 (s, 4H), 3.72 (t, 2H, J = 7.6 Hz), 3.44 (s, 3H), 2.62 (t, 2H, J = 7.6 Hz). $^{13}\text{C}\{^1\text{H}\}$ NMR (125 MHz, DMSO- d_6) δ = 171.0, 153.6, 132.7, 133.0, 132.7, 132.5, 131.8, 131.4, 128.7, 127.65, 127.62, 126.7, 126.5, 125.6, 124.7, 113.6, 51.6, 48.7, 30.0, 19.3. MS (ESI $^+$) calcd for $\text{C}_{33}\text{H}_{29}\text{N}_2\text{O}_2^+$ [M-Br] $^+$ of m/z = 485.2, found m/z = 485.0. ATR-IR (cm^{-1}): 1727 (C=O).

2.4.10. Synthesis of 2-methyl-1,3-bis(naphthalen-2-ylmethyl)benzimidazolium bromide (**11**)

—2-Methyl-benzimidazole (0.50 g, 3.78 mmol) was dissolved in acetonitrile (6 mL), potassium hydroxide (0.25 g, 4.46 mmol) was added and the mixture was refluxed for 30 min. 2-(Bromomethyl)naphthalene (0.84 g, 3.80 mmol) was added, and the mixture was refluxed overnight. The reaction mixture was filtered hot to remove a white solid, presumed to be potassium bromide. 2-(Bromomethyl)naphthalene (0.84 g, 3.80 mmol) was added to the filtrate and refluxed overnight. The resulting precipitate was collected via vacuum filtration of the hot reaction mixture. The solid in the funnel was washed with diethyl ether and air dried to yield a white solid **11** (1.54 g, 82%). Mp: 279 – 282 °C. Anal. Calcd for $\text{C}_{30}\text{H}_{25}\text{BrN}_2$: C, 73.02; H, 5.11; N, 5.68%. Found: C, 72.74; H, 5.32; N, 5.64%. ^1H NMR (500 MHz, DMSO- d_6) 8.02 – 7.89 (m, 10H), 7.60 – 7.52 (m, 8H), 6.05 (s, 4H), 3.13 (s, 3H). $^{13}\text{C}\{^1\text{H}\}$ NMR (125 MHz, DMSO- d_6) 152.8, 132.7, 132.5, 131.6, 131.2, 128.7, 127.7, 127.6, 126.6, 126.5, 126.3, 126.1, 125.0, 113.4, 48.6, 11.3. MS (ESI $^+$) calcd for $\text{C}_{30}\text{H}_{25}\text{N}_2^+$ [M-Br] $^+$ of m/z = 413.2, found m/z = 413.3.

Crystal data for the solvate of **11-Cl**: $\text{C}_{30}\text{H}_{25}\text{ClN}_2\cdot\text{H}_2\text{O}$, M = 466.99, triclinic, a = 10.1897(17) Å, b = 10.4212(17) Å, c = 11.4397(19) Å, α = 80.323(2)°, β = 86.985(2)°, γ = 76.344(2)°, V = 1163.5(2) Å 3 , T = 100(2) K, space group P-1, Z = 2, 9361 reflections measured, 4658 independent reflections (R_{int} = 0.0250). The final R_1 values were 0.0396 ($I > 2\sigma(I)$). The final $wR(F^2)$ values were 0.1052 ($I > 2\sigma(I)$). The final R_1 values were 0.0451 (all data). The final $wR(F^2)$ values were 0.1099 (all data). A single crystal was obtained by slow evaporation of a concentrated solution of **11-Cl** in acetone/water (1:1).

2.4.11. Synthesis of 2-(2-carboxyethyl)-1,3-bis(naphthalen-2-ylmethyl)benzimidazolium chloride (**12**)

—A solution of **10** (0.25 g, 0.442 mmol) in 9 M aqueous HCl (6 mL) was heated at reflux for 3 d. The solution was filtered via vacuum filtration to isolate the precipitate, and the solid was air-dried to yield a white powder **12** (0.15 g, 66%). Mp: 242 – 245 °C. Anal. Calcd for $\text{C}_{32}\text{H}_{27}\text{ClN}_2\text{O}_2\cdot\text{H}_2\text{O}$: C, 73.20; H, 5.57; N, 5.34%. Found: C, 74.22; H, 5.48; N, 5.45%. HRMS (ESI $^+$) calcd for $\text{C}_{32}\text{H}_{27}\text{N}_2\text{O}_2^+$ [M-Cl] $^+$ of m/z = 471.2072, found m/z = 471.2096. ^1H NMR (500 MHz, DMSO- d_6) 12.60 (bs, 1H), 7.99 (s, 1H), 7.98 (s, 1H), 7.95 (m, 4H), 7.87 (m, 4H), 7.59 (m, 2H), 7.55 (m, 4H), 7.49 (m, 2H), 6.14 (s, 4H), 3.71 (t, 2H, J = 7.7 Hz), 2.62 (t, 2H, J = 7.7 Hz). $^{13}\text{C}\{^1\text{H}\}$ NMR (125 MHz, DMSO- d_6) 172.0, 154.0, 132.7, 132.5, 131.9, 131.4, 128.7, 127.7, 127.6, 126.7, 126.6, 126.5, 125.6, 124.7, 113.6, 48.8, 30.5, 19.5. ATR-IR (cm^{-1}): 1704 (C=O).

Crystal data for **12**: $\text{C}_{32}\text{H}_{27}\text{ClN}_2\text{O}_2$, M = 507.01, triclinic, a = 8.0368(6) Å, b = 12.690(2) Å, c = 12.996(3) Å, α = 106.523(7)°, β = 100.163(7)°, γ = 92.637(7)°, V = 1244.2(4) Å 3 , T = 100(2) K, space group P-1, Z = 2, 19911 reflections measured, 5013 independent reflections (R_{int} = 0.0379). The final R_1 values were 0.0390 ($I > 2\sigma(I)$). The final $wR(F^2)$

values were 0.0902 ($I > 2\sigma(I)$). The final R_1 values were 0.0545 (all data). The final $wR(F^2)$ values were 0.0989 (all data). A single crystal was obtained by slow evaporation of a concentrated solution of **12** in acetonitrile/methanol (1:1).

2.4.12. Synthesis of 2-ethyl-1,3-bis(naphthalen-2-ylmethyl)benzimidazolium bromide (**13**)

—o-Phenylenediamine (0.40 g, 3.70 mmol) and propionic acid (0.34 mL, 4.54 mmol) were added to 4 M aqueous hydrochloric acid (5 mL) to synthesize 2-ethylbenzimidazole according to literature procedure.[15] 2-Ethyl-benzimidazole (0.11 g, 0.75 mmol) was dissolved in acetonitrile (6 mL), potassium hydroxide (70.0 mg, 1.25 mmol) was added, and the mixture was refluxed for 30 min. 2-(Bromomethyl)naphthalene (0.17 g, 0.77 mmol) was added and the mixture was refluxed overnight. The reaction mixture was filtered hot to remove a white solid, presumed to be potassium bromide. 2-(Bromomethyl)naphthalene (0.17 g, 0.77 mmol) was added to the filtrate and refluxed overnight. The resulting precipitate was collected via vacuum filtration of the hot reaction mixture. The solid was washed with diethyl ether and air dried to yield a white solid **13** (0.15 g, 39%). Mp: 268 – 270 °C. Anal. Calcd for $C_{31}H_{27}BrN_2$: C, 73.37; H, 5.36; N, 5.52 %. Found: C, 72.44; H, 53.49; N, 5.48. 1H NMR (500 MHz, DMSO- d_6) 7.98 – 7.89 (m, 10H), 7.59 – 7.54 (m, 8H), 7.48 (m, 2H), 6.10 (s, 4H), 3.53 (q, 2H, $J = 7.3$ Hz), 1.07 (t, 3H, $J = 7.5$ Hz). $^{13}C\{^1H\}$ NMR (125 MHz, DMSO- d_6) 156.0, 132.7, 132.5, 131.9, 131.4, 128.7, 127.7, 127.6, 126.7, 126.5, 125.7, 124.7, 113.6, 48.6, 17.6, 11.3. MS (ESI $^+$) calcd for $C_{31}H_{27}N_2^+ [M-Br]^+$ of $m/z = 427.6$, found $m/z = 427.4$.

Crystal data for **13**: $C_{31}H_{27}BrN_2$, $M = 507.46$, triclinic, $a = 9.5820(10)$ Å, $b = 10.1627(11)$ Å, $c = 14.0507(15)$ Å, $\alpha = 79.7760(10)^\circ$, $\beta = 89.5660(10)^\circ$, $\gamma = 64.9650(10)^\circ$, $V = 1216.5(2)$ Å 3 , $T = 100(2)$ K, space group P-1, $Z = 2$, 9895 reflections measured, 4898 independent reflections ($R_{int} = 0.0124$). The final R_1 values were 0.0210 ($I > 2\sigma(I)$). The final $wR(F^2)$ values were 0.0547 ($I > 2\sigma(I)$). The final R_1 values were 0.0215 (all data). The final $wR(F^2)$ values were 0.0551 (all data). A single crystal was obtained by slow evaporation of a concentrated solution of **13** in acetonitrile/methanol (1:1).

2.4.13. Synthesis of 1,3-bis(naphthalen-2-ylmethyl)-2-propylbenzimidazolium bromide (**14**)

—o-Phenylenediamine (0.40 g, 3.70 mmol) and butyric acid (0.42 mL, 4.57 mmol) were added to 4 M aqueous hydrochloric acid (5 mL) to synthesize 2-propylbenzimidazole according to literature procedure.[15] 2-Propyl-benzimidazole (0.30 g, 1.87 mmol) was dissolved in acetonitrile (7 mL), potassium hydroxide (0.17 g, 3.03 mmol) was added, and the mixture was refluxed for 30 min. 2-(Bromomethyl)naphthalene (0.42 g, 1.90 mmol) was added and the mixture was refluxed overnight. The reaction mixture was filtered hot to remove a white solid, presumed to be potassium bromide. 2-(Bromomethyl)naphthalene (0.42 g, 1.90 mmol) was added to the filtrate and refluxed overnight. The resulting precipitate was collected via vacuum filtration of the hot reaction mixture. The solid was washed with diethyl ether and air dried to yield a white solid **14** (0.23 g, 23%). Mp: 264 – 266 °C. Anal. Calcd for $C_{32}H_{29}BrN_2$: C, 73.70; H, 5.61; N, 5.37%. Found: C, 73.18; H, 5.57; N, 5.40%. 1H NMR (500 MHz, DMSO- d_6) 7.98 – 7.88 (m, 10H), 7.60 – 7.54 (m, 6H), 7.48 (m, 2H), 6.09 (s, 4H), 3.47 (m, 2H), 1.44 (m, 2H), 0.88 (t, 3H, $J = 7.3$ Hz). $^{13}C\{^1H\}$ NMR (125 MHz, DMSO- d_6) 154.9, 132.7, 132.5, 131.9, 131.4,

128.7, 127.7, 127.6, 126.7, 126.5, 125.8, 124.7, 113.7, 48.7, 25.4, 20.5, 13.4. MS (ESI⁺) calcd for C₃₂H₂₉N₂⁺ [M-Br]⁺ of m/z = 441.6, found m/z = 441.4.

2.4.14. Synthesis of 2-butyl-1,3-bis(naphthalen-2-ylmethyl)benzimidazolium bromide (15)—o-Phenylenediamine (0.40 g, 3.70 mmol) and pentanoic acid (0.50 mL, 4.60 mmol) were added to 4 M aqueous hydrochloric acid (5 mL) to synthesize 2-butylbenzimidazole according to literature procedure.[15] 2-Butyl-benzimidazole (0.17 g, 0.98 mmol) was dissolved in acetonitrile (5 mL), potassium hydroxide (0.09 g, 1.60 mmol) was added and the mixture was refluxed for 30 min. 2-(Bromomethyl)naphthalene (0.22 g, 1.00 mmol) was added and the mixture was refluxed overnight. The reaction mixture was filtered hot to remove a white solid, presumed to be potassium bromide. 2-(Bromomethyl)naphthalene (0.22 g, 1.00 mmol) was added to the filtrate and refluxed overnight. The resulting precipitate was collected via vacuum filtration of the hot reaction mixture. The solid was washed with diethyl ether and air dried to yield a cream solid **15** (0.29 g, 55%). Mp: 266 – 268 °C. Anal. Calculated for C₃₃H₃₁BrN₂: C, 74.01; H, 5.83; N, 5.23%. Found: C, 73.74; H, 5.82; N, 5.25%. ¹H NMR (500 MHz, DMSO-*d*₆) 8.00 – 7.88 (m, 10H), 7.61 (m, 2H), 7.55 (m, 4H), 7.46 (m, 2H), 6.09 (s, 4H), 3.45 (m, 2H), 1.25 (m, 4H), 0.53 (t, 3H, J = 6.5 Hz). ¹³C{¹H} NMR (125 MHz, DMSO-*d*₆) 155.0, 132.7, 132.5, 131.9, 131.4, 128.7, 127.7, 127.6, 126.7, 126.5, 125.9, 124.7, 113.6, 48.7, 28.5, 23.7, 21.7, 13.1. MS (ESI⁺) calcd for C₃₃H₃₁N₂⁺ [M-Br]⁺ of m/z = 455.6, found m/z = 455.4.

Crystal data for **15**: C₃₃H₃₁BrN₂, M = 535.51, monoclinic, a = 27.1716(10) Å, b = 9.7618(3) Å, c = 19.4068(7) Å, α = 90°, β = 92.1326(17)°, γ = 90°, V = 5144.0(3) Å³, T = 100(2) K, space group P2(1)/c, Z = 8, 41594 reflections measured, 10427 independent reflections (R_{int} = 0.1032). The final R₁ values were 0.0694 (I > 2σ(I)). The final wR(F²) values were 0.1356 (I > 2σ(I)). The final R₁ values were 0.1215 (all data). The final wR(F²) values were 0.1521 (all data). A single crystal was obtained by slow evaporation of a concentrated solution of **15** in acetonitrile/methanol (1:1).

2.4.15. Synthesis of 2-heptyl-1,3-bis(naphthalen-2-ylmethyl)benzimidazolium bromide (16)—o-Phenylenediamine (0.40 g, 3.70 mmol) and octanoic acid (0.44 mL, 3.35 mmol) were added to 4 M aqueous hydrochloric acid (3 mL) to synthesize 2-heptylbenzimidazole. 2-Heptyl-benzimidazole (0.09 g, 0.42 mmol) was dissolved in acetonitrile (5 mL), potassium hydroxide (0.03 g, 0.53 mmol) was added and the mixture was refluxed for 30 min. 2-(Bromomethyl)naphthalene (0.11 g, 0.50 mmol) was added and the mixture was refluxed overnight. The reaction mixture was filtered hot to remove a white solid, presumed to be potassium bromide. 2-(Bromomethyl)naphthalene (0.11 g, 0.50 mmol) was added to the filtrate and refluxed overnight. The resulting precipitate was collected via vacuum filtration of the hot reaction mixture. The solid was washed with diethyl ether and air dried to yield a cream solid **16** (0.14 g, 57%). Mp: 229 – 232 °C. Anal. Calcd for C₃₆H₃₇BrN₂•H₂O: C, 72.60; H, 6.60; N, 4.70%. Found: C, 72.68; H, 6.34; N, 4.78%. HRMS (ESI⁺) calcd for C₃₆H₃₇N₂⁺[M-Br]⁺ of m/z = 497.2957, found m/z = 497.2950. ¹H NMR (500 MHz, DMSO-*d*₆) 8.02 – 7.91 (m, 6H), 7.88 (m, 4H), 7.63 (m, 2H), 7.56 – 7.53 (m, 4H), 7.44 (dd, 2H, J = 8.6, 1.7 Hz), 6.06 (s, 4H), 3.41 (m, 2H), 1.15 (m, 4H), 0.95 (m, 2H), 0.77 (m, 4H), 0.63 (t, 3H, J = 7.3 Hz). ¹³C{¹H} NMR (125 MHz, DMSO-*d*₆) 155.1,

132.7, 132.5, 132.0, 131.5, 128.7, 127.6, 126.7, 126.6, 126.5, 125.8, 124.6, 113.6, 48.6, 30.5, 28.4, 27.9, 26.5, 24.0, 21.7, 13.6.

2.4.16. Synthesis of 2-(2-methoxyethyl)-1,3-bis(naphthalen-2-ylmethyl)benzimidazolium bromide (**17**)

2-(2-Methoxyethyl)-benzimidazole (0.21 g, 1.19 mmol) was dissolved in acetonitrile (6 mL), sodium carbonate (0.21 g, 1.98 mmol) was added and the mixture was refluxed for 30 min. 2-(Bromomethyl)naphthalene (0.27 g, 1.22 mmol) was added and the mixture was refluxed overnight. The reaction mixture was filtered hot to remove a white solid, presumed to be sodium bromide. 2-(Bromomethyl)naphthalene (0.27 g, 1.22 mmol) was added to the filtrate and refluxed overnight. The resulting precipitate was collected via vacuum filtration of the hot reaction mixture. The solid was washed with diethyl ether and air dried to yield a cream solid **17** (0.20 g, 31%). Mp: 243 – 246 °C. Anal. Calcd for $C_{32}H_{29}BrN_2O \cdot H_2O$: C, 69.19; H, 5.63; N, 5.04%. Found: C, 69.54; H, 5.36; N, 5.10%. HRMS (ESI⁺) calculated for $C_{32}H_{29}N_2O^+ [M-Br]^+$ = 457.2280, found m/z = 457.2278. ¹H NMR (500 MHz, DMSO-*d*₆) 8.00 – 7.94 (m, 4H), 7.89 – 7.84 (m, 6H), 7.57 – 7.54 (m, 5H), 7.48 (m, 3H), 6.12 (s, 4H), 3.88 (t, 2H, J = 5.6 Hz), 3.56 (t, 2H, J = 5.6 Hz), 3.03 (s, 3H). ¹³C{¹H} NMR (125 MHz, DMSO-*d*₆) 153.3, 132.7, 132.4, 131.9, 131.2, 128.7, 127.7, 127.7, 127.6, 126.7, 126.6, 126.5, 125.6, 124.6, 113.7, 68.0, 58.2, 48.9, 24.9.

Crystal data for **17**: $C_{32}H_{29}BrN_2O$, $M = 537.48$, triclinic, $a = 8.8985(8)$ Å, $b = 17.1053(15)$ Å, $c = 18.9826(16)$ Å, $\alpha = 63.486(4)^\circ$, $\beta = 88.991(4)^\circ$, $\gamma = 88.822(4)^\circ$, $V = 2584.8(4)$ Å³, $T = 100(2)$ K, space group P-1, $Z = 4$, 38791 reflections measured, 10457 independent reflections ($R_{int} = 0.0648$). The final R_1 values were 0.0776 ($I > 2\sigma(I)$). The final $wR(F^2)$ values were 0.1958 ($I > 2\sigma(I)$). The final R_1 values were 0.1094 (all data). The final $wR(F^2)$ values were 0.2143 (all data). A single crystal was obtained by slow evaporation of a concentrated solution of **17** in acetone/methanol/ethyl acetate (1:1:1).

2.4.17. Synthesis of 2-(3-hydroxypropyl)-1,3-bis(naphthalen-2-ylmethyl)benzimidazolium bromide (**18**)

3-(Benzimidazol-2-yl)propan-1-ol (1.50 g, 8.51 mmol) was dissolved in acetonitrile (40 mL), potassium hydroxide (0.90 g, 16.0 mmol) was added, and the mixture was refluxed for 30 min. 2-(Bromomethyl)naphthalene (1.90 g, 8.59 mmol) was added and the mixture was refluxed overnight. The reaction mixture was filtered hot to remove a white solid, presumed to be potassium bromide. 2-(Bromomethyl)naphthalene (1.90 g, 8.59 mmol) was added to the filtrate, which was refluxed overnight. The resulting precipitate was collected via vacuum filtration of the hot mixture. The solid was washed with diethyl ether (10 mL) and air dried to yield a white powder **18** (2.20 g, 48 %). Mp: 229 – 231 °C. Anal. Calcd for $C_{32}H_{29}BrN_2O$: C, 71.51; H, 5.44; N, 5.21%. Found: C, 71.02; H, 5.61; N, 5.08%. ¹H NMR (500 MHz, DMSO-*d*₆) 7.99 – 7.88 (m, 10H), 7.58 – 7.50 (m, 8H), 6.09 (s, 4H), 4.78 (bs, 1H), 3.54 (m, 2H), 3.49 (m, 2H), 1.76 (m, 2H). ¹³C{¹H} NMR (125 MHz, DMSO-*d*₆) 155.3, 132.7, 132.5, 131.8, 131.4, 128.7, 127.7, 127.6, 126.7, 126.51, 126.45, 125.7, 124.7, 113.6, 59.2, 48.7, 29.7, 21.0. MS (ESI⁺) calcd for $C_{32}H_{29}N_2O^+ [M-Br]^+$: m/z = 457.2, found m/z = 457.1.

Crystal data for **18**: $C_{32}H_{29}BrN_2O$, $M = 537.48$, triclinic, $a = 10.3959(6)$ Å, $b = 10.4438(6)$ Å, $c = 12.8557(7)$ Å, $\alpha = 67.842(2)^\circ$, $\beta = 76.321(2)^\circ$, $\gamma = 79.887(2)^\circ$, $V = 1250.23(12)$ Å³, $T = 100(2)$ K, space group P-1, $Z = 2$, 27063 reflections measured, 5022 independent

reflections ($R_{\text{int}} = 0.0277$). The final R_1 values were 0.0221 ($I > 2\sigma(I)$). The final $wR(F^2)$ values were 0.0552 ($I > 2\sigma(I)$). The final R_1 values were 0.0237 (all data). The final $wR(F^2)$ values were 0.0561 (all data). A single crystal was obtained by slow evaporation of a concentrated solution of **18** in methanol.

2.4.18. Synthesis of 2-(3-hydroxypropyl)-1-(naphthalen-2-ylmethyl)-3-(quinolin-2-ylmethyl)benzimidazolium bromide (19)—3-(Benzimidazol-2-yl)propan-1-ol (0.40 g, 2.27 mmol) was dissolved in acetonitrile (11 mL) potassium hydroxide (0.15 g, 2.67 mmol) was added, and the mixture was refluxed for 30 min. 2-(Chloromethyl)quinoline (0.40 g, 2.26 mmol) was added, and the mixture was refluxed overnight. The reaction mixture was filtered hot to remove a white solid, presumed to be potassium chloride. 2-(Bromomethyl)naphthalene (0.50 g, 2.26 mmol) was added to the filtrate, which was refluxed overnight. The precipitate was collected via vacuum filtration of the hot mixture. The solid was washed with diethyl ether (5 mL) and air dried. The solid was triturated in a hot mixture of acetone and dichloromethane (1:1 v/v, 6 mL) and gravity filtered to yield a white powder **19** (0.57 g, 46%). Mp: 246 – 250 °C. Anal. Calcd for $C_{31}H_{28}BrN_3O$: C, 69.15; H, 5.24; N, 7.80%. Found: C, 69.07; H, 5.15; N, 7.72%. 1H NMR (500 MHz, DMSO- d_6) 8.50 (d, 1H, $J = 8.3$ Hz), 8.03 – 7.78 (m, 8H), 7.71 – 7.47 (m, 7H), 6.28 (s, 2H), 6.15 (s, 2H), 4.75 (t, 1H, $J = 4.8$ Hz), 3.53 – 3.48 (m, 4H), 1.86 (m, 2H). $^{13}C\{^1H\}$ NMR (125 MHz, DMSO- d_6) 156.0, 153.6, 146.6, 137.5, 132.7, 132.5, 132.0, 131.6, 131.1, 130.1, 128.8, 128.0, 127.7, 127.2, 126.9, 126.8, 126.6, 126.42, 126.39, 125.7, 124.5, 120.0, 113.5, 113.4, 59.2, 49.5, 48.4, 29.8.

Crystal data for **19**: $C_{31}H_{28}BrN_3O$, $M = 538.47$, triclinic, $a = 10.4596(3)$ Å, $b = 10.4601(3)$ Å, $c = 12.7946(4)$ Å, $\alpha = 113.0640(10)^\circ$, $\beta = 97.215(2)^\circ$, $\gamma = 99.6430(10)^\circ$, $V = 1241.21(6)$ Å³, $T = 100(2)$ K, space group P-1, $Z = 2$, 29636 reflections measured, 5003 independent reflections ($R_{\text{int}} = 0.0271$). The final R_1 values were 0.0295 ($I > 2\sigma(I)$). The final $wR(F^2)$ values were 0.0741 ($I > 2\sigma(I)$). The final R_1 values were 0.0310 (all data). The final $wR(F^2)$ values were 0.0750 (all data). A single crystal was obtained by slow evaporation of a concentrated solution of **19** in acetone/methanol/ethyl acetate (1:1:1).

2.4.19. Synthesis of 2-(3-hydroxypropyl)-1,3-bis(quinolin-2-ylmethyl)benzimidazolium chloride (20)—3-(Benzimidazol-2-yl)propan-1-ol (0.39 g, 2.21 mmol) was dissolved in acetonitrile (5 mL), sodium carbonate (0.24 g, 2.26 mmol) was added, and the mixture was refluxed for 30 min. 2-(Chloromethyl)quinoline (0.60 g, 3.38 mmol) was added and the mixture was refluxed overnight. The reaction mixture was filtered hot to remove a white solid, presumed to be sodium chloride. 2-(Chloromethyl)quinoline (0.60 g, 3.38 mmol) was added to the filtrate, which was refluxed overnight. The precipitate was collected via vacuum filtration of the hot mixture. The solid was washed with diethyl ether (5 mL) and air dried yield a white powder **20** (0.81 g, 72%). Mp: 256 – 260 °C. HRMS (ESI⁺) calcd for $C_{30}H_{27}N_4O^+ [M-Cl]^+$: $m/z = 459.2185$, found $m/z = 459.2193$. 1H NMR (500 MHz, DMSO- d_6) 8.51 (d, 2H, $J = 8.3$ Hz), 8.02 – 7.97 (m, 4H), 7.79 (d, 2H, $J = 8.6$ Hz), 7.72 – 7.67 (m, 4H), 7.62 – 7.59 (m, 2H), 7.55 – 7.52 (m, 2H), 6.35 (s, 4H), 4.95 (t, 1H, $J = 5.1$ Hz), 3.58 (t, 2H, $J = 7.7$ Hz), 3.46 (q, 2H, $J = 5.4$ Hz), 1.92 (m, 2H). $^{13}C\{^1H\}$ NMR

(125 MHz, DMSO-*d*₆) 156.5, 153.9, 146.7, 137.5, 131.4, 130.1, 128.4, 128.0, 127.2, 126.9, 126.3, 119.9, 113.4, 59.2, 49.7, 29.8, 21.0.

Crystal data for **20**•H₂O: C₃₀H₂₇Cl₁N₄O•H₂O, M = 513.02, monoclinic, a = 13.0575(11) Å, b = 19.4079(13) Å, c = 10.2667(8) Å, α = 90°, β = 90.609(3)°, γ = 90°, V = 2601.6(3) Å³, T = 100(2) K, space group P2(1)/c, Z = 4, 41453 reflections measured, 5281 independent reflections (R_{int} = 0.0376). The final R₁ values were 0.0336 (I > 2σ(I)). The final wR(F²) values were 0.0814 (I > 2σ(I)). The final R₁ values were 0.0421 (all data). The final wR(F²) values were 0.0866 (all data). A single crystal was obtained by slow evaporation of a concentrated solution of **20** in water/acetone (1:1).

2.4.20. Synthesis of 1,3-bis(naphthalen-2-ylmethyl)-1,3-dihydrobenzimidazol-2-one (**21**)

1,3-Dihydrobenzimidazol-2-one (0.52 g, 3.88 mmol) was dissolved in acetonitrile (5 mL), potassium hydroxide (0.32 g, 5.70 mmol) was added and the mixture was refluxed for 30 min. 2-(Bromomethyl)naphthalene (0.91 g, 4.12 mmol) was added and the mixture was refluxed overnight. The reaction mixture was filtered hot to remove a white solid, presumed to be potassium bromide. 2-(Bromomethyl)naphthalene (0.76 g, 3.44 mmol) was added to the filtrate and refluxed overnight. The resulting precipitate was collected via vacuum filtration of the hot reaction mixture. The solid was washed with diethyl ether (10 mL) and air-dried to yield a white powder **21** (0.29 g, 18%). Mp: 147 – 150 °C. HRMS (ESI⁺) calcd for C₂₉H₂₂N₂O•Na⁺[M+Na]⁺ of m/z = 437.1630, found m/z = 437.1617. ¹H NMR (500 MHz, DMSO-*d*₆) 7.91 – 7.84 (m, 8H), 7.50 (m, 6H), 7.16 (dd, 2H, J = 5.5, 3.1 Hz), 6.97 (dd, 2H, J = 5.5, 3.1 Hz), 5.30 (s, 4H). ¹³C{¹H} NMR (125 MHz, DMSO-*d*₆) 153.9, 134.5, 132.7, 131.3, 128.8, 128.4, 127.6, 127.5, 126.4, 126.0, 125.9, 125.4, 121.2, 108.4, 44.1. ATR-IR (cm⁻¹): 1683 (C=O).

Crystal data for **21**: C₂₉H₂₂N₂O, M = 414.49, monoclinic, a = 20.1385(6) Å, b = 7.0851(3) Å, c = 29.7407(11) Å, α = 90°, β = 104.9380(10)°, γ = 90°, V = 4100.1(3) Å³, T = 100(2) K, space group C2/c, Z = 8, 33990 reflections measured, 4161 independent reflections (R_{int} = 0.0323). The final R₁ values were 0.0749 (I > 2σ(I)). The final wR(F²) values were 0.1904 (I > 2σ(I)). The final R₁ values were 0.0963 (all data). The final wR(F²) values were 0.2102 (all data). A single crystal was obtained by slow evaporation of a concentrated solution of **21** in water/acetone/acetonitrile (1:1:1).

2.4.21. Synthesis of 1,3-bis(naphthalen-2-ylmethyl)-1,3-dihydrobenzimidazole-2-thione (**22**)

2-(Methylthio)benzimidazole (0.50 g, 3.04 mmol) was dissolved in acetonitrile (8 mL), potassium hydroxide (0.26 g, 4.99 mmol) was added and the mixture was refluxed for 30 min. 2-(Bromomethyl)naphthalene (0.74 g, 3.35 mmol) was added and the mixture was refluxed overnight. The reaction mixture was filtered hot to remove a white solid, presumed to be potassium bromide. 2-(Bromomethyl)naphthalene (0.76 g, 3.44 mmol) was added to the filtrate and refluxed overnight. The resulting precipitate was collected via vacuum filtration of the hot reaction mixture. The solid was washed with diethyl ether and air-dried to yield a white solid **22** (0.92 g, 70%). Mp: 201 – 204 °C. Anal. Calcd for C₂₉H₂₂N₂S: C, 80.90; H, 5.15; N, 6.51%. Found: C, 80.49; H, 5.39; N, 6.40%. ¹H NMR (500 MHz, DMSO-*d*₆) 7.91 – 7.84 (m, 8H), 7.56 – 7.50 (m, 6H), 7.41 (m, 2H), 7.16 (m, 2H), 5.83 (s, 4H). ¹³C{¹H} NMR (125 MHz,

DMSO-*d*₆) 170.1, 133.8, 132.6, 132.3, 131.4, 128.8, 128.3, 127.6, 127.5, 126.4 126.1, 125.3, 123.1, 110.0, 47.6. MS (ESI⁺) calcd for C₂₉H₂₂N₂S•Na⁺[M+Na]⁺ of m/z = 453.1, found m/z = 453.5. ATR-IR (cm⁻¹): 1223 (C=S).

2.4.22. Synthesis of 1,3-bis(quinolin-2-ylmethyl)benzimidazolium chloride (23)

—The synthesis of **23** was previously published.[9]

2.4.23. Synthesis of 2-methyl-1,3-bis(quinolin-2-ylmethyl)benzimidazolium chloride (24)

—2-Methyl-benzimidazole (0.50 g, 3.78 mmol) was dissolved in acetonitrile (7 mL), potassium hydroxide (0.24 g, 4.28 mmol) was added, and the mixture was refluxed for 30 min. 2-(Chloromethyl)quinoline (0.68 g, 3.83 mmol) was added, and the mixture was refluxed overnight. The reaction mixture was filtered hot to remove a white solid, presumed to be potassium chloride. 2-(Chloromethyl)quinoline (0.68 g, 3.83 mmol) was added to the filtrate which was refluxed overnight. The resulting precipitate was collected via vacuum filtration of the hot reaction mixture. The solid was washed with diethyl ether and air dried to yield a cream powder **24** (0.80 g, 46%). Mp: 279 – 282 °C. Calculated for C₂₈H₂₃ClN₄•(H₂O): C, 71.71; H, 5.37; N, 11.95%. Found: C, 70.63; H, 5.50; N, 12.02%. HRMS (ESI⁺) calcd for C₂₈H₂₃N₄⁺ [M-Cl]⁺: m/z = 415.1923, found m/z = 415.1961. ¹H NMR (500 MHz, CD₃OD-*d*₄) 8.42 (d, 2H, J = 8.3 Hz), 7.96 – 7.92 (m, 4H), 7.73 – 7.58 (m, 10H), 6.21 (s, 4H), 3.12 (s, 3H). ¹³C{¹H} NMR (125 MHz, CD₃OD-*d*₄) 154.5, 149.0, 139.4, 133.3, 131.5, 130.0, 129.2, 128.5, 128.1, 120.8, 114.2, 51.2, 12.0.

Crystal data for the solvate of **24**•4(H₂O): C₂₈H₂₃ClN₄•4(H₂O), M = 532.02, orthorhombic, a = 14.5122(2) Å, b = 14.0615(2) Å, c = 24.8411(3) Å, α = 90°, β = 90°, γ = 90°, V = 5069.16(12) Å³, T = 100(2) K, space group Pbca, Z = 8, 29696 reflections measured, 5137 independent reflections (R_{int} = 0.0420). The final R₁ values were 0.0373 (I > 2σ(I)). The final wR(F²) values were 0.0860 (I > 2σ(I)). The final R₁ values were 0.0497 (all data). The final wR(F²) values were 0.0935 (all data). A single crystal was obtained by slow evaporation of a concentrated solution of **24** in water/acetone (1:1).

2.4.24. Synthesis of 5,6-dimethyl-1,3-bis(quinolin-2-ylmethyl)benzimidazolium chloride (25)

—5,6-Dimethyl-benzimidazole (0.41 g, 2.80 mmol) was dissolved in acetonitrile (6 mL), potassium hydroxide (0.18 g, 3.21 mmol) was added, and the mixture was refluxed for 30 min. 2-(Chloromethyl)quinoline (0.50 g, 2.81 mmol) was added, and the mixture was refluxed overnight. The reaction mixture was filtered hot to remove a white solid, presumed to be potassium chloride. 2-(Chloromethyl)quinoline (0.50 g, 2.81 mmol) was added to the filtrate which was refluxed overnight. The resulting precipitate was collected via vacuum filtration of the hot reaction mixture. The solid was washed with diethyl ether and air dried to yield a cream powder **25** (0.80 g, 62%). Mp: 248 – 250 °C. Anal. Calcd for C₂₉H₂₅ClN₄•2(H₂O): C, 69.52; H, 5.83; N, 11.18%. Found: C, 68.92; H, 4.91; N, 10.93%. HRMS (ESI⁺) calcd for C₂₉H₂₅N₄⁺ [M-Cl]⁺: m/z = 429.2079, found m/z = 429.2025. ¹H NMR (500 MHz, DMSO-*d*₆) 10.12 (s, 1H), 8.51 (d, 2H, J = 8.3 Hz), 8.02 (d, 2H, J = 7.8 Hz), 7.82 – 7.72 (m, 8H), 7.63 (m, 2H), 6.19 (s, 4H), 2.32 (s, 6H). ¹³C{¹H} NMR (125 MHz, DMSO-*d*₆) 153.9, 146.8, 143.4, 137.6, 136.4, 130.1, 129.9, 128.5, 128.0, 127.2, 127.0, 119.9, 113.3, 51.2, 19.9.

2.4.25. Synthesis of 2-((2-(2-methoxyethoxy)ethoxy)methyl)benzimidazole (26)

—Synthesized according to literature procedure.[9]

2.4.26. Synthesis of 2-((2-(2-methoxyethoxy)ethoxy)methyl)-5,6-

dimethylbenzimidazole (27)—4,5-Dimethylbenzene-1,2-diamine (0.50 g, 3.67 mmol) was dissolved in 2-[2-(2-Methoxyethoxy)ethoxy]acetic acid (0.68 mL, 4.41 mmol) and was refluxed overnight. The solution was extracted in dichloromethane (2 × 32 mL). The organic layers were combined, dried with magnesium sulfate, and the volatiles were removed under reduced pressure to a brown, viscous oil. The oil was purified with column chromatography using a mobile phase of ethyl acetate/hexanes (1:1) to yield a clear, orange liquid. The orange oil was dissolved in dichloromethane (5 mL) and stirred for 1 h with sodium bicarbonate. Deionized water (10 mL) was added to the residue, and the organic layer was extracted with dichloromethane (3 × 10 mL). The organic layers were combined, dried with magnesium sulfate, and the volatiles were removed under reduced pressure to yield a viscous, orange oil **27** (0.46 g, 45%). HRMS (ESI⁺) calcd for C₁₅H₂₃N₂O₃⁺ [M+H]⁺ of m/z = 279.1709, found [M+H]⁺ of m/z = 279.1713. ¹H NMR (500 MHz, DMSO-*d*₆) δ 12.18 (s, 1H), 7.33 (s, 1H), 7.30 (s, 1H), 4.67 (s, 2H), 3.63 (m, 2H), 3.57 (m, 2H), 3.52 (m, 2H), 3.42 (m, 2H), 3.24 (s, 3H), 2.30 (s, 6H). ¹³C{¹H} NMR (125 MHz, DMSO-*d*₆) δ 150.4, 129.9, 71.3, 69.7, 66.2, 58.0, 19.8.

2.4.27. Synthesis of 2-((2-(2-methoxyethoxy)ethoxy)methyl)-1,3-

bis(naphthalen-2-ylmethyl)benzimidazolium bromide (28)—Compound **26** (1.37 g, 5.47 mmol) was dissolved in acetonitrile (20 mL), potassium hydroxide (0.38 g, 6.77 mmol) was added, and the mixture was refluxed for 30 min. 2-(Bromomethyl)naphthalene (1.21 g, 5.47 mmol) was added to the flask and the mixture was refluxed overnight. The reaction mixture was filtered hot to remove a white solid, presumed to be potassium bromide. 2-(Bromomethyl)naphthalene (1.21 g, 5.47 mmol) was added to the filtrate, which was refluxed overnight. The resulting precipitate was filtered hot, washed with acetonitrile and diethyl ether, and dried in air to yield an off-white solid **28** (0.68 g, 20%). Mp: 195 – 198 °C. Anal. Calculated for C₃₅H₃₅BrN₂O₃: C, 68.74; H, 5.77; N, 4.58%. Found: C, 68.46; H, 5.68; N, 4.60%. HRMS (ESI⁺) calculated for C₃₅H₃₅N₂O₃⁺ [M-Br]⁺ = 531.2648, found m/z = 531.2621. ¹H NMR (500 MHz, DMSO-*d*₆) 8.00 – 7.88 (m, 10H), 7.62 (m, 3H, J = 6.1, 2.9 Hz), 7.55 (m, 5H), 6.14 (s, 4H), 5.45 (s, 2H), 3.78 (m, 2H), 3.40 (m, 2H), 3.32 (m, 2H), 3.22 (m, 2H), 3.09 (s, 3H). ¹³C{¹H} NMR (125 MHz, DMSO-*d*₆) 149.5, 132.6, 132.5, 131.7, 131.1, 128.6, 127.7, 127.6, 127.2, 126.63, 126.57, 126.4, 125.0, 114.1, 71.0, 70.5, 69.42, 69.40, 59.8, 57.9, 49.2.

2.4.28. Synthesis of 2-((2-(2-methoxyethoxy)ethoxy)methyl)-3-(naphthalen-2-ylmethyl)-1-(quinolin-2-ylmethyl)benzimidazolium bromide (29)—Synthesized according to literature procedure[9].

2.4.29. Synthesis of 2-((2-(2-methoxyethoxy)ethoxy)methyl)-1,3-bis(quinolin-2-ylmethyl)benzimidazolium chloride (30)—Compound **26** (0.54 g, 2.16 mmol) was dissolved in acetonitrile (2 mL), potassium hydroxide (0.18 g, 3.21 mmol) was added, and the mixture was refluxed for 30 min. 2-(Chloromethyl)quinoline (0.39 g, 2.20 mmol) was

added to the flask, and the mixture was refluxed overnight. The reaction mixture was filtered hot to remove a white solid, presumed to be potassium chloride. 2-(Chloromethyl)quinoline (0.39 g, 2.20 mmol) was added to the filtrate, which was refluxed overnight. The resulting precipitate was filtered hot, and washed with acetonitrile and diethyl ether at room temperature to yield an off-white solid. The white solid was purified by column chromatography on silica gel using a mobile phase of methanol/dichloromethane (10:90) to yield an off-white solid **30** (0.64 g, 52%). Mp: 203 – 206°C. Anal. Calculated for $C_{33}H_{33}ClN_4O_3$: C, 69.65; H, 5.84; N, 9.84%. Found: C, 69.10; H, 5.82; N, 9.84%. 1H NMR (500 MHz, DMSO- d_6) δ 8.50 (d, 2H, J = 8.3 Hz), 8.12 (m, 2H), 8.01 (d, 2H, J = 8.1 Hz), 7.76 (m, 2H), 7.68 – 7.60 (m, 8H), 6.39 (s, 4H), 5.47 (s, 2H), 3.65 (m, 2H), 3.23 – 3.19 (m, 6H), 3.09 (s, 3H). $^{13}C\{^1H\}$ NMR (125 MHz, DMSO- d_6) 153.8, 151.2, 146.7, 137.5, 131.5, 130.0, 128.4, 127.9, 127.2, 127.0, 126.9, 119.8, 113.7, 70.9, 70.3, 69.3, 69.2, 61.2, 57.9, 50.1.

Crystal data for the solvate of **30**•(**H₂O**)(**C₃H₈O**): $C_{33}H_{33}ClN_4O_3 \cdot (H_2O)(C_3H_8O)$, $M = 647.19$, monoclinic, $a = 13.3734(5)$ Å, $b = 12.2699(6)$ Å, $c = 21.1045(9)$ Å, $\alpha = 90^\circ$, $\beta = 90.419(2)^\circ$, $\gamma = 90^\circ$, $V = 3463.0(3)$ Å³, $T = 100(2)$ K, space group P2(1)/n, $Z = 4$, 29417 reflections measured, 7038 independent reflections ($R_{int} = 0.0433$). The final R_1 values were 0.0501 ($I > 2\sigma(I)$). The final $wR(F^2)$ values were 0.1217 ($I > 2\sigma(I)$). The final R_1 values were 0.0695 (all data). The final $wR(F^2)$ values were 0.1354 (all data). A single crystal was obtained by slow evaporation of a concentrated solution of **30** in isopropanol.

2.4.30. Synthesis of 2-((2-(2-methoxyethoxy)ethoxy)methyl)-5,6-dimethyl-1,3-bis(naphthalen-2-ylmethyl)benzimidazolium bromide (31)—Compound **27** (1.00 g, 3.59 mmol) was dissolved in acetonitrile (10 mL) with potassium hydroxide (0.24 g, 4.31 mmol) and was refluxed for 30 min. 2-(Bromomethyl)naphthalene (0.79 g, 3.59 mmol) was added and the mixture was refluxed overnight. The reaction mixture was filtered hot to remove a white precipitate, presumed to be potassium bromide. The filtrate was stirred with 2-(bromomethyl)naphthalene (0.79 g, 3.59 mmol) and refluxed overnight. The volatiles were removed under reduced pressure, and the residue was washed in a mixture of diethyl ether and acetone (4:1 v/v, 265 mL). A solid impurity was removed via a room temperature vacuum filtration. The volatile components of the filtrate were removed under reduced pressure and the resulting residue was purified by column chromatography on silica gel using a mobile phase of methanol/chloroform (5:95) to yield an off white residue. The residue was washed with diethyl ether (20 mL) at room temperature to afford a cream solid **31**. (0.48 g, 21%). Mp: 163–166 °C. Anal. Calcd for $C_{37}H_{29}BrN_2O_3$: C, 69.48; H, 6.15; N, 4.38%. Found: C, 69.28; H, 6.12; N, 4.34%. 1H NMR (400 MHz, DMSO- d_6) δ 7.97 – 7.87 (m, 8H), 7.81 (s, 2H), 7.57 – 7.51 (m, 6H), 6.08 (s, 4H), 5.35 (s, 2H), 3.71 (m, 2H), 3.35 (m, 2H), 3.29 (m, 2H), 3.20 (m, 2H), 3.09 (s, 3H), 2.32 (s, 6H). $^{13}C\{^1H\}$ NMR (125 MHz, DMSO- d_6) δ 148.1, 137.1, 132.7, 132.5, 131.9, 129.7, 128.6, 127.8, 127.6, 126.7, 125.6, 126.1, 125.0, 71.0, 70.8, 69.40, 60.39, 57.9, 48.9, 20.0. MS (ESI+) calcd for $C_{37}H_{29}N_2O_3^+$ [M-Br]⁺ of $m/z = 559.3$, found [M-Br]⁺ of $m/z = 559.2$.

3. Results and Discussion

3.1 Synthesis and Characterization

Equation 1 outlines the syntheses of the C⁵(C⁶) – substituted *N,N'*-bis(naphthylmethyl)-benzimidazolium salts **2–6** from commercially available benzimidazole reagents with yields ranging from 62 to 84% with the exception of **3**, which has a 19% yield. The lipophilic salts **2–5** were prepared by stirring the select benzimidazole ring (5-methoxybenzimidazole, methyl benzimidazole-5-carboxylate, 5,6-dimethyl-benzimidazole, and dimethyl benzimidazole-5,6-dicarboxylate) with a minimum of 1.4 molar equivalents of base and one molar equivalent of 2-(bromomethyl)naphthalene in acetonitrile at reflux to yield a mono-substituted intermediate. Following the removal of a white precipitate, a second equivalent of 2-(bromomethyl)naphthalene was added and the mixture was heated at reflux overnight to yield salts **2–5**. In the case of **6**, 5-benzimidazolecarboxylic acid was reacted with 4.2 equivalents of sodium bicarbonate for one hour, followed by 5.6 molar equivalents of 2-(bromomethyl)naphthalene in acetonitrile at reflux for 3 days.

Equation 1

Synthesis of the C⁵(C⁶)-substituted *N,N'*-bis(naphthylmethyl)benzimidazolium salts **2–6**.

The hydrophilic imidazolium salts, **7** and **8** were synthesized as described in Equation 2. Compounds **5** and **6** were stirred for 3 days at reflux in 11 M hydrochloric acid to form the chloride salt **7** and mixed chloride/bromide salt **8** in moderate yields. Acidification of **6** with hydrochloric acid led to a mixture of chloride and bromide anions in **8**. Acidification of **6** with aqueous hydrobromic acid was attempted, as well as anion exchange, but the intended salt with a single bromide anion could not be isolated for **8**.

Equation 2

Synthesis of hydrophilic C⁵(C⁶)–substituted *N,N'*-bis(naphthylmethyl)-benzimidazolium salts, **7** and **8**.

The formation of the *N,N'*-bis(naphthylmethyl)benzimidazolium salts **2–8** was confirmed by ¹H NMR, ¹³C NMR and IR spectroscopy. A distinctive resonance for the C⁵(C⁶) substituted benzimidazolium salts (**2–8**) was the C² proton, which was observed within the range of 9.84–10.30 ppm and was consistent with the C² proton of **1** (10.14 ppm).[5] In the ¹H NMR spectra of **2–8**, appearance of singlet resonances in the range of 5.50–6.09 ppm corresponded to the methylene linker between the naphthalene groups and the nitrogen atoms of the benzimidazole ring. Furthermore, the ¹H NMR spectra of **2** and **3** showed singlet resonances at 3.83 ppm and 3.86 ppm, which corresponded to the methyl carbon in the ether and ester substituents at the C⁵ position. The ¹H NMR spectra of **4** and **5** show singlet resonances at 2.31 ppm and 3.84 ppm, which indicated the methyl alkyl and methyl ester groups at the C⁵(C⁶) positions of the benzimidazolium cation, respectively. The methylene of the naphthylmethyl ester at the C⁵ position was observed at 5.53 ppm in the ¹H NMR spectrum of **6**. Transformation of **5** to **7** was suggested by the ¹H NMR spectrum due to the loss of the proton resonance at 3.84 ppm corresponding to the two methyl substituents of **5** and the appearance of a broad resonance at 13.63 ppm indicative of a carboxylic acid. A shift in the C=O stretching frequencies from 1733 cm⁻¹ (**5**) to 1690 cm⁻¹ (**7**) was also observed by IR spectroscopy. In the ¹H NMR spectrum, the formation of **8** from **6** is suggested by the loss of the resonance at 5.53 ppm corresponding to the methylene separating the carbonyl from the naphthalene moiety in **6** and upfield shift of the C² proton from 10.22 ppm (**6**) to 9.84 ppm (**8**). Also, the C=O stretching frequency was shifted from 1719 cm⁻¹ (**6**) to 1703 cm⁻¹ (**8**) in the IR spectroscopy. The cationic portion of **8** was confirmed by single crystal X-ray crystallography (see supplemental information).

Compounds **9–11** were synthesized to compare the anti-cancer activity of benzimidazolium salts with methyl and ester substituents at the C² position to **3–5** with methyl and ester substituents at the C⁵ and C⁶ positions. The synthetic route of compounds **9–11** are outlined in Equation 3. Compounds **9–11** were prepared by the reaction of the corresponding commercially available C² substituted benzimidazole in acetonitrile at reflux with approximately 1.5 equivalents of base. Each deprotonated benzimidazole ring was alkylated with 1.1 equivalents of 2-(bromomethyl)naphthalene, to generate the mono-substituted benzimidazole, and a white solid that was removed by filtration. A second addition of 1.1 equivalents of 2-(bromomethyl)naphthalene was added to synthesize **9–11** in moderate yields (with the exception of **9** having a 26% yield).

Equation 3

Synthesis of hydrophobic C² substituted *N,N'*-bis(naphthylmethyl)-benzimidazolium bromide salts **9–11**.

Compounds **9** and **10** were stirred at reflux for 3 days in 9 M hydrochloric acid in order to transform the ester group at the C² position into the more hydrophilic carboxylic acid substituents (Equation 4). However, treatment of **9** with 9 M hydrochloric acid generated **11-Cl** as the final product, rather than the predicted 2-(carboxymethyl) group at the C² position. Compound **10** was transformed into **12**, with the 2-(carboxyethyl) group at the C² position.

Equation 4

Synthesis of C²-substituted N,N'-bis(naphthylmethyl)benzimidazolium chloride salts, **11-Cl** and **12**, post treatment with 9 M HCl.

Salts **9–12** were characterized by ¹H and ¹³C NMR spectroscopy, **9**, **10**, and **12** were characterized by IR spectroscopy, and **11-Cl** and **12** were characterized by single crystal X-ray crystallography to confirm their structure. The singlet resonances in the ¹H NMR spectra of **9–12** in the range of 6.05–6.14 ppm corresponds to the methylene linker between the naphthalene group and the nitrogen atom of the benzimidazolium cation. Formation of **11-Cl** from **9** was suggested by ¹H NMR spectroscopy by the disappearance of singlet resonances at 5.03 and 2.98 ppm in **9** and the emergence of a singlet at 3.13 ppm in **11-Cl**, which was indicative of the methyl carbon at the C² position. The structure of **11-Cl** was confirmed by single crystal X-ray crystallography. A single crystal for X-ray analysis was obtained by slow evaporation of a concentrated solution of **11-Cl** in a (1:1) mixture of acetone/water (Figure 3). Modification from **10** to **12** was detected in the ¹H NMR spectra by loss of the singlet at 3.44 ppm in **10** and appearance of a broad resonance at 12.6 ppm indicative of the carboxylic acid proton. IR spectroscopy also suggests the conversion was made by the shift of C=O frequencies from 1727 cm⁻¹ (**10**) to 1704 cm⁻¹ (**12**). The structure of **12** was confirmed by single crystal X-ray crystallography. A single crystal suitable for analysis was obtained by slow evaporation of a concentrated solution of **12** in a (1:1) mixture of acetonitrile and methanol (Figure 4).

Compounds **13–16**, bearing alkyl groups at the C² position, served as examples of highly lipophilic benzimidazolium salts and were synthesized to compare their anti-cancer activity to compounds with the hydrophilic functional groups described above. Compound **17**,

synthesized by a similar route with a methoxyethyl ether substituent at the C² position, was synthesized to increase hydrophilicity by adding the heteroatom to the alkyl chain. Cyclization of commercially available o-phenylenediamine with the corresponding aliphatic carboxylic acids in 4 M hydrochloric acid [15] yielded C²-substituted-benzimidazole ring intermediates, which were then alkylated with 2-(bromomethyl)naphthalene. Removal of the precipitate and the addition of the second equivalent of 2-(bromomethyl)naphthalene in acetonitrile at reflux overnight generated salts **13–17** in yields ranging from 24 to 57% (Equation 5).

Equation 5

Synthesis of the C²-substituted N,N'-bis(arylmethyl)benzimidazolium salts, **13–17**, from the commercially available reagent o-phenylenediamine.

The formation of **13–17** was confirmed by spectral analysis. The second alkylation and formation of **13–17** was suggested by the singlet resonances in the ¹H NMR spectra observed in the range of 6.06–6.12 ppm, which corresponded to the methylene linker between the naphthalene-based group and nitrogen atom of the benzimidazolium cation. The formation of the benzimidazolium cation places a positive charge on the N¹-C²-N³ bridge of the benzimidazole ring, resulting in the downfield shift of the methylene carbon bound to the C² position of salts **13–17**, which was indicated within the resonance range of 3.41–3.89 ppm in the ¹H NMR spectra. A distinctive resonance in the ¹H NMR spectrum of **17** was that of the methyl group in the methoxyethyl ether substituent at the C² position, which was observed at 3.03 ppm. The ¹³C NMR spectra indicated the chemical resonances of 11.3–30.5 ppm, which were characteristic of the alkyl chains at the C² position of benzimidazolium cation in salts **13–16**, while the methylene carbons bridging the benzimidazole nitrogen to the naphthalene ring have chemical shifts ranging from 48.6 to 48.7 ppm. Compound **17** has chemical shifts in the ¹³C NMR spectrum at 24.9 ppm likely due to the carbon directly bonded to the C² atom, 48.9 ppm for the bridging methylene, and 58.0 ppm and 58.2 ppm for the two ether carbons. The structures of **13**, **15**, and **17** were confirmed by single crystal X-ray crystallography. Crystals of **13** and **15** were both obtained from a (1:1) mixture of acetonitrile and methanol (Figure 5 and Figure 6, respectively). A crystal of **17** was isolated from a (1:1:1) mixture of acetone, ethyl acetate, and methanol (Figure 7).

In a similar synthetic route, and separate attempt to increase hydrophilicity when compared to **1**, a series of 2-(3-hydroxypropyl)-N,N'-bis(arylmethyl)-benzimidazolium salts, **18–20**, was synthesized to add hydrophilic substituents at the C² and the N¹(N³) positions of the benzimidazole ring (Equation 6). The starting benzimidazole, 3-(benzimidazol-2-yl)propan-1-ol, was synthesized from o-phenylenediamine by a literature procedure.[8]

Compound **18** was synthesized by deprotonating the benzimidazole and addition of 1 molar equivalent of 2-(bromomethyl)naphthalene to form the mono-alkylated intermediate. Compound **18** was isolated after a second addition of 2-(bromomethyl)naphthalene. Compounds **19** and **20** were synthesized by deprotonation of the starting benzimidazole and addition of one molar equivalent of 2-(chloromethyl)quinoline to form the mono-alkylated intermediate. The synthesis of **19** was achieved by addition of one molar equivalent of 2-(bromomethyl)naphthalene while **20** required a second addition of one molar equivalent of 2-(chloromethyl)quinoline.

Equation 6

Synthesis of 2-propan-1-ol substituted benzimidazolium salts **18–20**.

The distinctive alcohol resonance of **18–20** ranged from 4.78 to 4.95 ppm in the ^1H NMR spectra, whereas the resonances corresponding to the bridging methylenes between the aryl group and nitrogen atoms ranged from 6.09 to 6.35 ppm. The structure of compounds **18–20** were all confirmed by single crystal X-ray crystallography. Crystals of **18–20**, suitable for single crystal X-ray diffraction, were obtained by slow evaporation of a concentrated solution of the compounds in different solvent systems. A single crystal of **18** was grown from methanol (Figure 8). A single crystal of **19** was obtained from slow evaporation of a concentrated solution of a 1:1:1 mixture of methanol, ethyl acetate, and acetone (Figure 9). A single crystal of **20**, with a co-crystallized water molecule, was isolated from slow evaporation of a concentrated solution of water and acetone (1:1) (Figure 10).

Benzimidazoles with group 16 heteroatoms, oxygen and sulfur, bound directly to the C² position of the benzimidazole ring were investigated to determine their effect on the anti-cancer properties of neutral, naphthylmethyl substituted benzimidazoles. Compounds **21** and **22** were synthesized from the commercially available benzimidazoles 2-hydroxybenzimidazole and 2-(methylthio)benzimidazole. Each benzimidazole was reacted with approximately 1.5 molar equivalents of potassium hydroxide and 2.0 molar equivalents of 2-(bromomethyl)naphthalene, which was introduced in subsequent steps (Equation 7). After the second alkylation, loss of the proton bound to the group 6 heteroatom resulted in the neutral, substituted benzimidazole compounds **21** and **22**, instead of the benzimidazolium salt in 18% and 70% yield, respectively. The formation of the neutral compounds **21** and **22** was suggested by ^1H NMR, ^{13}C NMR, IR spectroscopy. High resolution mass spectrometry was used to further characterize **21**, whereas mass spectrometry and elemental analysis were employed to further characterize **22**. Appearance of a singlet resonance at 5.30 ppm and 5.83 ppm in the ^1H NMR spectra of **21** and **22**,

respectively corresponded to the methylene linker between the naphthalene group and nitrogen atom of the benzimidazole ring. These chemical shifts are significantly upfield compared to related benzimidazolium salts suggesting the absence of the positive charge in resonance between N¹-C²-N³. IR spectroscopy also suggested the carbonyl (C=O) and thio-carbonyl (C=S) moieties, versus the potential alcohol and thiol groups, which were noted by the frequencies at 1638 cm⁻¹ and 1223 cm⁻¹, respectively. Single-crystal X-ray analysis confirmed the structure of **21** (Figure 11). A single crystal of **21** was obtained by slow evaporation of a concentrated solution of the compound in a mixture of acetonitrile, acetone and water (1:1:1).

Equation 7

Synthesis of the N,N'-bis(naphthylmethyl)benzimidazole products, **21** and **22**, bearing select heteroatoms from Group 6 at the C² position.

To increase aqueous solubility, **23–25**, with two quinolylmethyl substituents, were prepared by stirring the corresponding benzimidazole starting material (benzimidazole, 2-methylbenzimidazole and 5,6-dimethylbenzimidazole) in acetonitrile at reflux overnight with 1.1 molar equivalents of potassium hydroxide and one molar equivalent of 2-(chloromethyl)quinoline (**23** was previously published).[9] Upon removal of a precipitate, a second equivalent of 2-(chloromethyl)quinoline was added and the mixture was heated at reflux overnight to produce the corresponding salts in moderate yields (Equation 8). Compounds **24** and **25** were characterized by ¹H NMR and ¹³C NMR spectroscopy and high resolution mass spectrometry. In the ¹H NMR spectra of **24 – 25**, the appearance of a singlet resonance observed in the range of 6.19–6.21 ppm, corresponding to the methylene linker between the quinoline groups and nitrogen atom of the benzimidazolium cation, is consistent with related compounds.[5] The methyl resonances of **24** and **25** were observed at 3.12 ppm and 2.32 ppm, by ¹H NMR spectroscopy and were indicative of the methyl alkyl substituents at the C² and C⁵(C⁶) positions, respectively. Compound **24**, was characterized by single-crystal X-ray crystallography (Figure 12). A single crystal, containing **24** and four water molecules, was obtained by slow evaporation of a concentrated solution of the compound in an acetone and water (1:1) mixture.

Equation 8

Synthesis of the N,N'-bis(quinolylmethyl)benzimidazolium chloride salt series, **23–25**.

Compounds **28–31**, with an extended ether chain at the C² position, were synthesized in an attempt to increase solubility in a different manner (Equation 9, the synthesis of **26** and **29** were previously published).[9] Cyclization of commercially available *o*-phenylenediamine and 4,5-dimethylbenzene-1,2-diamine with 2-(2-(2-methoxyethoxy)ethoxy)acetic acid generated the benzimidazole intermediates **26** and **27**, which were then alkylated with 2-(bromomethyl)-naphthalene or 2-(chloromethyl)quinoline in a two-step process to yield the **28**, **30**, and **31** in yields ranging from 20 to 52 %. Compounds **28**, **30**, and **31** were characterized by ¹H NMR and ¹³C NMR spectroscopy and elemental analysis. In the ¹H NMR spectra of **28**, **30**, and **31**, the appearance of singlet resonances in the range of 6.08–6.39 ppm corresponds to the methylene linker between the aryl groups and nitrogen atoms of the benzimidazolium cation. The protons of the methylene bridging the C² to the first oxygen of the ether chain appear significantly downfield, in the 5.35–5.47 ppm range, due to the adjacent electronegative oxygen atom and positive charge of the benzimidazolium cation. The ¹H NMR spectrum of **31** displayed the methyl groups at the C⁵ and C⁶ positions at a distinctive resonance between 2.30 and 2.33 ppm. Compound **30** was also characterized by single crystal X-ray crystallography to confirm the structure. A single crystal of **30**, with a co-crystallized water molecule and isopropanol molecule, was obtained by slow evaporation of a concentrated solution of the compound in isopropanol (Figure 13).

Equation 9

Synthesis of **28–31**.

3.2 In vitro Anti-tumor Activity and Structure Activity Relationship Study

Compound **1** has been reported to have in vitro activity comparable to that of the well-known chemotherapeutic agent cisplatin.[5] However, **1** was limited by poor aqueous solubility, and therefore does not have the potential to be systemically administered. Compounds **2–25** and **28–31** were synthesized to establish a SAR and discover benzimidazolium salts with the potential to be used clinically. Each compound was

specifically designed with functional groups of ranging hydrophilicities to understand how changes in functional groups affect the anti-cancer activity and aqueous solubility.

The in vitro activities of **2–25** and **28–31** were evaluated against the NSCLC cell lines NCI-H460, NCI-H1975, HCC827, and A549 (Table 1). The cells were exposed to the compounds for a duration of 72 hours at which time the optional MTT assay protocol was utilized in order to determine the IC₅₀ values (the concentration that causes 50% inhibition of cell growth, relative to the controls). Cisplatin was used as a positive control and had IC₅₀ values of 3 μ M (H460), 10 μ M (H1975), 6 μ M (HCC827) and 7 μ M (A549).

The range of IC₅₀ values indicated that the substituents at the C⁵ and/or C⁶ positions directly impact the in vitro activity. IC₅₀ values of the C⁵(C⁶)-substituted benzimidazolium salts **2–8** have shown that salts with highly lipophilic substituents exhibited higher anti-proliferative activities against the NSCLC cell lines. Compounds **2–8** all have a naphthylmethyl substituent at both the N¹ and N³ positions so direct comparisons can be made when changing substituents at the C⁵ and/or C⁶ positions. Compounds **2** and **4** had the lowest IC₅₀ values, with ether and dimethyl substituents respectively, with **4** being the most lipophilic in this group and having the lowest values collectively. Compound **6**, with a lipophilic naphthylester functional group, was also highly active. Compound **3**, with one methylester, had moderate activity whereas **5** with two methylesters had poor activity. Compound **8**, with one carboxylic acid, had poor activity, whereas **7**, with two carboxylic acids, was inactive against all four cell lines. This suggests that the inclusion of polar hydrophilic groups, like a carboxylic acid, causes a substantial decrease in anti-proliferative activity. Although there is a range of hydrophilicity in the substituents described above, all compounds have less than 0.5 mg/mL aqueous solubility. However, these comparisons give insight about what types of functional groups that can be added without hindering the anti-cancer activity.

Compounds **9–22**, with substituents in the C² position, showed varying activities against the four NSCLC cell lines. Compound **11**, with a methyl group, and **13–16**, with alkyl chains of increasing lengths, all had very high anti-cancer activities. Compound **16** with the longest alkyl chain and most lipophilicity, a heptyl chain, had the highest activity with IC₅₀ values in the sub-micromolar range for all four cell lines tested. Compound **17** with an ether moiety also had high anti-cancer activity comparable to **11** and **13–16**. Compounds **9** and **10** with ester moieties at the C² position proved to be active. These have added lipophilicity with the alkyl linkers between the C² carbon and the carbonyl, when compared to **3**, which suggests a cause for the higher activity. Compound **12**, with the carboxylic acid moiety, was inactive against the four NSCLC cell lines (IC₅₀ values: > 30 μ M) and supported the trend previously observed with carboxylic acid substituents at the C⁵(C⁶) positions. Compound **18**, with a propanol chain at the C² position, had less anti-cancer activity than **1** suggesting alkyl chains ending with an alcohol could be added to increase aqueous solubility without severely lessening activity.

Compounds **21** and **22**, substituted with group **6** heteroatoms at the C² position, were virtually inactive and had poor activity against all cell lines tested. Although these compounds are highly lipophilic from the naphthylmethyl substituents, they lack the positive

charge of the related benzimidazolium salts. These results suggest that the positive charge is essential to the anti-cancer activity of these benzimidazolium compounds.

Compounds **2–18**, **21**, and **22** were all substituted with naphthylmethyl groups at the N¹ and N³ positions with various functional groups at different positions of the benzimidazolium ring. Compounds **19** and **20** were substituted with differing arylmethyl groups at the N¹ and N³ positions in a separate attempt to increase water solubility. Compound **19** has one naphthylmethyl group and one quinolylmethyl group while **20** has two quinolylmethyl groups. The nitrogen heteroatom was added to increase the hydrogen bonding capability and also allow for the possibility of protonating to form a cation at a separate site to further increase solubility.[16] Compound **19**, with one quinoline group, had decreased activity from **18**, whereas **20** had even further decreased activity with moderate to poor results. This suggests that although a quinoline group could increase the solubility, it will decrease the activity.

Compounds **23–25** bearing quinolylmethyl groups at both the N¹ and N³ positions can be directly compared to the naphthylmethyl derivatives **1**, **4**, and **11** which have zero, one, and two methyl substituents around the benzimidazole ring respectively. The quinolylmethyl derivatives **23–25** had high anti-cancer activity comparable to cisplatin, **1**, **4**, and **11**. Compound **25** had the highest activity, even with the inclusion of two quinolylmethyl groups, which as seen previously, decreased the activity. This suggests that adding hydrophilicity with quinolylmethyl substituents should be combined with the inclusion of lipophilic groups such as methyl substituents to provide balance between water solubility and the lipophilicity needed for anticancer activity. Although water solubilizing groups were present in various compounds discussed above, **23–25** are the first compounds discussed herein that have aqueous solubility above 0.5 mg/mL (see supplemental information). With higher aqueous solubilities and high anti-cancer activity, **23–25** have clinical potential.

To complete the SAR study we have established thus far, **28–31** were synthesized to combine the best functional groups and substituents in an attempt to discover the idealized benzimidazolium salt with sufficient aqueous solubility for systemic administration and high anti-proliferative effects. Compounds **28–31** all have a methyl-terminated diethylene oxide chain at the C² position since ether substitution will enhance aqueous solubility without severely lessening anti-cancer activity. The arylmethyl group at the N¹ and N³ position was also varied to determine the most effective compound. All four compounds, **28–31**, exhibited high anti-proliferative activity comparable to that of cisplatin. The most lipophilic salt **31** had IC₅₀ values in the sub-micromolar range against the H460 and H1975 cell lines while **29** and **30** had sub-micromolar values against the H1975 cell line. In combination with the high anti-cancer values, **28–31** had higher aqueous solubilities with **30** being soluble at over 40 mg/mL.

3.3 NCI-60 Human Tumor Cell Line Screen

The National Cancer Institute's (NCI) Developmental Therapeutic Program (DTP) agreed to test **1**, **3–5**, **10**, **11**, **13–18**, **20–25**, and **28–30** in their 60 human tumor cell line one-dose assay. Details about the procedures used to test compounds can be found on the DTP

website. Briefly, in the one-dose assay, cells are plated at a concentration based on their doubling rate and exposed to the tested compound at one concentration (10 μ M). Results are then given as a percent growth relative to the starting cell density. A negative growth percentage means there were less cells alive at the end of the experiment than at the beginning and the compound is considered lethal at that concentration.

As expected due to the high lipophilicity, **4**, **15**, and **16** were the most active compounds with negative growth values for all NSCLC cell lines tested and an average growth rate of negative 58.98%, negative 77.96%, and negative 77.07 respectively (Table 2). Also highly lipophilic, **11**, **14**, and **17** had an average growth rate of negative 26.84%, negative 30.74%, and negative 23.19% respectively, against the NSCLC cell lines tested. Compounds **1**, **10**, **25**, **28**, and **29** also had high activity against all cell lines tested with lethal growth rates for several cell lines and an average growth rate of 1.29%, 9.86%, 11.83%, 13.61%, and 12.40% respectively. Compounds **3**, **18**, **23**, and **30** with hydrophilic groups had moderate activities with average growth rates of 45.23%, 33.13%, 44.24% and 24.73% respectively. Compounds **5**, **13**, **20**, and **24** all had poor activity with average growth rates of 87.03%, 56.02%, 80.52%, and 81.95%, respectively. Finally, **21** and **22**, the urea and thiourea compounds respectively, were virtually inactive with average growth percentages of 77.63% and 84.80% respectively.

The results from the one-dose assay follow the same trend we observed from the SAR described above. Compounds **4**, **15**, and **16**, three of the most lipophilic compounds, had the highest activities. The slightly less lipophilic derivative **11** (having one methyl substituent versus two methyl substituents in **4**) had slightly less activity. The highly lipophilic derivative **14**, with a propyl group at the C² position, also had high activity suggesting the position on the benzimidazole ring is less important than the identity of the added substituents in affecting activity. Compound **17** with an ether chain at the C² had excellent values suggesting the ether modification causes a mild change in anti-cancer activity while helping to increase solubility. Cells exposed to **1** had an average growth rate close to 0% showing virtually complete inhibition of growth which is expected due to the lipophilic nature of the compound. Compounds **23** and **24**, with two quinolylmethyl groups at the N¹ and N³ positions versus two naphthylmethyl groups as in **1** and **11**, had significantly less activity due to the loss of lipophilicity. Also, the loss in activity from **4** to **25**, was even more drastic than the loss from **1** to **23**. Compounds **18** and **20**, with a propanol chain at the C² position also had a decrease in activity when compared to **1**, with a proton at the C² position. Compound **29** with an extended ether chain at the C² position and one naphthylmethyl and one quinolylmethyl substituent bound to the nitrogens, had moderate anti-cancer activity slightly worse than **1**; however, the aqueous solubility of **29** is significantly higher than **1**. Compounds **28** and **30** also had high anti-cancer activity comparable to **1**. Compounds **3**, **5** and **10**, all with ester substituents, had drastically different activities. Compound **10** had the highest activity and was the most lipophilic, whereas **3** with one ester had higher activity than **5** with two ester substituents. Compounds **21** and **22**, neutral benzimidazole compounds, were virtually inactive further suggesting the importance of the positive charge for high anti-cancer activity. The most unexplainable result came from **13**, with an ethyl chain at the C² position and naphthylmethyl substituents at both nitrogens. This compound

had poor activity considering the C²-methyl (**11**), -propyl (**14**), -butyl (**15**), and -heptyl (**16**) compounds had such high anti-cancer activity. These results confirm the claim that lipophilicity increases activity and a positively charged compound is necessary for high anti-cancer activity. Several direct comparisons were made by changing one specific group and although hydrophilic substitutions can increase water solubility they will also decrease the activity.

The NCI's DTP will further test compounds if the compound is lethal against several cell lines in the one-dose assay. The second stage of the 60 cell line screen is the five-dose assay. In the five-dose assay, the DTP exposed all 60 cell lines to **1**, **4**, **11**, **14**, **15**, **18**, **23**, **25**, and **29** at five doses (10 nM, 100 nM, 1 μ M, 10 μ M, and 100 μ M) and then determined the cellular density to compare with control cells. Three different values are given as results to the five-dose assay (Table 3 and Table 4). The GI50 is the concentration that causes growth inhibition of 50% of cells relative to control, the TGI is the concentration of total growth inhibition meaning the number of live cells at the beginning and end of the assay are equivalent, and the LC50 is the lethal concentration of 50% of cells relative to the initial cell count. Compounds **1**, **4**, **11**, **14** and **15** were highly active with GI50, TGI, and LC50 values all in the sub-micromolar and low micromolar range (Figure 14). However, multiple LC50 and TGI values were not recorded because the growth percentage was higher at the 100 μ M dose than at the 10 μ M dose. No explanation was given to us from the DTP and we cannot explain this trend. The more hydrophilic derivatives **18**, **23**, and **25** had GI50 values in the sub-micromolar to low micromolar range (a table of values can be found in the supplemental information section). However, the TGI and LC50 values ranged from the low micromolar to high micromolar range. Although the hydrophilic compounds were able to slow the growth of all the NSCLC lines tested as shown by the GI50, the complete inhibition and lethality concentrations were significantly higher than the concentrations of **1**, **4**, **11**, **14**, and **15** needed to achieve inhibition and lethality. Compound **29** had results intermittent between the highly lipophilic compounds and the more hydrophilic compounds with results in the sub-micromolar and low micromolar range for the GI50 and TGI with low to mid- micromolar values for the LC50 (Figure 14). The Results from the five-dose assays were consistent with results from MTT assay experiments in our lab and the one-dose assays done by the DTP. The more lipophilic compounds had higher anti-cancer activity while the more hydrophilic compounds had lower anti-cancer activity further confirming the SAR developed with the in vitro assays described above.

3.4 Preliminary In Vivo Toxicity Study

Due to the high anti-cancer activity of **29** and the ability to be solubilized by a chemical excipient, 2-hydropropyl- β -cyclodextrin (2-HP β CD), **29** was chosen for a preliminary in vivo toxicity study using C57BL/6 mice. 2-HP β CD is an FDA approved excipient that is considered generally regarded as safe (GRAS) and used in several drug formulations.[17] Compound **29** was dissolved in the vehicle control solution, a 20% (w/v) solution of 2-HP β CD dissolved in sterile PBS. Six week old C57BL/6 mice were allowed to acclimate to their cages for 5 days prior to any injections, with 3 animals per cage. The vehicle control group was injected on days 0, 7, 14, 21, 24, and 29 by intraperitoneal (IP) injection. The vehicle control group was injected with 100 μ L of the vehicle control solution. The

experimental group was only injected on day 0 by IP injection. The experimental group was injected with 100 μ L of a 20 mg/kg dose (assuming an average mass of 20 g for each mouse) of **29** dissolved in the vehicle solution. The weight and behavior was closely monitored for each mouse for the duration of the study (Figure 15).

A sharp decrease in weight was observed for two of the three mice injected with **29** over the course of the first two days following injection. These two mice had also lost most of their strength and were therefore euthanized (Figure 16). The third mouse had minimal weight loss after the first injection and by day 16, had gained 6% in mass from the original weight. This mouse was sacrificed on day 16 because it was the lone surviving mouse. Compound **29** proved to be quite toxic at the treated dose with a survival rate of 33% after the lone injection. The results from this preliminary toxicity study can be used as a starting point for future in vivo toxicity studies to determine the maximum tolerated dose to maintain 100% survival and guide a lung xenograft model to determine the efficacy in an in vivo model. All mice injected with the vehicle control gained weight on a consistent basis and survived for the duration of the experiment.

In this work, a series of *N,N'*-bis(arylmethyl)benzimidazolium salts with hydrophilic and hydrophobic substituent variations were synthesized, characterized and evaluated against four NSCLC cell lines. An in vitro SAR was established after combining the IC₅₀ values and structural details of compounds **2–25** and **28–31**. Results suggested that highly lipophilic compounds, that are positively charged, possess potent anti-cancer activity. However, these compounds are limited by their poor water solubilities. Unfortunately adding highly hydrophilic substituents significantly decreases the in vitro anti-cancer properties of benzimidazolium salts. In attempt to increase water solubility, mildly hydrophilic groups such as ethers and quinoline moieties were added to increase the aqueous solubility resulting in highly active compounds with aqueous solubility. The NCI's DTP also tested multiple compounds against their 60 human tumor cell line screen in the one-dose and five-dose assays. The results were consistent with those determined in our lab. The more lipophilic compounds had higher activity, while less lipophilic compounds had less potent activity. The NCI-60 human tumor screen also confirmed the need for a positive charge to have high activity considering the two neutral compounds were inactive. Compound **29**, with high anti-cancer activity and the ability to be solubilized by 2-HP β CD, was used for a preliminary in vivo toxicity study. The compound proved to be toxic at the dose tested with a 33% survival rate days after the lone injection. However, this provides information for dosing regimens that can be used in future toxicity studies and in vivo lung xenograft models to determine the compounds clinical potential and guide the synthesis of future derivatives. Future experiments will also involve more in vitro studies to determine mode and mechanism of cell death.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

NCI	National Cancer Institute
IC₅₀	inhibitory concentration 50%
NSCLC	non-small cell lung cancer
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
SAR	structure-activity relationship
GI₅₀	growth inhibition 50%
TGI	total growth inhibition
LC₅₀	lethal concentration 50%
DTP	Developmental Therapeutics Program
2-HPβCD	2-hydropropyl- β -cyclodextrin

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Highlights

1. Hydrophobic and hydrophilic benzimidazolium salts were synthesized/characterized
2. Benzimidazolium salts were tested for their anti-cancer properties
3. Lipophilic benzimidazolium salts have higher anti-cancer activity
4. NCI's DTP tested 21 compounds in the NCI-60 human tumor cell line screen

Figure 1.
Examples of imidazolium salts, Lepidiline A, Lepidiline B and tetrahydrobenzodifuran benzimidazolium salt, with in vitro anti-cancer activity against the A549 NSCLC cell line.

Figure 2.
Schematic representation of N,N'-bis(naphthylmethyl)imidazolium bromide salt, **1**.

Figure 3.

Thermal ellipsoid plot of **11-Cl** in the crystal structure of **11-Cl·(H₂O)**. All thermal ellipsoids have been drawn at the 50% probability level. Hydrogen atoms and carbon atom labels have been removed for clarity. A water solvate molecule is not shown for clarity.

Figure 4.
Thermal ellipsoid plot of **12**. All thermal ellipsoids have been drawn at the 50% probability level. Hydrogen atoms and carbon atom labels have been removed for clarity.

Figure 5.

Thermal ellipsoid plot of **13**. All thermal ellipsoids have been drawn at the 50% probability level. Hydrogen atoms and carbon atom labels have been removed for clarity.

Figure 6.
Thermal ellipsoid plot of **15**. All thermal ellipsoids have been drawn at the 50% probability level. Hydrogen atoms and carbon atom labels have been removed for clarity.

Figure 7.

Thermal ellipsoid plot of **17**. All thermal ellipsoids have been drawn at the 50% probability level. Hydrogen atoms and carbon atom labels have been removed for clarity.

Figure 8.

Thermal ellipsoid plot of **18**. All thermal ellipsoids have been drawn at the 50% probability level. Hydrogen atoms and carbon atom labels have been removed for clarity.

Figure 9.

Thermal ellipsoid plot of **19**. All thermal ellipsoids have been drawn at the 50% probability level. Hydrogen atoms and carbon atom labels have been removed for clarity.

Figure 10.

Thermal ellipsoid plot of **20** in the structure of **20•(H₂O)**. All thermal ellipsoids have been drawn at the 50% probability level. Hydrogen atoms, carbon atom labels, and the co-crystallized water solvent molecule have been removed for clarity.

Figure 11.

Thermal ellipsoid plot of **21**. All thermal ellipsoids have been drawn at the 50% probability level. Hydrogen atoms and carbon atom labels have been removed for clarity.

Figure 12.

Thermal ellipsoid plot of **24** in the structure of **24•4(H₂O)**. All thermal ellipsoids have been drawn at the 50% probability level. Hydrogen atoms, four solvate water molecules and carbon atom labels have been removed for clarity.

Figure 13.

Thermal ellipsoid plot of **30** in the structure of **30**•(**H₂O** + **C₃H₈O**). All thermal ellipsoids have been drawn at the 50% probability level. Hydrogen atoms and carbon atom labels have been removed for clarity. A water solvate molecule and isopropanol molecule are not shown for clarity.

Figure 14.

Plot of growth curves for NSCLC cell lines exposed to 1, 4, 11, 14, 15, and 29 at five different concentrations.

Weight gain % chart for mice exposed to 29 **Figure 15.**

Average weight gain % for mice injected with **29** and the vehicle control.

Figure 16.

Kaplan-Meier curve for animals injected with **29** and the vehicle control. 4. Conclusion

Table 1

IC₅₀ values of synthesized N,N'-bis(arylmethyl)benzimidazolium salts **1–25** and **28–31**.

Compound	IC ₅₀ values(μM)			
	NCI-H460	NCI-H1975	HCC827	A549
cisplatin	3	11	6	8
1	3	3	3	5
2	4	2	4	6
3	9	7	12	16
4	2	2	2	3
5	26	15	20	23
6	4	4	7	7
7	> 30	> 30	> 30	> 30
8	16	23	> 30	12
9	7	6	8	8
10	4	2	4	8
11	3	2	4	4
12	> 30	> 30	> 30	> 30
13	3	< 1	4	3
14	2	< 1	< 1	2
15	2	< 1	2	2
16	< 1	< 1	< 1	< 1
17	3	2	3	3
18	4	4	6	10
19	4	3	11	12
20	11	7	17	25
21	> 30	19	> 30	> 30
22	> 30	> 30	> 30	> 30
23	4	3	6	10
24	3	2	7	9
25	2	< 1	3	4
28	3	3	5	5
29	3	< 1	6	3
30	4	< 1	4	9
31	< 1	< 1	2	2

Results from the DTP's one dose assay in the NCI-60 human tumor cell line screen for the NSCLC cell lines tested. Cells were exposed to compounds at a single dose (10 μ M) and results are given as a growth percent relative to controls.

Table 2

Compound	Growth percentage										
	A549/ATCC	EKVX	HOP-62	HOP-92	NCI-H226	NCI-H23	NCI-H322M	NCI-H460	NCI-H522	Average	
1	30.92	13.90	3.98	-27.65	19.65	-0.74	34.60	-34.24	-28.78	1.29	
3	74.97	71.02	63.76	-10.74	61.32	34.63	57.21	28.64	26.26	45.23	
4	-71.27	-64.27	-73.03	-80.82	-25.54	-71.85	-8.28	-76.75	*	-58.98	
5	92.23	94.28	78.84	84.65	77.67	93.15	90.58	94.51	77.34	87.03	
10	38.29	42.10	18.99	-34.39	17.66	-7.39	36.58	6.29	-29.37	9.86	
11	8.96	9.57	-61.97	-68.17	-9.12	-29.46	9.37	-73.90	*	-26.84	
13	74.18	76.67	81.32	21.85	64.91	28.77	90.72	53.64	12.10	56.02	
14	-19.44	-4.84	-55.44	-48.12	-4.44	-12.93	4.72	-76.06	-60.15	-30.74	
15	-72.31	-84.62	-76.77	-70.71	-72.70	-83.93	-82.25	-81.11	-77.24	-77.96	
16	-77.50	-73.52	-70.58	-84.36	-70.78	-76.88	-85.97	-72.99	-81.07	-77.07	
17	4.72	4.70	-19.12	-53.10	-7.12	-24.31	5.25	-65.32	-54.42	-23.19	
18	68.38	47.58	37.52	-24.30	48.48	19.04	49.91	18.43	*	33.13	
20	93.22	97.76	87.52	47.91	79.78	74.97	91.67	91.82	60.05	80.52	
21	85.16	79.55	62.84	79.90	83.94	77.67	81.59	74.57	73.41	77.63	
22	87.60	93.12	81.86	83.40	73.45	93.11	92.63	69.84	88.23	84.80	
23	53.98	74.67	53.77	-8.16	37.12	31.99	78.13	32.38	*	44.24	
24	87.89	102.12	82.74	51.97	89.64	75.84	94.92	95.81	56.61	81.95	
25	30.35	39.64	4.38	-27.26	9.61	1.58	36.00	0.34	*	11.83	
28	33.14	47.43	17.15	-32.14	17.17	5.56	40.53	10.27	-16.61	13.61	
29	22.48	31.03	11.66	-18.24	28.11	1.03	26.79	7.94	0.79	12.40	
30	39.38	71.61	20.84	-22.15	37.87	12.75	47.41	16.54	-1.70	24.73	

* DTP did not test this compound on this cell line.

Table 3

Results from the DTP's five-dose assay in the NCI-60 human tumor cell line screen for **1**, **4**, and **11** against the nine NSCLC cell lines tested. Cells were exposed to 1 at 5 different concentrations and results are given in molar concentrations for the GI50, TGI, and LC50.

Cell Line	GI50, TGI, and LC50 values (μM) from the five-dose assay								
	1			4			11		
	GI50	TGI	LC50	GI50	TGI	LC50	GI50	TGI	LC50
A549/ATCC	1.87	3.88	8.01	1.42	2.95	n/a	1.76	3.40	6.55
EKVZ	1.47	2.95	n/a	1.37	2.88	n/a	1.78	4.08	n/a
HOP-62	1.54	2.98	n/a	1.03	2.47	n/a	1.69	3.24	n/a
HOP-92	0.192	1.57	4.93	0.587	2.53	n/a	0.0317	2.06	5.03
NCI-H226	1.46	3.25	n/a	1.39	3.42	n/a	1.44	3.20	n/a
NCI-H23	1.05	2.51	n/a	0.872	2.28	n/a	n/a	2.76	n/a
NCI-H322M	1.49	2.89	n/a	1.14	2.42	n/a	1.56	3.00	5.78
NCI-H460	1.35	2.91	n/a	0.662	n/a	n/a	1.47	3.02	n/a
NCI-H522	1.25	2.82	n/a	1.25	2.99	n/a	0.0360	2.27	n/a

Table 4

Results from the DTP's five-dose assay in the NCI-60 human tumor cell line screen for **14**, **15**, and **29** against the nine NSCLC cell lines tested. Cells were exposed to 1 at 5 different concentrations and results are given in molar concentrations for the GI50, TGI, and LC50.

Cell Line	GI50, TGI, and LC50 values (μM) from the five-dose assay								
	14			15			29		
	GI50	TGI	LC50	GI50	TGI	LC50	GI50	TGI	LC50
A549/ATCC	0.619	2.04	5.09	1.04	2.42	n/a	0.748	10.6	37.1
EKVZ	0.653	2.33	n/a	1.17	n/a	n/a	1.27	10.8	50.9
HOP-62	0.537	2.03	n/a	1.05	2.55	n/a	0.468	2.52	9.53
HOP-92	0.192	0.911	n/a	0.298	1.35	n/a	0.207	0.718	5.22
NCI-H226	0.373	1.80	n/a	1.08	n/a	n/a	0.874	4.83	45.2
NCI-H23	0.354	1.60	n/a	0.371	1.70	n/a	0.386	10.3	53.5
NCI-H322M	1.04	2.31	n/a	1.22	2.57	n/a	1.08	7.09	28.6
NCI-H460	0.337	1.36	n/a	0.436	n/a	n/a	0.424	1.56	5.70
NCI-H522	0.300	1.05	n/a	0.289	1.35	n/a	0.248	0.936	4.59