Synthesis, characterization, and in vitro SAR evaluation of N,N'-Bis(arylmethyl)-C²-alkyl substituted imidazolium salts

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Abstract

A series of C²-alkyl substituted N,N'-bis(arylmethyl) imidazolium salts were synthesized, characterized, and tested for their in vitro anti-cancer activity against multiple non-small cell lung cancer cell lines by our group and the National Cancer Institute's-60 human tumor cell line screen to establish a structure-activity relationship. Compounds are related to previously published N,N'-bis(arylmethyl)imidazolium salts but utilize the historical quinoline motif and anion effects to increase the aqueous solubility. Multiple derivatives displayed high anti-cancer activity with IC₅₀ values in the nanomolar to low micromolar range against a panel of non-small cell lung cancer cell lines. Several of these derivatives have high aqueous solubilities with potent anti-proliferative properties and are ideal candidates for future in vivo xenograft studies and have high potential to progress into clinic use.

Cancer is a disease that kills millions of people each year. Lung cancer is the leading cause of cancer related deaths, accounting for one in every four cancer deaths.¹ Treatments are based upon the type of lung cancer and physical characteristics of the cancer cells. These treatments include surgery, radiation, targeted therapies, and chemotherapy. However, the 5-year survival rate for lung cancer is only 17% suggesting that existing treatments are inadequate for the proper treatment of this disease.

Using molecular hybridization to discover new chemotherapeutics has received significant attention recently.²⁻⁵ Quinoline motifs have been researched for decades as potential chemotherapeutics (Figure 1).⁶ The first compound with a quinoline motif and antitumor properties was discovered in 1966 and named camptothecin.⁷ Camptothecin prevents the replication of cancerous cells and can induce apoptosis by inhibiting topoisomerase I, a DNA-replicating enzyme.⁸ This pentacyclic alkaloid has shown potent activity against several human tumor cell lines, but is limited by poor aqueous solubility and severe side effects.⁹ However, other camptothecin derivatives are Food and Drug Administration (FDA) approved for the treatment of cancer malignancies. This includes irinotecan, which is approved for the treatment of pancreatic cancers,¹⁰ and topotecan, which is FDA approved as a second line treatment for relapsed small-cell lung cancer.¹¹

Figure 1. Structure of the quinoline-based antineoplastic compounds camptothecin, topotecan, and irinotecan.

Imidazolium salts are another class of compounds that have received substantial attention for their anti-tumor properties against a variety of human tumor cell lines.¹² In particular, naphthylmethyl-substituted imidazolium salts, such as **IS29** with a proton at the C² position, were shown to have high anti-proliferative effects against non-small cell lung cancer (NSCLC) (Figure 2).^{13–16} However, the clinical use of **IS29** was limited by poor water solubility. The quinoline motif is structurally similar to a naphthalene substituent but has a nitrogen heteroatom to increase the hydrogen bonding capabilities and increase aqueous solubility, which has been shown previously by our group.^{13,15,16} Also, the nitrogen atom could potentially be protonated to further increase the solubility if necessary.¹⁷ Therefore, the historical quinoline motif and the novel, highly-active naphthylmethyl-based imidazolium salt could be combined to produce potent chemotherapeutics with high aqueous solubility.

Figure 2. Structure of the naphthylmethyl substituted imidazolium salt **IS29**.

Presented herein is the synthesis, characterization, and in vitro structure-activity relationship (SAR) study of a series of C²-alkyl and N¹(N³)-naphthylmethyl and/or quinolylmethyl substituted imidazolium salts. Various alkyl substituents at the C² position were used to fine tune the lipophilic nature of compounds to optimize activity. Quinolylmethyl groups at the N¹ and/or N³ positions, which replaced naphthylmethyl groups of previous derivatives, and the incorporation of chloride anions versus bromide anions increased aqueous solubility making these novel derivatives clinically relevant for future studies.

Compound **1** was synthesized to evaluate the effect on the anti-cancer properties of the steric bulk and lipophilicity of a phenyl group at the C² (Figure 3). Commercially available 2-phenyl imidazole was deprotonated with sodium hydride and reacted with 2-(bromomethyl)naphthalene. The resulting naphthalene-substituted imidazole was reacted with a second molar equivalent of 2-(bromomethyl)naphthalene to synthesize **1** in moderate yield, 58%, after recrystallization from acetone. The formation of **1** was suggested by the resonance at 5.48 ppm in the ¹H NMR corresponding to the methylene linker bridging the naphthalene rings to the imidazole ring. The structure of the cationic portion of **1**, as the hexafluorophosphate salt, was confirmed by single crystal X-ray crystallography. To obtain a single crystal of **1-PF₆**, **1** was dissolved in a water/methanol mixture. Ammonium hexafluorophosphate was added to the mixture to form a white precipitate which was collected by filtration. The white solid was dissolved in acetonitrile and a single crystal of **1-PF₆** grew from slow evaporation of this concentrated solution (See supplementary data).

Figure 3. Synthetic scheme for **1**, **3**, **4-Br**, **4-Cl**, and **5-12** (top row) and synthetic scheme for **2** from **IS29** (bottom Row). Structures of the aryl group substituents (R₁ and R₃) are shown to the right of the synthesis for **2**. The identities of R₁, R₂, R₃, and X are identified in the table.

Compound **2** was synthesized to evaluate the anti-cancer properties of a N¹, N³-naphthylmethyl substituted imidazolium salt with a hydroxyl group at the C² position in attempt to increase the aqueous solubility when compared to other N,N'-bisnaphthylmethyl imidazolium salts, such as **1**. A modified literature procedure was used to synthesize **2** (Figure 3).¹⁸ Previously published **IS29** was reacted with 37% formalin and N,N'-diisopropylethylamine to form **2** in moderate yield, 69%, after recrystallization from acetonitrile. The formation of **2** was suggested by the disappearance of the C² proton of **IS29** at 9.50 ppm in the ¹H NMR spectrum and appearance of a triplet resonance at 6.27 ppm and doublet resonance at 5.03 ppm in the ¹H NMR spectrum of **2**. There was also a downfield shift of the methylene resonance bridging the naphthalene rings and imidazole in the ¹H NMR spectrum of **IS29** to **2** from 5.62 ppm to 5.71 ppm, respectively. The structure of **2** was confirmed by single crystal X-ray crystallography (Figure 4). A single crystal was grown from slow evaporation of a concentrated solution of **2** in acetonitrile.

Figure 4. Thermal ellipsoid plot of **2** with thermal ellipsoids drawn to the 50% probability level. Carbon labels and hydrogen atoms, except for the hydroxyl proton, have been removed for clarity.

In a separate attempt to increase aqueous solubility, compounds **3**, **4-Br**, **4-Cl**, and **5-7**, with a quinolylmethyl substituent at the N¹ position and a naphthylmethyl substituent at the N³ position, were synthesized by procedures developed in the Youngs' lab^{13,15,19–21} and evaluated for their anti-cancer properties. The nitrogen heteroatom of the quinoline moiety increases the hydrophilicity of the compound; whereas the naphthalene ring helps to maintain the high anti-cancer activity. Compounds **3** and **4-Br** were synthesized by deprotonating the appropriate imidazole with KOH and addition of approximately 0.5 and 0.85 molar equivalents of 2-(chloromethyl)quinoline (Figure 3). Excess imidazole was used to ensure substitution at both nitrogens did not occur. The excess imidazole and generated inorganic salts were washed away with a basic aqueous solution. Once it was confirmed that only the mono-substituted imidazole was present by ¹H NMR spectroscopy, the intermediate was reacted with 2-(bromomethyl)naphthalene to yield **3** and **4-Br** in moderate to poor yield, 50% and 23% respectively.

Compounds **3** and **4-Br** were characterized by ^1H and ^{13}C NMR spectroscopies, high resolution mass spectrometry, and melting point analysis. The most notable resonances in the ^1H NMR for **3** and **4-Br** were two singlet resonances in each spectrum corresponding to the methylene protons bridging the imidazole ring to the quinoline or naphthalene substituent. The chemical shift of these singlet resonances ranged from 5.88 ppm to 5.72 ppm. The appearance of two singlets in this region was indicative of the asymmetrically substituted imidazolium salts differing from the observance of only one singlet resonance in the ^1H NMR spectrum of a symmetrically substituted imidazolium salt, such as **1** or **2**.

Compound **4-Cl** was synthesized for multiple reasons: (1) to crystallize the cationic portion of '**4-Br**' to confirm the structure of the cation and (2) to determine the differences in solubility and anti-cancer activity of the bromide salt versus the chloride salt. 2-Ethylimidazole was deprotonated with potassium hydroxide and reacted with 2-(bromomethyl)naphthalene. Following filtration and removal of a white solid, the mono-alkylated species was reacted with 2-(chloromethyl)quinoline to afford a white solid, **4-Cl**, in poor yield (Figure 3).

Compound **4-Cl** was characterized by ^1H NMR, ^{13}C NMR, and single crystal X-ray crystallography. The two resonances corresponding to the methylenes bridging the aryl group to the nitrogens of the imidazole ring were in the appropriate region of the ^1H NMR spectrum, 5.76 ppm and 5.90 ppm, and the ^{13}C NMR, 50.8 ppm and 52.2 ppm. A single crystal suitable for X-ray analysis was obtained by slow evaporation of **4-Cl** in a concentrated solution of acetonitrile (Figure 5).

Figure 5. Thermal ellipsoid plot of **4-Cl·H₂O** with thermal ellipsoids drawn to the 50% probability level. Carbon labels and protons, except for those bound to the water, were removed for clarity.

The asymmetrically substituted derivatives **5** and **6** were more difficult to isolate due to solubility issues of the deprotonated imidazoles (Figure 3). After deprotonation of the imidazole and addition of the first aryl halide, a mixture of the starting imidazole, mono-alkylated intermediate, and the imidazolium salt were present. Therefore, a phase transfer catalyst, tetrabutylammonium bromide, was used to help solubilize all reagents and produce the mono-alkylated intermediate without any starting material present. In the first alkylation leading to **6**, excess 2-(bromomethyl)naphthalene was used to ensure the starting imidazole would be completely consumed. However, this produced imidazolium salt impurities to form. Acetone was used to solubilize the mono-alkylated intermediate leaving the imidazolium salt insoluble which was removed by filtration. After confirming the mono-substituted intermediate was the only species present by ¹H NMR spectroscopy, 2-(chloromethyl)quinoline was added to synthesize **5** and **6** in moderate to poor yield, 46% and 17% respectively.

Compounds **5** and **6** were characterized by ¹H NMR and ¹³C NMR spectroscopies, high resolution mass spectrometry, and melting point analysis. Resonances in the ¹H NMR spectra used to suggest the products were the two singlets in each spectrum ranging from 5.78 ppm to

5.94 ppm corresponding to the protons bridging the imidazole ring to the quinoline and naphthalene rings. This is consistent with the spectra of **3**, **4-Br**, and **4-Cl** discussed above. Compound **5** was also characterized by single crystal X-ray crystallography (see supplementary data). A single crystal of **5**, with a co-crystallized acetonitrile molecule, was grown from slow evaporation of a concentrated solution of **5** dissolved in acetonitrile.

Compound **7** was synthesized by reacting 1-(naphthalen-2-ylmethyl)-2-phenylimidazole with excess 2-(chloromethyl)quinoline in refluxing acetonitrile overnight (Figure 3). The product precipitated from addition of ether and acetone to the reaction mixture to produce **7** in moderate yield, 58%. ^1H NMR and ^{13}C NMR spectroscopies, melting point analysis, and high resolution mass spectrometry were used to characterize **7**. Two singlet resonances with a chemical shift of 5.70 ppm and 5.57 ppm in the ^1H NMR spectrum suggested the formation of **7**.

In attempt to synthesize highly hydrophilic compounds, **8-12** were synthesized with quinolylmethyl substituents at both the N^1 and N^3 positions (Figure 3). Compounds **8-12** were synthesized by deprotonation of the appropriate imidazole with potassium hydroxide and addition of one molar equivalent of 2-(chloromethyl)quinoline to form the mono-substituted intermediate. After filtration to remove a white precipitate, a second molar equivalent was added to yield **8-12** in poor yields, 9–37%.

Compounds **8-12** were characterized by high resolution mass spectrometry, ^1H NMR and ^{13}C NMR spectroscopies, and melting point analysis. The most notable resonance in the ^1H NMR spectra of **8-12** was the resonance corresponding to the methylene bridging the quinoline groups to the imidazole ring ranging from 5.76 to 5.99 ppm. These values are slightly downfield when compared to resonances corresponding to the bridging methylenes of **1** at 5.48 ppm. This

downfield shift is most likely due to the more electronegative nitrogen atom in the quinoline moiety when compared to the naphthalene ring.

Compounds **8**, **10** (as the hydrate **10•1.5(H₂O)**) and the cationic portion of **9** and **11** as the hexafluorophosphate salts (**9-PF₆** and **11-PF₆•1.5(H₂O)**) were analyzed by single crystal X-ray crystallography to confirm their structures (See supplementary data). A single crystal of **8** was grown from slow evaporation of a concentrated solution of deuterated dimethyl sulfoxide. A single crystal of **10•1.5(H₂O)** was grown from a concentrated solution of water. Compounds **9-PF₆** and **11-PF₆** were obtained similar to **1-PF₆** as described above by anion exchange of **9** and **11** from a chloride anion to a hexafluorophosphate anion. A single crystal of **9-PF₆**, was obtained by slow evaporation of a concentrated solution of **9-PF₆** in acetonitrile and ethanol (1:1). Whereas a single crystal of **11-PF₆•1.5(H₂O)** was obtained by slow evaporation of a concentrated solution of **11-PF₆** in acetone and ethanol (1:1).

The anti-cancer properties of **1-3**, **4-Br**, **4-Cl**, and **5-12** against several NSCLC cell lines (NCI-H460, NCI-H1975, HCC827, and A549) were evaluated by the MTT assay, as done previously by our group,^{13,15,16} to create a SAR of C² substituted imidazolium salts with a highly lipophilic substituent (naphthylmethyl) or a more hydrophilic substituent (quinolylmethyl) at the N¹ and/or N³ positions (Table 1). Briefly, cells were grown to confluency and plated in 96-well plates at 5,000-7,000 cells per well depending on the cell line. Cells were allowed to adhere to the plate overnight. Following incubation, cells were exposed to **1-3**, **4-Br**, **4-Cl**, and **5-12** at four concentrations in growth medium, 1, 4, 16, and 32 μ M. Cells were incubated for 72 hours and the optional MTT assay protocol was followed to determine the IC₅₀ value of each compound for the four cell lines tested (the concentration of compound that inhibits 50% of cell growth relative to control cells). Results were compared to cisplatin and **IS29** to establish a SAR of various

alkyl groups at the C² position and naphthylmethyl and quinolylmethyl substituents at the N¹ and/or N³ positions.

Table 1. IC₅₀ values of **1-3**, **4-Br**, **4-Cl**, **5-12**, **IS29**, and cisplatin determined by the MTT assay.

Compound	IC ₅₀ value (μM)			
	NCI-H460	NCI-H1975	HCC827	A549
1	3	2	2	4
2	14	7	12	> 30
3	2	< 1	9	3
4-Br	2	< 1	5	3
4-Cl	2	< 1	2	3
5	3	2	6	3
6	3	2	6	3
7	3	2	6	4
8	8	2	13	15
9	6	< 1	9	11
10	5	1	7	10
11	5	< 1	7	7
12	8	2	8	14
IS29	4	6	9	n/a
cisplatin	2	6	3	7

Compounds **1-3**, **4-Br**, **4-Cl**, and **5-12** had varying degrees of solubility in aqueous solution (Table 2). Compounds were dissolved in water to a concentration of 1 mM prior to

diluting in growth medium to the testing concentrations. However, due to their low solubility in pure water (< 0.5 mg/mL), compounds **1** and **2** were dissolved in DMSO before diluting with water to a final concentration of 1 mM and 1% (v/v) DMSO. The highest final concentration of DMSO in growth medium was 0.032% (v/v) after diluting the stock concentration to the desired testing concentrations. Compounds **3**, **4-Br**, **4-Cl**, and **5-7** had varying solubilities in water. All compounds were soluble at a concentration of 1 mM. However, the more lipophilic derivatives **5-7** had an aqueous solubility greater than 10 mM, whereas **4-Br** had a solubility of 5 mM and **3** had a solubility < 5 mM. It is interesting to note that the solubility of the **5-7** is higher than the solubility of **3** and **4-Br** in water considering **5-7** has more lipophilicity at the C² position. However, the solubility of **4-Cl** is also greater than 10 mM and therefore at least 2-fold higher than the bromide derivative **4-Br**. This suggests the anion significantly affects the solubility properties of these imidazolium salts. Compounds **3** and **4-Br** could be solubilized by 2-hydroxypropyl- β -cyclodextrin (2-HP β CD), a chemical excipient that is considered ‘generally regarded as safe’ (GRAS) by the FDA and approved in several drug formulations,²² at a concentration above 10 mM. Compounds **8-12** had aqueous solubilities of greater than 80 mM. Therefore, there was no need to use DMSO for the testing of **3**, **4-Br**, **4-Cl**, and **5-12**. These compounds were dissolved in pure water to a concentration of 1 mM and then diluted in growth medium to the desired concentrations. Cisplatin was dissolved in water without the use of DMSO by stirring a 1 mM solution for several hours at room temperature prior to diluting with growth medium.

Table 2. Aqueous solubilities of compounds **1-3**, **4-Br**, **4-Cl**, and **5-12** in mM.

Compound	Aqueous solubility (mM)

1	< 0.5
2	< 0.5
3	< 5
4-Br	~ 5
4-Cl	> 10
5	> 10
6	> 10
7	> 10
8	> 80
9	> 80
10	> 80
11	> 80
12	> 80

Compound **1** was synthesized to assess the anti-cancer activity of a C²-phenyl substituted imidazolium salt with naphthylmethyl substituents at the N¹ and N³ positions. This derivative gave insight suggesting how the steric bulk and lipophilicity at the C² position affects the anti-cancer activity. The IC₅₀ values of **1** for the four NSCLC cell lines tested were in the low micromolar range. This suggests that a large amount of steric bulk can be added to the C² position without negatively altering the anti-cancer properties of N¹ and N³ naphthylmethyl substituted imidazolium salts. Unfortunately, this compound, like many other lipophilic imidazolium salts with high anti-cancer activity, was limited by poor aqueous solubility as described above.

Compound **2** with naphthylmethyl substituents at both the N¹ and N³ positions and a hydroxymethyl group at the C² position was synthesized to evaluate the anti-proliferative properties of a hydrophilic hydroxyl group at the C² position. Compound **2** had moderate to poor activity against all four NSCLC cell lines. The hydroxyl group was added to the C² position to increase aqueous solubility, whereas naphthylmethyl substituents were at the N¹ and N³ positions to maintain high anti-cancer activity. However, the hydroxyl group decreased the activity drastically in all but the H1975 cell line when compared to **IS29**, which has a hydrogen at the C² position. Unfortunately this chemical modification not only reduced the anti-proliferative effects when compared to related derivatives, but also did not significantly add aqueous solubility considering the aqueous solubility of **2** was also quite poor. Results of the MTT assay for **2** were similar to another previously published related compound with hydroxymethyl groups at the C⁴ and C⁵ positions suggesting that alcohols at any position of the imidazole ring reduce the anti-cancer activity of imidazolium salts against NSCLC.

To increase solubility when compared to **IS29**, **3**, **4-Br**, **4-Cl**, and **5-7** were synthesized with a naphthylmethyl at the N¹ position and a quinolylmethyl substituent at the N³ position. The quinolylmethyl substituent is structurally and sterically similar to the naphthylmethyl substituent, but it has a nitrogen heteroatom in the planar, aromatic ring system to increase the hydrogen bonding capabilities of the imidazolium salt and consequently increase the aqueous solubility. Compounds **3**, **4-Br**, **4-Cl**, and **5-7** all had high anti-cancer activity against the four NSCLC lines tested with IC₅₀ values in the nanomolar range for **3**, **4-Br**, and **4-Cl** against the H1975 cell line and the low micromolar range for all other compounds and cell lines. Although these compounds are more hydrophilic, these values are similar to the more lipophilic derivatives **1** and **IS29**.

The IC₅₀ values of **4-Br** and **4-Cl** were almost identical (2 μ M (H460), < 1 μ M (H1975), 5 μ M (HCC827), and 3 μ M (A549) for **4-Br** and 2 μ M (H460), < 1 μ M (H1975), 2 μ M (HCC827), and 3 μ M (A549) for **4-Cl**). This suggests that the anion has virtually no effect on the compounds anti-cancer properties. Considering **4-Cl** had over 2-fold higher aqueous solubility than **4-Br**, **4-Cl** not only has a clinically relevant solubility but it also has high-anti cancer activity. This suggests that it is desirable for imidazolium salts to be chloride salts versus bromide salts since chloride derivatives have higher solubility an equal anti-proliferative effects.

To maximize solubility from heteroatoms in the bicyclic ring systems, derivatives **8-12** were synthesized, with quinolylmethyl substituents at both the N¹ and N³ positions, and tested for their anti-proliferative properties. Compounds **8-12** had high anti-cancer activity against the H1975 cell line with IC₅₀ in the high nanomolar to low micromolar range. These highly water-soluble derivatives also had high to moderate activity against the other three NSCLC cell lines with values in the low micromolar range.

Two SARs can be established from the MTT assay results discussed. The first involves substituents at the C² position. A series of compounds were tested with various alkyl groups at the C² position with the substituents at the N¹ and N³ positions remaining constant. The hydroxyl group at the C² positions clearly causes a reduction in anti-cancer activity when comparing **2** with **1** and **IS29**. However, when comparing **1** to **IS29**, there does not seem to be a substantial effect on the anti-cancer activity of a lipophilic phenyl substituent at the C² position, although **1** does have slightly lower IC₅₀ values. This trend, that the length of the alkyl chain does not have a significant effect on anti-cancer activity, remained constant when comparing **3**, **4-Br**, **4-Cl**, and **5-7** with **8-12**. The second SAR considers the different aryl substituents at the N¹ and N³ positions. Although the solubilities of **3**, **4-Br**, **4-Cl**, and **5-7** were increased when compared to **1**,

the IC₅₀ values were comparable. However, a difference in activity was observed when comparing the highly hydrophilic derivatives **8-12** to the more lipophilic compounds considering the more hydrophilic derivatives displayed less potent anti-cancer properties.

The National Cancer Institute's (NCI) Developmental Therapeutics Program (DTP) tested multiple compounds in the NCI-60 human tumor cell line screen. This allowed us to expand upon the SAR determined in our lab to include several more NSCLC cell lines than the ones we have available for use. The first stage of this program involves a one dose assay where all 60 cell lines are exposed to the compound at one dose (10 μ M). Experimental procedures can be found on the DTP website (https://dtp.cancer.gov/discovery_development/nci-60/methodology.htm) to describe how the experiment was performed in greater detail. The growth percentage values are determined relative to growth with no drug added and the initial cell count. This allows growth inhibition (growth percentage values between 0 and 100) and lethality values (growth percentage values between 0 and -100) to be determined.

The DTP tested **1-3**, **4-Br**, **5**, **8-11**, and **IS29** in their one-dose assay (Table 3). Compound **1** had the lowest average growth percentage of all the compounds tested with an average lethality rate of -26.99%. Compound **2** with a hydroxyl group at the C² position had a high average growth rate at 60.67% while the more hydrophilic derivatives **3**, **4-Br**, and **5** had average growth rates between 7.64 and 15.98%. The highly hydrophilic derivative **8** had the highest average growth rate of 80.66% while **9-11** had lower average growth rates ranging from 24.96% to 31.84%.

The results from the one-dose assay confirm the SAR established with the MTT assay described above. The most lipophilic derivative **1** had the highest anti-cancer activity as expected while the hydroxyl group in **2** caused a reduction in activity when compared to the other N,N'-

bis-naphthylmethyl derivative **1**. The slightly more hydrophilic derivatives **3**, **4-Br**, and **5** had low average growth percentage values and were all comparable to each other. This also suggests that the difference in lipophilicity and steric bulk of a methyl, ethyl, and propyl group do not have a significant influence on the anti-cancer activity. More drastic effects were seen with the highly hydrophilic derivatives **8-11** whereas **8** had very poor inhibitory effects with an average growth rate of 80.66%. The more lipophilic derivatives **9-11** had lower growth percentage values compared to **8** but higher than the related C² alkyl N¹/N³ asymmetrically substituted derivatives **4-Br** and **5**. The overall trend observed with the MTT assay described above and results from the one-dose assay is that lipophilicity increases activity.

Table 3. Growth percentage values for multiple NSCLC cell lines from the NCI-60 human tumor cell line one-dose assay against **1-5**, **8-11**, and **IS29**.

Compound	Growth %									Average
	A549/ATCC	EKVX	HOP-62	HOP-92	NCI-H226	NCI-H23	NCI-H322M	NCI-H460	NCI-H522	
1	12.48	3.50	-44.84	-69.65	-9.03	-38.74	8.84	-78.47	n/a	-26.99
2	68.58	83.29	75.01	-2.81	61.63	67.69	78.91	53.09	n/a	60.67
3	33.51	48.29	13.28	-6.10	12.73	2.01	26.02	10.17	3.95	15.98
4-Br	37.29	15.92	1.85	-20.13	19.65	-4.20	34.78	11.08	-27.43	7.64
5	39.11	41.63	8.38	-18.63	12.30	-11.55	25.72	7.73	-0.78	11.54
8	88.63	80.66	90.55	74.47	88.12	69.84	102.72	95.03	35.94	80.66
9	57.44	31.88	40.89	-11.85	48.43	16.01	73.14	34.82	-3.51	31.84
10	62.82	58.14	51.67	16.03	37.98	13.04	72.08	34.24	22.76	40.97
11	44.83	50.89	25.25	4.31	26.68	4.77	41.44	16.51	10.02	24.96

IS29	67.63	51.65	42.12	-13.30	52.09	41.65	62.77	39.60	-11.71	38.06
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The NCI's DTP also tested **1**, **3**, **4-Br**, **5**, and **9-11**, and **IS29** in their five-dose assay (Table 4, Table 5 and Figure 6, full plots of the results for all compounds tested can be found in the supplementary data), whereas **2** and **8** were not considered active enough in the one-dose assay for the DTP to test them in the five-dose assay. The experimental details are the same for the five-dose assay as for the one-dose assay, except cells are exposed to each compound at five doses (10 nM, 100 nM, 1 μ M, 10 μ M, and 100 μ M) versus one dose. Results are also given in a different format and described as the GI50 (growth inhibition of 50% of cells relative to control cells), TGI (total growth inhibition), and LC50 (lethal concentration of 50% of cells relative to the initial cell count at the time of drug addition).

Compound **1**, with a phenyl substituent at the C² position and naphthylmethyl substituents at the N¹ and N³ positions had GI50 values from the mid nanomolar range (602 nM) to the low micromolar range (1.78 μ M), all TGI values in the low micromolar range (2.28 μ M – 3.52 μ M), and LC50 values for only the NCI-H23 and NCI-H322M cell line in the low micromolar range (6.14 μ M and 5.61 μ M respectively). **IS29** with two naphthylmethyl groups and a proton at the C² position have GI50 values in the nanomolar to low micromolar range (172 nm – 8.40 μ M), TGI values in micromolar range (1.39 μ M – 22.9 μ M) and LC50 values in the micromolar range (15.2 μ M - 71.9 μ M). Compounds **3**, **4-Br**, and **5**, with methyl, ethyl, and propyl chains at the C² position respectively, a naphthylmethyl at the N¹ position, and a quinolylmethyl substituent at the N³ position had similar results with GI50 values in the low nanomolar range (184 nM) to low micromolar range (3.93 μ M), TGI values in the mid nanomolar range (671 nM) to the low micromolar range (27.6 μ M), and LC50 values all in the

micromolar range (4.59 μM - > 100 μM). Compounds **9-11**, with an ethyl, propyl, and butyl chain at the C² position respectively, and quinolylmethyl substituents at both the N¹ and N³ positions, had GI50 values from the low nanomolar range (18.3 nM) to the low micromolar range (12.5 μM), TGI values from the mid nanomolar range (521 nM) to unmeasured values in the micromolar range (> 100 μM), and LC50 values of (19.7 μM) to unmeasured values > 100 μM .

All compounds tested in the five-dose assay had good GI50 values comparable to the IC₅₀ values determined in our lab. As the water solubility increases from **1**, with the lowest solubility, to **9-11**, with the highest solubilities, the TGI and LC50 values also increase, meaning the more water soluble derivatives have inferior anti-cancer activities. This follows the trend observed in both in vitro assays described above that lipophilicity is key to the anticancer activity observed with this class of imidazolium salts against NSCLC. When comparing **3**, **4-Br**, and **5** to each other, although they have similar results from the five-dose assay, on average, the most lipophilic derivative **5** has the lowest values or the highest anti-cancer properties. This is consistent when comparing derivatives **9**, **10**, and **11** to each other. Compound **11** has the highest anti-cancer activity of this group and is the most lipophilic. Direct comparisons can also be made with **4-Br** to **9** and with **5** to **10**. Derivatives **4-Br** and **5** are more lipophilic than **9** and **10** and again have the higher anti-cancer activities further suggesting the need for lipophilicity when considering highly active compounds.

Table 4. GI50, TGI, and LC50 values (μM) from the NCI-60 human tumor cell line five-dose assay for **1**, **3**, **4-Br**, and **5**. Results are shown for all the NSCLC cell lines tested by the DTP.

Cell Line	GI50, TGI, and LC50 values (μM) from the five-dose assay			
	1	3	4-Br	5

	GI50	TGI	LC50	GI50	TGI	LC50	GI50	TGI	LC50	GI50	TGI	LC50
A549/ATCC	1.78	3.52	n/a	1.31	13.1	43.5	0.997	14.1	46.1	1.88	11.8	37.3
EKVZ	1.58	3.43	n/a	3.93	27.6	> 100	0.442	7.34	38.7	2.16	14.9	52.7
HOP-62	1.46	3.23	n/a	1.53	6.66	34.5	1.40	7.02	32.1	1.63	5.38	26.1
HOP-92	0.602	2.28	n/a	0.518	3.81	27.0	0.184	0.671	7.04	0.188	1.12	9.13
NCI-H226	1.26	2.97	n/a	1.86	10.9	47.5	1.06	4.87	39.4	1.20	7.60	39.7
NCI-H23	0.775	2.35	6.14	1.83	13.4	62.0	0.311	3.23	37.0	0.479	10.6	52.0
NCI-H322M	1.42	2.82	5.61	3.27	14.7	40.4	2.86	13.8	38.1	3.46	15.0	39.6
NCI-H460	1.37	2.87	Na	0.959	14.4	47.0	0.456	10.8	49.5	0.658	10.6	46.5
NCI-H522	1.07	2.58	n/a	0.271	1.46	12.4	0.275	0.986	13.3	0.249	1.13	4.59

Table 5. GI50, TGI, and LC50 values (μM) from the NCI-60 human tumor cell line five-dose assay for **9**, **10**, **11**, and **IS29**. Results are shown for all the NSCLC cell lines tested by the DTP.

Cell Line	GI50, TGI, and LC50 values (μM) from the five-dose assay											
	9			10			11			IS29		
	GI50	TGI	LC50	GI50	TGI	LC50	GI50	TGI	LC50	GI50	TGI	LC50
A549/ATCC	5.27	> 100	> 100	6.78	> 100	> 100	4.76	18.5	47.6	7.97	22.9	55.3
EKVZ	0.343	41.5	> 100	12.5	> 100	> 100	4.00	22.0	72.4	2.51	15.5	4.75
HOP-62	4.66	31.5	> 100	4.28	19.4	62.6	2.80	12.0	39.8	3.26	17.0	45.9
HOP-92	0.0183	0.521	19.7	0.399	3.23	23.2	0.339	3.36	20.1	0.160	1.39	15.2
NCI-H226	2.56	30.8	> 100	3.40	34.8	> 100	2.37	13.6	60.3	1.72	14.3	71.9
NCI-H23	0.257	8.66	> 100	0.865	15.0	> 100	0.480	7.27	43.6	0.901	10.1	39.4
NCI-H322M	6.33	41.9	> 100	4.68	41.9	> 100	3.57	16.3	42.0	8.40	21.3	47.3

NCI-H460	0.511	11.9	45.7	2.56	11.7	39.8	2.17	11.6	38.5	1.27	10.4	42.0
NCI-H522	0.0837	1.49	37.9	0.601	4.30	27.1	0.435	4.19	32.0	0.393	3.11	26.8

Figure 6. Growth percentage charts from the NCI-60 human tumor cell line five-dose assay for compounds **1**, **3**, **4-Br**, and **5**. Only results for the NSCLC cell line tested are shown.

In conclusion, a series of C²-alkyl substituted imidazolium salts with water solubilizing groups at the C² and N¹/N³ positions were synthesized and evaluated for their anti-cancer properties against NSCLC to establish a SAR. The highly lipophilic derivative **1** was extremely potent against a panel of NSCLC cell lines tested in our lab and by the NCI's DTP. However,

this compound is limited by poor aqueous solubility without the use of a chemical excipient. The slightly more hydrophilic derivative **2** had poor anti-cancer properties against the NSCLC cell lines despite the limited water solubility observed with a hydroxyl functional group at the C² position. Although **3** and **4-Br** had high anti-cancer activity, these also had poor solubility, whereas **4-Cl** and **5-7** had potent anti-proliferative effects and solubility sufficient for systemic administration. The solubility difference between the chloride and bromide derivative of **4** was quite drastic with **4-Cl** having over a 2-fold increase in solubility compared to **4-Br** while both compounds had comparable anti-cancer activity suggesting use of the chloride anion is more effective in designing a chemotherapeutic imidazolium salt. Future studies will include more in vitro assays to determine the mechanism of action and in vivo toxicity and lung xenograft models for these compounds due to properties they demonstrated. Compounds **8-12** were highly soluble in aqueous solution but were less active than the more lipophilic derivatives. This concludes the SAR study suggesting lipophilicity will increase activity; however, asymmetrically substituted compounds with one naphthylmethyl and one quinolylmethyl substituent at the N¹ and N³ positions with a chloride anion can produce highly active compounds with a clinically relevant solubility. Although several compounds discussed herein merit further studies and show potential for clinical use, this will also help guide future derivatives to continue to optimize these highly active imidazolium salts as prospective chemotherapeutics.

Supplementary data. Full details of the Materials and Methods can be found in the supplementary data. The ¹H and ¹³C NMR for compounds **1-3**, **4-Br**, **4-Cl**, and **5-12**; X-ray crystal structures of **1-PF₆**, **5**, **8**, **9-PF₆**, **10**, and **11-PF₆**; full plots of the five-dose assays against the nine NSCLC cell lines tested for compounds **1-3**, **4-Br**, **5**, **8-11**, and **IS29**; and abbreviations used in this manuscript are also included in the supplementary data. Crystallographic

Information Files (CIF) for compounds **1-PF₆**, **2**, **4-Cl**, **5**, **8**, **9-PF₆**, **10**, and **11-PF₆** (CCDC #'s 1503023-1503030) 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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References

1. American Cancer Society. *Cancer Facts Fig.* **2016**, Atlanta: American Cancer Society; 2016.
2. Liu, L.-X.; Wang, X.-Q.; Zhou, B.; Yang, L.-J.; Li, Y.; Zhang, H.-B.; Yang, X.-D. *Sci. Rep.* **2015**, *5*, 13101.
3. Xu, X.; Yu, C.; Chen, W.; Li, Y.; Yang, L.-J.; Li, Y.; Zhang, H.-B.; Yang, X.-D. *Org. Biomol. Chem.* **2015**, *13*, 1550–1557.
4. Zhou, B.; Liu, L.-X.; Deng, G.-G.; Chen, W.; Li, M.-Y.; Yang, L.-J.; Li, Y.; Yang, X.-D.; Zhang, H.-B. *Org. Biomol. Chem.* **2016**, *14*, 9423–9430.
5. Liu, J.-M.; Wang, M.; Zhou, Y.-J.; Yan, J.-M.; Yang, L.-J.; Li, Y.; Zhang, H.-B.; Yang,

- X.-D. *RSC Adv.* **2015**, *5*, 63936–63944.
6. Afzal, O.; Kumar, S.; Haider, M. R.; Ali, M. R.; Kumar, R.; Jaggi, M.; Bawa, S. *Eur. J. Med. Chem.* **2014**, 1–40.
 7. Wall, M. E.; Wani, M. C.; Cook, C. E.; Palmer, K. H. *J. Am. Chem. Soc.* **1966**, *88*, 3888–3890.
 8. Hsiang, Y. H.; Hertzberg, R.; Hecht, S.; Liu, L. F. *J. Biol. Chem.* **1985**, *260*, 14873–14878.
 9. Zhou, L.; Lichent, D.; Chen, X.; Li, X.; Li, Z.; Wen, Y.; Li, Z.; He, X.; Wei, Y.; Zhao, X.; Qian, Z. *Oncol. Rep.* **2010**, *24*, 941–948.
 10. Huang, H. C.; Mallidi, S.; Liu, J.; Chiang, C. Te; Mai, Z.; Goldschmidt, R.; Ebrahim-Zadeh, N.; Rizvi, I.; Hasan, T. *Cancer Res.* **2016**, *76*, 1066–1077.
 11. Hu, G.; Zekria, D.; Cai, X.; Ni, X. *Phytochem. Rev.* **2015**, *14*, 429–441.
 12. Riduan, S. N.; Zhang, Y. *Chem. Soc. Rev.* **2013**, *42*, 9055–9070.
 13. Wright, B. D.; Deblock, M. C.; Wagers, P. O.; Duah, E.; Robishaw, N. K.; Shelton, K. L.; Southerland, M. R.; DeBord, M. A.; Kersten, K. M.; McDonald, L. J.; Stiel, J. A.; Panzner, M. J.; Tessier, C. A.; Paruchuri, S.; Youngs, W. J. *Med. Chem. Res.* **2015**, *24*, 2838–2861.
 14. Sun, C.-J.; Chen, W.; Li, Y.; Liu, L.-X.; Wang, X.-Q.; Li, L.-J.; Zhang, H.-B.; Yang, X.-D. *RSC Adv.* **2014**, *4*, 16312–16319.
 15. Shelton, K. L.; DeBord, M. A.; Wagers, P. O.; Southerland, M. R.; Taraboletti, A.; Robishaw, N. K.; Jackson, D. P.; Tosanovic, R.; Kofron, W. G.; Tessier, C. A.; Paruchuri, S.; Shriver, L. P.; Panzner, M. J.; Youngs, W. J. *Tetrahedron* **2016**, *72*, 5729–5743.
 16. Shelton, K. L.; DeBord, M. A.; Wagers, P. O.; Southerland, M. R.; Williams, T. M.;

- Robishaw, N. K.; Shriver, L. P.; Tessier, C. A.; Panzner, M. J.; Youngs, W. J.; Williams, T. M.; Robishaw, N. K.; Shriver, L. P.; Tessier, C. A.; Panzner, M. J.; Youngs, W. J. *Bioorg. Med. Chem.* **2016**. <http://dx.doi.org/10.1016/j.bmc.11.009>.
17. Shukla, V.; Scholar, R. *J. Med. Pharm. Allied Sci.* **2016**, *1*, 18–38.
18. Agelis, G.; Resvani, A.; Koukoulitsa, C.; Tumova, A.; Slaninova, J.; Kalavrizioti, D.; Spyridaki, K.; Afantitis, A.; Melagraki, G.; Siafaka, A.; Gkini, E.; Megariotis, G.; Grdadolnik, S. G.; Papadopoulos, M. G.; Vlahakos, D.; Maragoudakis, M.; Liapakis, G.; Mavromoustakos, T.; Matsoukas, J. *Eur. J. Med. Chem.* **2013**, *62*, 352–370.
19. Youngs, W. J.; Panzner, M. J.; Deblock, M. C.; Tessier, C. A.; Wright, B. D.; Wagers, P. O.; Robishaw, N. K. Azolium and purinium salt anticancer and antimicrobial agents **2015**. US patent US20140142307 A1, May 22, 2014.
20. Wagers, P. O.; Tiemann, K. M.; Shelton, K. L.; Kofron, W. G.; Panzner, M. J.; Wooley, K. L.; Youngs, W. J.; Hunstad, D. a. *Antimicrob. Agents Chemother.* **2015**, *59*, AAC.00881-15.
21. Ornelas-Megiatto, C.; Shah, P. N.; Wich, P. R.; Cohen, J. L.; Tagaev, J. A.; Smolen, J. A.; Wright, B. D.; Panzner, M. J.; Youngs, W. J.; Fréchet, J. M. J.; Cannon, C. L. *Mol. Pharm.* **2012**, *9*, 3012–3022.
22. Brewster, M. E.; Loftsson, T. *Adv. Drug Deliv. Rev.* **2007**, *59*, 645–666.

Graphical Abstract

Synthesis, characterization, and in vitro SAR evaluation of N,N'-Bis(arylmethyl)-C²-alkyl substituted imidazolium salts

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