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Discovery of Novel Dimeric Pyridinium Bromide Analogues Inhibits Cancer Cell Growth by Activating Caspases and Downregulating Bcl-2 Protein

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Cite This: *ACS Omega* 2023, 8, 13243−13251

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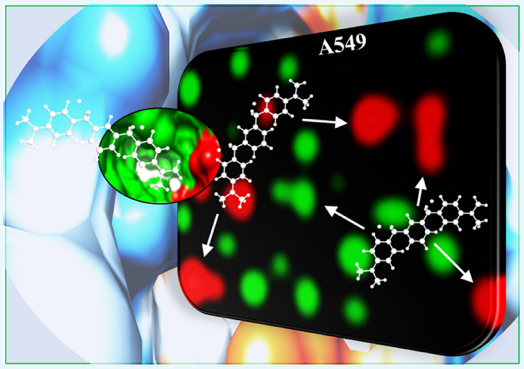
ABSTRACT: Flexible dimeric substituted pyridinium bromides with primary and tertiary amines are prepared by conventional and solvent-free methods. The formation of compounds **2** and **4** is much easier than that of compounds **1** and **3** because of the benzyl carbon which is more electropositive than the primary alkyl carbon. The newly synthesized dimeric pyridinium compounds are optimized using DFT and B3LYP 6-31 g(d,p). The *in vitro* antiproliferative activity is studied in lung (A549) and breast cancer cell lines (MDA-MB 231). Among the four compounds, 1,1′-(1,3-phenylene bis(methylene)bis 2-aminopyridinium bromide **4** showed potent anticancer activity when compared to the standard drug 5-fluorouracil. 1,1′-(1,3-Phenylene bis(methylene)bis 2-aminopyridinium bromide **4** is not toxic to normal cell lines 3T3-L1 and MRC-5 cell lines. Also, 1,1′-(1,3-phenylene bis(methylene)bis 2-aminopyridinium bromide **4**-induced apoptosis in cancer cell lines is examined using AO/EB and Hoechst staining, which is further supported by cell cycle analysis. Western blot analysis showed that 1,1′-(1,3-phenylene bis(methylene)bis 2-aminopyridinium bromide **4** induces apoptosis through the extrinsic apoptotic pathway by upregulating caspase 3 and caspase 9. This compound also downregulates intrinsic apoptotic proteins, including Bcl-2, Bcl-x, and Bad. From the present study results, it is confirmed that 1,1′-(1,3-phenylene bis(methylene)bis 2-aminopyridinium bromide **4** has potent anticancer activity when compared to other compounds.

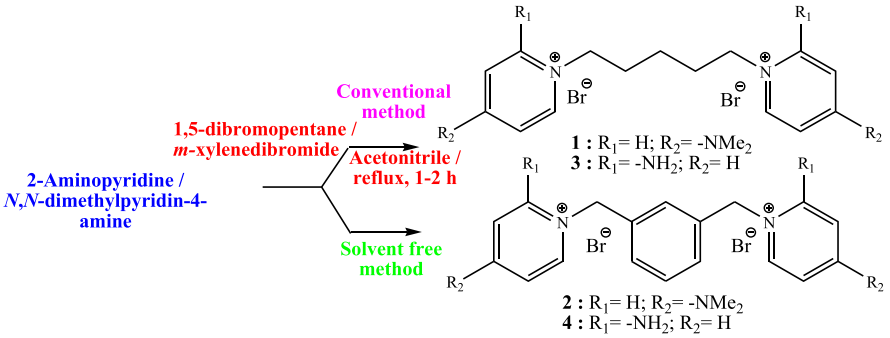


1. INTRODUCTION

Ionic liquids are salts with the melting point below 100 °C, containing an organic cation/inorganic cation and inorganic anion/organic cation, exhibiting unique properties like high thermal stability, high viscosity, excellent conductive behaviors, and high vapor pressure.[1−3] The biological efficacy of second- and third-generation ionic liquids may differ from one organism to another due to the solubility in solution and hydrophobic and hydrophilic interactions with the solvent. The toxicity of ionic liquids increases progressively with the increasing alkyl chain length of the substituents of imidazolium moieties which destabilizes the cell membrane by mechanistic studies[4,5] and mammalian cell cultures.[6−12] The imidazolium cation with chloride (Cl^-) and tetrafluoroborate (BF_4^-) anions is completely soluble in water. However, the pyrrolidinium/piperidinium cation exhibits moderate-to-poor solubility in water for various counterions.[13,14] The solid form of drugs has many drawbacks such as low bioavailability, low solubility, and polymorphic change. The properties of active pharmaceutical ingredients containing imidazolium salts are very important to overcome the difficulties of the solid form of drug molecules.[15−18] Hough-Troutman and co-workers reported a biologically active quaternary ammonium cation with various synthetic sweet anions and investigated its limited water-soluble good inhibitory activity against *Tribolium confusum*, *Sitophilus granaries*, and *Trogoderma granarium*.[19] Anti-bacterial activity and melittin behaviors of pyrrolidinium-based ionic liquids with longer alkyl chain (C_{12}) against human pathogenic bacteria showed better efficacy than the shorter alkyl chain (C_4). The cytotoxicity of pyrrolidinium-based ionic liquids studied on the HEK 293 cell line using MTT assay showed a lesser toxicity.[20] Vineet *et al.* studied the antitumor screening of ammonium and phosphonium cations with five different anions against the NCI 60 human tumor cell line. Phosphonium-based ionic liquids with a higher alkyl chain

Received: January 26, 2023
Accepted: March 16, 2023
Published: March 27, 2023

Scheme 1. Synthesis of Flexible Dimeric Pyridinium Bromides from Conventional and Solvent-Free Reaction Conditions



length showed a more significant effect than ammonium-based ionic liquids.[21] Taurine-based ionic liquids can deliver biopolymers such as dextran (or) insulin, which play an important role in drug delivery systems.[22] The cytotoxicity evaluation of imidazolium- and pyridinium-based ionic liquids is studied against the human Hep-2 cell line. The higher alkyl chain (C_8) with the ester functional imidazolium/pyridinium chloride showed cytotoxicity, with IC_{50} values of 0.181 and 5.75 after 72 h of incubation period, whereas a shorter alkyl chain (C_8) with the ester functional imidazolium/pyridinium chloride showed a less cytotoxicity response.[23] The antibacterial, antifungal, and anticancer activities of ether-based monomeric imidazolium bromide are studied. Benzyl/phenoxy ethyl imidazolium bromide showed good activity against *P. aeruginosa* strains.

Phenyl alkyl substituted imidazolium bromide showed better antifungal response against *C. albicans* strains. Benzyl/phenyl propyl/phenoxy propyl imidazolium bromide showed only 50−60% anticancer response against the MD-MB-435 cell line.[24] Guzmán *et al.* studied the antibacterial activities and molecular dynamic simulations against halogenated and hydrophobic substituted flexible alkyl imidazolium salts. The hydrophobic unit of *tert*-butyl-*N*-methyl phenolic imidazolium salt showed excellent efficacy against *S. aureas*, *E.coli*, and *P. aeruginosa* under minimal inhibitory concentrations.[25] Ferraz *et al.* prepared active pharmaceutical ingredients containing anions with highly hydrophilic imidazolium/ammonium/phosphonium/pyridinium ionic liquids and studied their antibacterial activity against sensitive and resistant *Escherichia coli* and *Staphylococcus aureus* under minimal inhibitory concentrations.[26] Recently, our laboratory started the synthesis of some novel imidazolium-/pyridinium-type of ionic liquids under conventional[27]/solvent-free silica-supported muffle furnace reaction conditions[28] and reported their antibacterial[29] and catalytic behaviors.[30] Pradip and co-workers reported the *in vitro* anticancer studies of pyridine thiazole-based transition metal complexes against u937 human monocyclic tumor cells. The pyridine thiazole-based manganese-incorporated complex showed better activity with the IC_{50} value of 4.374 ± 0.02 μM than the nickel- and cobalt-incorporated complexes, the IC_{50} values of which are 5.583 and 11.63 ± 0.01 μM, respectively.[31] Using the AMDET lab, they studied the chemical reactivity, promiscuity risks of the substituted pyrazole derivatives, and drug-like behaviors of pyrazole derivatives.[32] Pyridine−urea-based anticancer drug molecules are prepared and their anticancer activity is studied against the breast cancer MCF-7 cell line. 4-Chloro 3-trifluoro arene urea derivatives and 3-fluoro arene urea derivatives also showed better anti proliferative response against colon cancer, leukemia, renal cancer, melanoma, and CNS lines compared to electron-donating substituents containing arene urea derivatives.[33] The tolyl-substituted pyrazoline derivative showed effective anticancer response against AsPC-1 and U251 cell lines at low concentrations. However, the electron-donating group-containing pyrazoline derivative response against the same anticancer cell line is not appreciable.[34] Supaluk and co-workers listed out the important heterocyclic molecules in drug discovery and development aspects in which mono-substituted pyridine coumarine derivatives showed excellent anticancer response against HepG2, di-substituted pyridine derivative showed anticancer response against MCF-7 cell line with GI50 values from 0.16 to 0.28 μM, triaryl pyridine derivatives showed interesting response of Topo II and cytotoxicity activities in triaryl pyridines, with the OH group at m- or p-position on the 2-phenyl ring, benzoyl urea-substituted pyrimidine derivatives showed significant antitumor activity, and tetra-substituted pyrimidine derivatives showed significant anticancer activity against lung cancer HOP-92 and leukemia MOLT-4 cell lines.[35] Mari *et al.* described the antimalarial, antiviral, anti-inflammatory, antimicrobial, and anticancer properties of some novel substituted pyridine and pyrimidine derivatives in the review article.[36] Based on the literature, we wish to prepare a water-soluble longer alkyl chain/flexible aryl unit as a linker moiety containing amino-substituted dimeric pyridinium bromides using an easily available starting material under a conventional and solvent-free silica-supported muffle furnace method. All the synthesized dimeric pyridinium bromides contain lipophilic and lipophobic moieties; thus, these molecules are potential candidates for anticancer studies.

2. RESULTS AND DISCUSSION

2.1. Chemistry. *2.1.1. Synthesis of Dimeric Pyridinium Bromides from the Conventional Method.* Synthesis of dimeric primary/tertiary amine-substituted pyridinium bromides is done, using flexible longer alkyl chains as the linker unit, by a conventional method.[30] 1,5-Dibromopentane reacted with 2.02 equiv of 2-amino pyridine/*N*,*N*-dimethyl-4-aminopyridine in dry CH_3CN for quaternization reaction under refluxing conditions for 1−2 h to get dimeric substituted pyridinium bromides **1** and **3** as colorless precipitates (Scheme 1). After the disappearance of 1,5-dibromo pentane, the reaction mixture is washed thrice with CH_3CN to remove the unreacted excess substituted pyridine and dried. After the purification process, the reaction mixture afforded 88% of dimeric substituted pyridinium bromides **1** and **3**.

Similarly, *m*-xylene dibromide is treated with 2.02 equiv of 2-aminopyridine/*N*,*N*-dimethyl-4-aminopyridine at room temperature with stirring for 10−18 min, affording a colorless

https://doi.org/10.1021/acsomega.3c00526
ACS Omega 2023, 8, 13243−13251

Table 1. Preparation of Dimeric Pyridinium Bromides under Different Conditions

		synthesis of flexible dimeric pyridinium bromides							
		conventional method				solvent-free method			
s. no.	compound	room temperature (min)	yield (%)	reflux (min)	yield	room temperature (min)	yield (%)	thermal condition	yield (%)
1	**1**			60	87			30 min	94
2	**2**	60	75	20	92	5	95	not required	
3	**3**			60	88			20 min	92
4	**4**	15	80	10	80	5	96	not required	

Table 2. Physicochemical Properties of Dimeric Pyridinium Bromides 1−4

physicochemical parameters	1	2	3	4
lipophilicity (XLOGP3)	2.92	3.48	1.98	2.54
size (M_W; g/mol)	314.47	348.48	258.36	292.38
molar reactivity	100.79	111.01	81.18	91.41
polarity (TPSA; Å^2)	14.24	14.24	59.80	59.80
solubility (Log*S*)	−3.49	−4.31	−2.76	−3.59
saturation (fraction; Csp3)	0.47	0.27	0.33	0.11
flexibility (number of rotatable bonds)	8	6	6	4
HBA(N + O)	4	4	4	4
HBD(NH + OH)	0	0	4	4
BBB permeant	yes	yes	yes	yes
log Kp (skin permeation; cm/s)	−6.15	−5.95	−6.47	−6.28
P-gp substrate	yes	yes	yes	yes
log Po/w	2.26	2.49	1.31	1.54
bioavailability score	0.55	0.55	0.55	0.55
CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4	CYP2D6 (yes)	CYP2D6 (yes)		CYP2D6 (yes)

precipitate (Scheme 1). The precipitate is washed with dry CH_3CN to remove excess substituted pyridine, which afforded 92% of yield, as shown in Table 1. All the synthesized compounds are thoroughly characterized by spectral and analytical data.

2.1.2. Synthesis of Dimeric Pyridinium Bromides from the Greener Method. We attempted the preparation of dimeric pyridinium bromides **1−4** from the solvent-free silica-supported muffle furnace method. The required equivalence of the substituted pyridine and dihaloalkane/*m*-xylene dibromide is mixed with 5 g of 60−120 mesh silica gel, followed by gentle grinding using a mortar and pestle. We observed the interesting results during simple grinding: when *m*-xylene dibromide is grinded with 2-amino pyridine/*N*,*N*′-dimethyl amino pyridine, the reaction is completed without thermal support with a higher yield, whereas 1,5-dibromo pentane is grinded with substituted pyridine and 5 g of silica gel and kept in a muffle furnace at 100 °C for 20−30 min to get a higher yield. Based on the above observation, we conclude that the solvent-free silica-supported muffle furnace method is superior to the conventional method for the preparation of dimeric pyridinium bromides **1−4** due to the higher yield, less reaction time, toxic solvents being avoided for the preparation, and easy separation methodology.

2.2. In Silico Pharmacokinetics. The bioavailability aspects of dimeric pyridinium bromides **1−4** are evaluated by Swiss Drug Design Tools.[37] An overview on the applications of the Swiss ADME web tool in the design and development of anticancer, antitubercular, and antimicrobial agents: A medicinal chemist's perspective[38] and the bioavailability radar showed six physicochemical indices such as lipophilicity (XLOGP3), size, polarity, solubility, saturation, and flexibility (Table 2 and Figure 1). The pink area represents the biophysical range, which accounts for the drug-like nature.

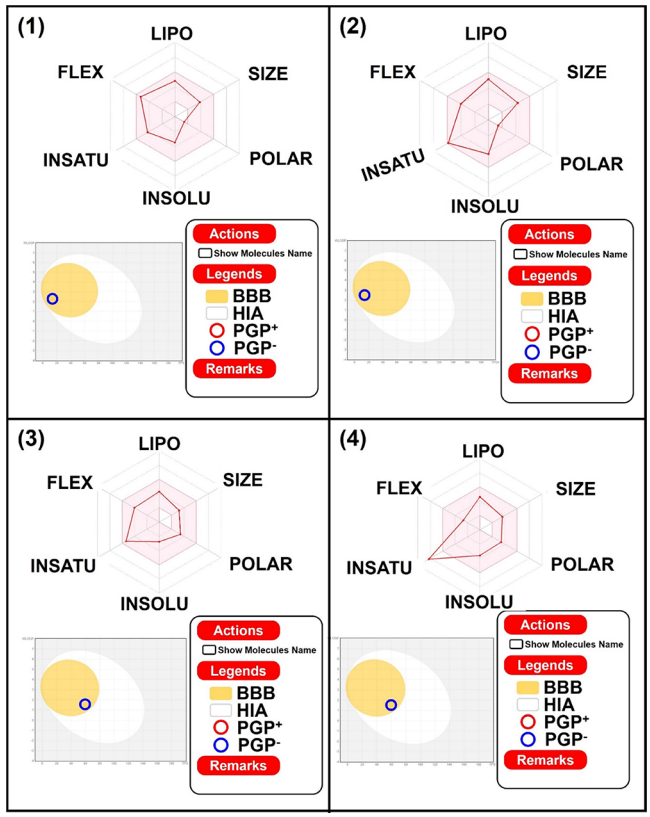


Figure 1. Drug likeness parameters of dimeric pyridinium bromides **1−4** assessed using Swiss ADME. LIPO = lipophilicity; POLAR = polarity; FLEX = flexibility; INSATU = insaturation or saturation as per the fraction of carbons in sp3 hybridization; INSOLU = solubility.

https://doi.org/10.1021/acsomega.3c00526
ACS Omega 2023, 8, 13243−13251

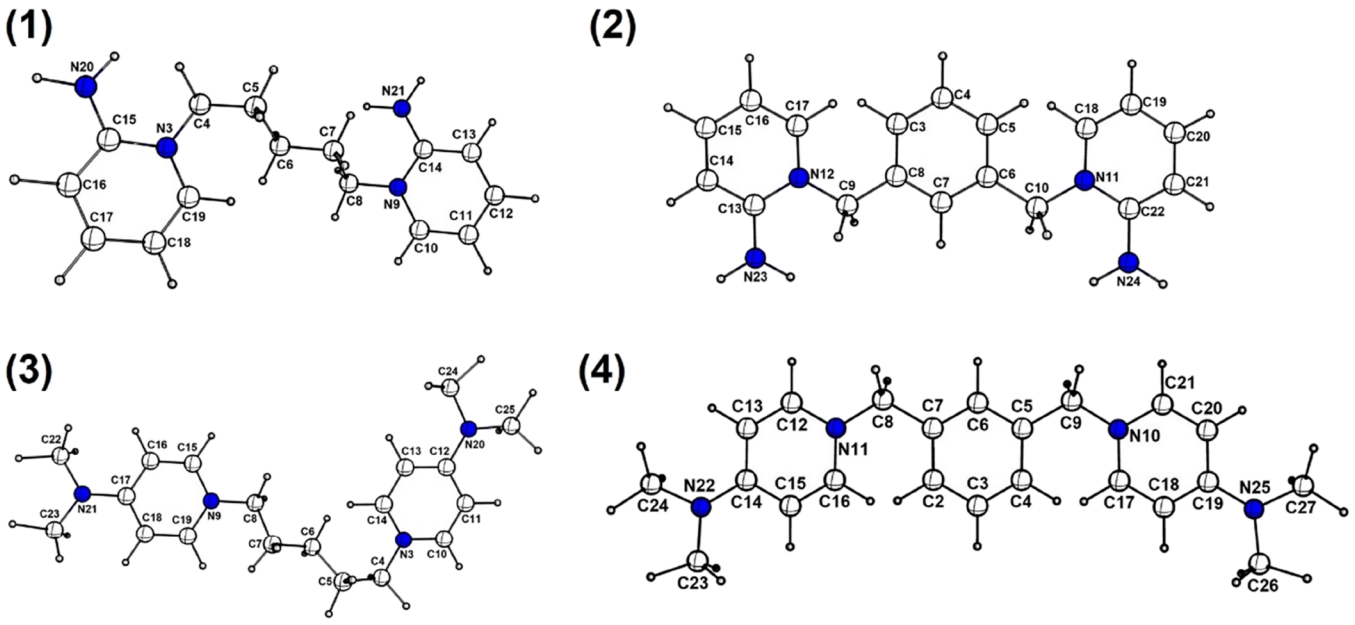


Figure 2. Optimized molecular structures of dimeric pyridinium bromides **1**−**4**.

Table 3. IC_{50} Values of the Dimeric Pyridinium Bromides 1−4 against Two Cancer Cell Lines (A549 and MDA-MB-231) and Normal (3T3-L1) Cell Lines

	IC_{50} (μM)				
	A549		MDA-MB-231		3T3-L1
compound	24 h	72 h	24 h	72 h	72 h
1	1255 ± 1.01			54.67 ± 0.11	>100
2	757 ± 0.54			28.35 ± 0.03	61.96 ± 0.52
3	1000 ± 0.38			35.87 ± 0.17	>100
4	487 ± 0.01	11.25 ± 0.01	524 ± 0.02	19.12 ± 0.81	56.87 ± 0.24
5-fluorouracil	747 ± 0.13	17.01 ± 0.41	885 ± 0.76	29.01 ± 0.01	41.35 ± 1.05

From the radar images, it is observed that compounds **1**−**3** are within the limits but only 1,1′-(1,3-phenylene bis(methylene)-bis 2-aminopyridinium bromide **4** is slightly above the saturation limit. All the compounds showed blue spots in the egg points leading to their efficient efflux by the "permeability glycoprotein" or P-glycoprotein (P-gp). P-glycoprotein is an efflux transporter pump present in many organelles and plays a key role in drug transport. Compounds are predicted to easily cross the blood−brain barrier (BBB), which is a major barrier to drug delivery to the central nervous system (CNS). Also, the skin permeability coefficient (log Kp) is calculated, which appeared to relate with the lipophilicity and molecular size. Low skin penetration is indicated by the highly negative values of log Kp assessed for all compounds (−6.47 to −5.95 cm/s).

According to the results, all newly designed dimeric pyridinium bromides **1**−**4** obey Lipinski's five rules; thus, these compounds are expected to have better drug-like properties. The dimeric pyridinium bromide derivatives are found to have molecular weights below 500, indicating that these molecules are easier to transport, diffuse, and absorb than larger molecules. All the compounds showed four hydrogen-bond acceptors (nHA), and dimeric pyridinium bromides **3** and **4** showed only four donor (nHD) atoms, while **1** and **2** have no donor atoms, which are less than 10 and 5, respectively (Table 2 and Figure 1). All the compounds showed the number of rotatable bonds between 4 and 8 which is within the range. Compounds with TPSA values above 140 have poor intestinal absorption, while TPSA values below 90 must cross the blood−brain barrier. The TPSA values of dimeric pyridinium bromides **1** and **2** are similar, which are 14.24, and **3** and **4** also showed the same value of 59.80, which is below the normal limit. From the results shown in Table 2, it can be seen that all the compounds are within the parameter range of $M_W \leq 500$ Da, log$P$ < 5, nHBD ≤ 5, nHBA ≤ 10, and TPSA < 140 Å^2. These results confirm that the compounds obey Lipinski's five rules, which leads to the adherence to criteria for oral drugs.

2.3. Optimized Molecular Structure. Density functional theory (DFT) calculations have been extensively used to elucidate the molecular structure in the absence of single-crystal XRD data, providing optimized structures with bond lengths and angles.[39,40] The newly synthesized dimeric pyridinium compounds have been optimized by the DFT/B3LYP 6-31G(d,p) basis set, and the optimized structure of the compounds is shown in Figure 2. The calculated bond length and bond angle values are compared with previously reported X-ray data; interestingly, the calculated bond length and bond angle values are in good agreement with the reported X-ray data.[41−43] The calculated bond lengths for compounds **1** and **3** (dimeric pyridinium moieties) N3−C4; C4−C5; C5−C6; C6−C7; C7−C8; and C8−N9 are found to be 1.47, 1.50, 1.48, 1.52, 1.46, and 1.45, which are almost similar to the reported crystal data. The bond angle values are also very close to the reported values of N3−C4−C5; C4−C5−C6; C6−C7−

https://doi.org/10.1021/acsomega.3c00526
ACS Omega 2023, 8, 13243−13251

C8; and C8−C9−N9 and are found to be 112.1, 113.9, 114.2, and 114.[41,42] Similarly, the dimeric pyridinium cations **2** and **4** are also compared with the previously reported crystal data,[37] which are consistent with the reported X-ray data of bond lengths and bond angles. These findings also supported the proposed molecular structure of the compounds.

2.4. Anticancer Properties. *2.4.1. MTT Assay.* This new dimeric pyridinium bromide produced excellent antimicrobial activity, which is very interesting, and no reports of antiproliferative activity are available for the dimeric pyridinium bromides. Therefore, these compounds are subjected to antiproliferative activity against two different cancer cell lines: human lung adenocarcinoma (A549) and human breast adenocarcinoma (MDA-MB-231), and the normal lines 3T3-L1 are tested by MTT assay with the standard drug 5-fluorouracil.[44−47] The antiproliferative activity is evaluated at two different incubation times, short (24 h) and long (72 h).

After 24 h of incubation, all compounds showed better antiproliferative activity in a time-dependent manner, with increasing incubation time and decreased cell viability against A549 and MDA-MB-231 cell lines (Table 3). Interestingly, 1,1′-(1,3-phenylene bis(methylene)bis 2-aminopyridinium bromide **4** is significantly ($p < 0.01$; IC_{50}: 487 ± 0.01) more potent than the commercially used drug 5-fluorouracil (IC_{50}; 747 ± 0.13) against A549. Likewise, in MDA-MB-231 cells, 1,1′-(1,3-phenylene bis(methylene)bis 2-aminopyridinium bromide **4** exhibited a significantly ($p < 0.01$; IC_{50}: 524 ± 0.02) more efficacy than the commercially used drug 5-fluorouracil (IC_{50}: 885 ± 0.76). The dimeric pyridinium bromide **2** (IC_{50}: 757 ± 0.54) is also close to the standard drug 5-fluorouracil against A549.

After 72 h incubation, dimeric pyridinium bromides **1**−**4** substantially increased the antiproliferative activity against all the tested cell lines (Table 3). Remarkably, the compound **4** is significantly ($p < 0.01$; IC_{50}: 11.25 ± 0.01) more potent than the commercially used drug 5-fluorouracil (IC_{50}: 17.01 ± 0.41) and other dimeric pyridinium bromides **1**−**3** against A549. Also, in MDA-MB-231 cells, 1,1′-(1,3-phenylene bis(methylene)bis 2-aminopyridinium bromide **4** revealed significantly ($p < 0.001$; IC_{50}: 19.12 ± 0.81) more efficacy than the commercially used drug 5-fluorouracil (IC_{50}: 29.01 ± 0.01) and other compounds. Compound **2** (IC_{50}: 28.35 ± 0.03) is also close to the standard drug 5-fluorouracil against MDA-MB-231. Crucially, the newly synthesized dimeric pyridinium bromides showed higher IC_{50} values against normal cells, which means less toxicity in normal cells (3T3-L1) and high toxicity against cancer cells (Table 3).

Among the four compounds, 1,1′-(1,3-phenylene bis(methylene)bis 2-aminopyridinium bromide **4** showed the highest antiproliferative activity because of their hydrophobic $-CH_3$ and $-CH_2$ substituents. Dimeric pyridinium bromides **1** and **3** have shown lower antiproliferative activity over the standard drug 5-fluorouracil, and other dimeric pyridinium bromides **2** and **4**, which may be a flexible aliphatic pentane moiety. Furthermore, the observed antiproliferative activity can also be associated with lipophilicity; 1,1′-(1,3-phenylene bis(methylene)bis 2-aminopyridinium bromide **4** showed high hydrophobic values over the other compounds. This hydrophobicity of compounds may promote increased uptake of the compounds by the cells, thereby increasing the antiproliferative activity.

2.4.2. Apoptosis. 2.4.2.1. AO/EB Staining. We are interested in investigating the morphological changes in A549 cells stained with acridine orange (AO) and ethidium bromide (EB) after treatment with 1,1′-(1,3-phenylene bis(methylene)bis 2-aminopyridinium bromide **4** (15 μM) for 24 h. No significant apoptotic morphology is observed in control cells in dual staining with acridine orange and ethidium bromide. However, in the 1,1′-(1,3-phenylene bis(methylene)-bis 2-aminopyridinium bromide **4**-treated group, reduced cell density with late apoptotic morphology and green apoptotic cells containing apoptotic bodies and red necrotic cells are observed (Figure 3A,B).

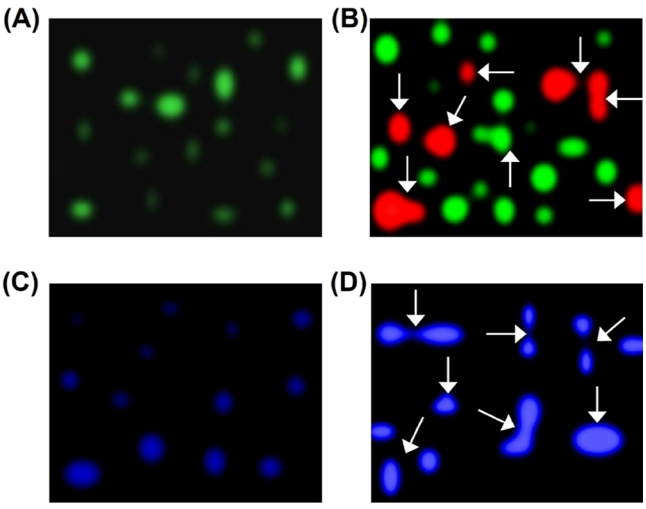


Figure 3. Fluorescence microscopy images of A549 cells treated with dimeric pyridinium bromide **4** and AO/EB staining (A). (C) Control. (B) Dimeric pyridinium bromide **4** with Hoechst 33258 staining. (D) Dimeric pyridinium bromide **4**.

2.4.2.2. Hoechst 33258 Staining. To further confirm the induction of apoptosis, the A549 cells are also treated with 1,1′-(1,3-phenylene bis(methylene)bis 2-aminopyridinium bromide **4** (15 μM) and stained with Hoechst 33258 for 24 h, exhibiting the apoptotic highlights such as chromatin fragmentation, cytoplasmic vacuolation, nuclear swelling, and cytoplasmic blebbing (Figure 3C,D). This result also supports the results observed from AB/EB staining.

2.4.3. Cell Cycle Arrest. Cell cycle analysis plays an important role in the apoptosis in cancer;[44,47] therefore, cell cycle analysis is achieved by flow cytometry against A549 cells treated with 1,1′-(1,3-phenylene bis(methylene)bis 2-amino-pyridinium bromide **4** (15 μM) for 24 h. After the treatment of A549 cells with1,1′-(1,3-phenylene bis(methylene)bis 2-aminopyridinium bromide **4**, a significant increase in the SubG0/G1 phase occurred from 0.5% in the control to 5.5%. Similarly, S (7.8% in the control to 11.5%) and G2/M (15.6% in the control to 24.8%) phases also increased. The G0/G1 phase (51.3% in the control to 36.8%) considerably decreased (Figure 4). This result indicates that the compound **4** induces apoptosis and does not arrest the DNA synthesis phase.

2.4.4. Western Blot Analysis. In general, apoptosis occurs *via* two important apoptotic pathways, namely, the extrinsic (receptor-dependent) and intrinsic (mitochondrial) pathways.[44,47] Among these, caspases involved with the extrinsic and Bcl-2 family proteins are involved with the intrinsic pathways,[48] and these two family proteins play an important

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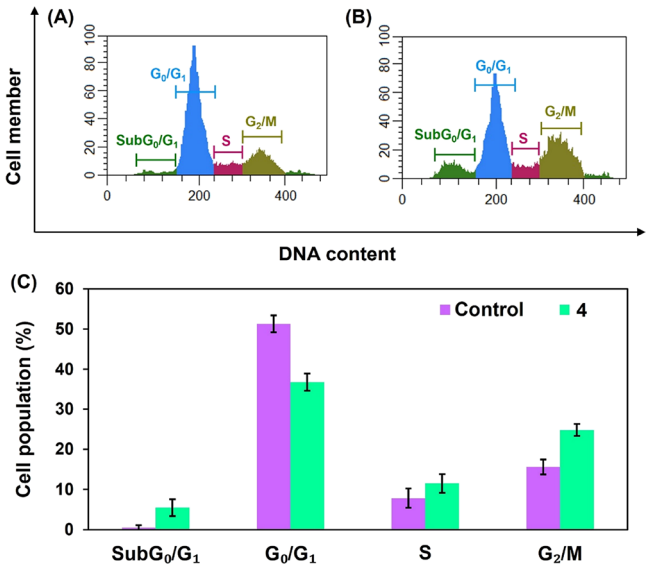

Figure 4. Cell cycle progression of A549 cells for 24 h: (A) Control, (B) dimeric pyridinium bromide **4**, and (C) cell population.

role in regulating the process of apoptosis.[49] Dimeric pyridinium bromide **4** showed the highest anticancer activity in A549 cells among the four compounds; therefore, this dimeric pyridinium bromide **4** is preferred for western blot investigation. After 24 h of treatment with 1,1′-(1,3-phenylene bis(methylene)bis 2-aminopyridinium bromide **4** (15 μM) in A549 cells, the expressions of the extrinsic pathway proteins, caspase-3 and caspase-9, showed a substantial upregulation compared to the control (Figure 5). On the other hand, the

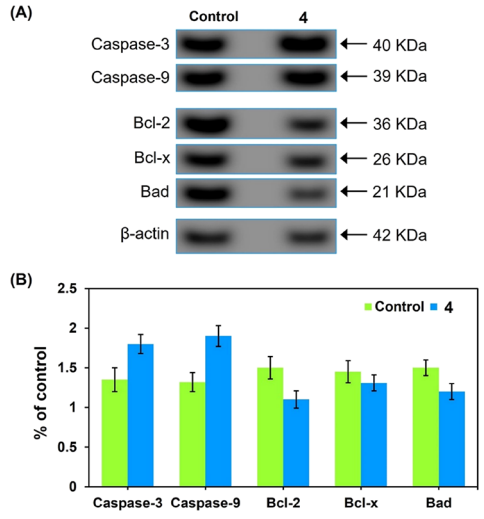

Figure 5. (A and B) Western blot analysis of caspase 3, caspase 9, Bcl-x, Bcl-2, and Bad against A549 cells treated with 1,1′-(1,3-phenylene bis(methylene)bis 2-aminopyridinium bromide **4** for 24 h.

intrinsic pathway proteins Bcl-2, Bcl-x, and Bad showed a substantial downregulation compared to the control (Figure 5). These results support that the compounds are able to induce apoptosis in A549 cells by activating the caspase family proteins.

3. CONCLUSIONS

Water-soluble amino-substituted flexible dimeric pyridinium bromides are prepared from conventional and solvent-free silica-supported muffle furnace methods. Solvent-free method has more merits than the conventional method, such as easy workup procedure, less reaction time, and higher yield. Toxic organic solvents are totally avoided. We have examined the cytotoxicity of our newly synthesized compounds against A549 lung cancer cell lines. 1,1′-(1,3-Phenylene bis(methylene)bis 2-aminopyridinium bromide **4** showed the highest cytotoxicity response compared to other compounds with the standard 5-fluorouracil drug. Apoptosis studies confirmed the cell death, which is further confirmed by cell cycle analysis. These novel dimeric pyridinium bromides inhibit cancer cell growth by activating the caspases and downregulating the Bcl-2 protein.

4. EXPERIMENTAL SECTION

4.1. General Procedure for the Synthesis of Dimeric Pyridinium Bromide. 1,5-Dibromopentane/*m*-xylene dibromide (1.0 equiv) and *N*,*N*-dimethyl 4-amino pyridine/2-amino pyridine (2.02 equiv) are dissolved in 80 mL of dry CH_3CN at room temperature/refluxed for the required duration to obtain dimeric pyridinium bromides **1**−**4** in quantitative yields after purification.

*4.1.1. 1,1′-(Pentane-1,5-diyl) Bis(4-(dimethylamino) Pyridinium Bromide **1**.* Yield: 3.0 g (87%); mp 88−90 °C; ^{1}H NMR (400 MHz; D_2O) δ (ppm): 7.89−7.91 (d, J = 8 Hz, 2H); 6.77−6.79 (d, J = 4 Hz, 2H); 4.70 (s, 4H); 4.06−4.02 (t, J = 4 Hz, 4H); 3.12 (s, 12H); 1.70−1.77 (quint., J = 8 Hz, 4H); 1.20−1.26 (quint., J = 8 Hz, 2H), ^{13}C NMR (100 MHz,D_2O) δ = 21.7, 29.2, 39.4, 57.1, 107.5, 141.3, 156.3, MS: *m/z:* 157; anal. calculated for $C_{19}H_{30}N_4Br_2$: C, 48.10; H, 6.32; N, 11.81; found: C, 48.07. 10; H, 6.28; N, 11.78.

*4.1.2. 1,1′-(1,3-Phenylene Bis(methylene)bis(4-(dimethylamino) Pyridinium Bromide **2**.* Yield: 1.5 g (92%); mp 68−70 °C;^{1}H NMR (400 MHz; D_2O) δ (ppm) 7.93−7.95 (d, J = 8 Hz, 2H); 7.28−7.29 (d, J = 8 Hz, 4H), 7.40−7.48 (quint., J = 8 Hz, 1H) 7.18 (s,1H), 6.77−6.79 (d, J = 8 Hz, 4H); 5.24 (s, 4H); 3.11 (s, 12H), ^{13}C NMR (100 MHz, D_2O): δ = 39.4, 59.5, 107.7, 127.0, 128.5, 130. 1, 135.9, 141.4, 156.4, MS: *m/z*; 185 anal. calculated for $C_{24}H_{26}N_4Br_2$: C, 54.33; H, 4.90; N, 10.56; found: C, 54.28; H, 4.87; N, 10.54.

*4.1.3. 1,1′-(Pentane-1,5-diyl)bis 2-Aminopyridinium Bromide **3**.* Yield 3.5 g (88%); liquid; ^{1}H NMR (400 MHz; D_2O) δ (ppm): 7.81−7.83 (d, J = 8 Hz, 2H); 7.71−7.75 (t, J = 4 Hz, 2H); 6.99−7.01 (d, J = 8 Hz, 2H); 6.89−6.94 (t, J = 8 Hz, 2H); 4.08−4.13 (t, J = 8 Hz, 4H); 1.61−1.67 (quint., J = 8 Hz, 4H); 1.33−1.39 (quint., J = 4 Hz, 2H), ^{13}C NMR (100 MHz; D_2O): δ = 22.08, 26.1, 53.5, 113.8, 115. 1, 139.4, 142.5, 153.8, MS: *m/z:*129; anal. calculated for $C_{15}H_{22}N_4Br_2$: C, 43.06; H, 5.26; N, 13.39; found: C, 43.02; H, 5.23; N, 13.36.

*4.1.4. 1,1′-(1,3-Phenylene Bis(methylene)bis 2-Aminopyridinium Bromide **4**.* Yield: 2 g (80%); liquid; ^{1}H NMR (400 MHz; D_2O) δ (ppm): 7.83−7.85 (t, J = 8 Hz, 2H), 7.79−7.81 (d, J = 8 Hz, 2H), 7.41−7.45 (t, J = 8 Hz, 2H), 7.25−7.27 (d, J = 8 Hz, 2H), 7.00−7.03 (d, J = 4 Hz, 2H), 6.86−6.90 (quart., J = 8 Hz, 1H), 6.45 (s, 1H), 5.34 (s, 4H); ^{13}C NMR (100 MHz, D_2O): δ = 56.0, 114.1, 115.3, 123.4, 127.4, 128.9, 154.6, 143.4, 139.6, 133.1, MS: *m/z*: 146; anal. calculated for $C_{18}H_{20}N_4Br_2$: C, 47.78; H, 4.42; N, 12.38; found: C, 47.74; H, 4.48; N, 12.34.

https://doi.org/10.1021/acsomega.3c00526
ACS Omega 2023, 8, 13243−13251

4.2. Procurement of Cell Line. To evaluate the cytotoxicity in cancer cells, A549 lung adenocarcinoma and MDA-MB-231 breast adenocarcinoma cell lines are used. The cells are procured from the National Center for Cell Sciences (NCCS). The A549 cells are maintained in Dulbecco's minimal essential media (DMEM), and MDA-MB-231 are maintained in Eagle's minimum essential medium (MEM) supplemented with fetal bovine serum (FBS) and antibiotics in a T25 flask. The cells are incubated at 37 °C and humidified with 5% CO_2. Once confluency is reached, the cells are trypsinized and used for further experiments.

4.2.1. MTT Assay. 5×10^3 cells are seeded in a 96-well plate and incubated for growth under the conditions mentioned above. Once confluency is reached, the cells are treated with different concentrations of dimeric pyridinium bromides **1**−**4** and incubated for 24 h. After incubation, the media is removed, and 20 μL of MTT is added and incubated for further 3 h. After 3 h, MTT is removed, and DMSO is added to dissolve the formazan crystals. The absorbance is measured at 570 nm, and the percentage of cell death is calculated using the formula

$$\%\text{of cell death} = \text{absorbance of control} - \text{absorbance of treated} / \text{absorbance of control} \times 100$$

4.2.2. Acridine Orange (AO) and Ethidium Bromide (EtBr) Staining. 5×10^4 cells are seeded in a six-well plate and incubated to reach confluency. The cells are treated with two different concentrations including dimeric pyridinium bromides **1**−**4**. The cells are incubated for 24 h. After the incubation period, the media is removed, and the cells are stained with 10 μL of 1 mg/mL of AO, and EtBr is added into each well and observed under a fluorescence microscope at 10× magnification (Nikon Eclipse Ti).

4.2.3. Screening of Toxicity in Normal Cell Line. To screen the toxicity in the normal cell line, 3T3-L1, a fibroblast cell line is used. Cells are maintained in DMEM, supplemented with 5% FBS and antibiotics. To check the toxicity, 3T3-L1 cells are treated with four different compounds at various concentrations for 24 h. After 24 h, MTT assay is performed as described above.

4.2.4. Geometry Optimization Study. DFT calculation, apoptosis, cell cycle analysis, and Wester docking studies are carried out by following the procedure reported in the earlier publications.[31−33,36]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c00526.

^{1}H and ^{13}C NMR spectra of dimeric pyridinium bromides **1**, **2**, **3**, and **4** (PDF)

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Notes

The authors declare no competing financial interest.

The authors declare the following competing financial interest(s): S.G., K.G., and R.L. are co-inventors of the patent application IP 202141061136, which describes antibacterial and antifungal efficacies.

■ ACKNOWLEDGMENTS

K.G. thanks Dr. M. Sugunalakshmi, Senior Principal Scientist, Polymer Science & Technology, CLRI, Chennai-600020, and Dr. M. Bakthadoss, Professor in Chemistry, Pondicherry University, for their constant support. S.G. thanks D. Sathish, Indian Institute of Technology, Madras, for his constant support. M.M.A. and M.A. thank Deanship of Research, King Khalid University, Saudi Arabia, for the large research group under the grant number R. G. P. 2/210/1444.

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