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Brief Article

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Carboranes Increase the Potency of Small Molecule Inhibitors of Nicotinamide Phosphoribosyltranferase

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Supporting Information

ABSTRACT: Herein we report the use of carboranes to significantly increase the potency of small molecule inhibitors of nicotinamide phosphoribosyltranferase (Nampt), an enzyme that is central to metabolism and cell survival. We compare the inclusion of carborane with other similarly sized substituents and demonstrate that, compared with their purely organic counterparts, these molecules exhibit up to 10-fold greater antiproliferative activity against cancer cells in vitro and a 100-fold increase in Nampt inhibition.

INTRODUCTION

Over the past decade, there has been an increasing interest in the use of carboranes as pharmacophoric units in drug design.[1] Carboranes, which are icosahedral clusters composed of boron, carbon, and hydrogen, demonstrate high chemical stability and may be readily incorporated into small molecules as highly symmetrical analogues of aromatic hydrocarbons.[2] Because each of the 12 B−H or C−H vertices may be derivatized through extensively developed substitution chemistry, these clusters may serve as rigid scaffolds upon which to build molecules with well-defined, three-dimensional conformations.[3] While early biomedical applications of carborane chemistry focused on the development of boron-delivery agents for use in boron neutron capture therapy (BNCT),[4] more recent work has focused on the incorporation carboranes into the structures of new pharmacological agents. In such examples, the hydrophobic nature of carboranes was exploited for the development of novel bioactive molecules.[5−8] In addition to hydrophobic interactions, it has been elucidated that carboranes bind strongly with biomolecules through a unique, strong form of hydrogen bonding.[9] This dihydrogen, or proton−hydride, bond may occur between a hydrogen on a typical proton donor and a hydrogen bound to an electropositive boron atom.[10] The strength of these individual dihydrogen bonds has been calculated to average 20 kJ mol^{-1} and may total more than 67 kJ mol^{-1} for a single carborane cluster bound to multiple donors and over 200 kJ mol^{-1} for metallacarboranes.[9] Consequently, these noncovalent interactions may be much stronger than typical biomolecular hydrogen bonds. Therefore, the use of the carborane moiety in drug design might prove superior to similarly sized organic groups in some circumstances.

Before now, no group has systematically compared the three isomers of carborane with other similarly sized organic groups in the structure of a drug molecule with the purpose of improving activity. Here, we report such a comparison. For this purpose, we chose to construct inhibitors of nicotinamide phosphoribosyltranferase (Nampt). This protein is the first and rate limiting enzyme in the mammalian NAD^+ recycling pathway, converting nicotinamide to nicotinamide mononucleotide (NMN). Owing to the many disparate physiological roles and cellular compartmentalization of NAD^+, this vital enzyme has been given different names (visfatin, pre-B cell colony enhancing factor (PBEF), NAmPRTase, and Nampt). It has recently been elucidated that Nampt activity plays a central role in metabolism, cellular proliferation, cell survival, and inflammatory response, making this enzyme a new and intriguing target for the treatment of many diseases, including cancer, Alzheimer's, diabetes, and arthritis.[11−15]

FK866 **1**, a molecule identified through library screening methods, is the first known specific and highly potent small molecule inhibitor of Nampt.[16] Although this drug has no primary effect on cellular energy metabolism, its activity causes a gradual depletion of cellular NAD^+ levels, inducing apoptosis.[16] The molecular basis for the inhibition of Nampt by **1** is understood, as the crystal structure for the drug−enzyme complex has been determined.[17] Located near the protein dimer interface, each of the two active sites is accessible only through a narrow tunnel, the dimensions of which appeared to be ideally suited for a drug molecule bearing a carborane moiety. When **1** is bound to Nampt, its benzoylpiperidine moiety partially blocks this tunnel.[17] While Nampt possesses residues at the benzoylpiperidine−enzyme interface that could potentially form hydrogen bonds with a ligand, no such drug−enzyme interactions are apparent. We postulated that a drug presenting a carborane moiety in this position might prove superior to benzoylpiperidine or other similarly sized organic groups, owing to the size and hydrophobicity of carboranes, as well as their potential to form strong dihydrogen bonds with the available amino acid residues. Among the adjacent residues is an arginine, which has been calculated to form strong interactions with carboranes.[9] Figure 1 depicts a molecular model of the binding of **1** in the

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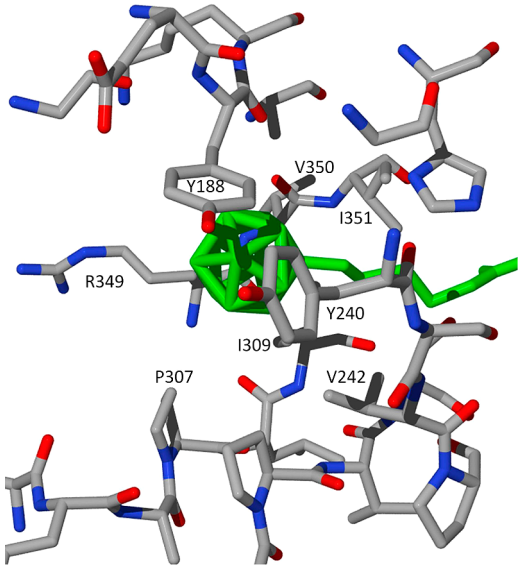

Figure 1. Molecular model of the binding of **1** (green) in the active site of Nampt. Here, the benzoylpiperidine moiety of **1** has been replaced with a carborane to illustrate the residues immediately surrounding this volume.

active site of Nampt where the benzoylpiperidine moiety has been replaced by a carborane. In this model, the carborane is surrounded by several hydrophobic residues, including tyrosine, valine, and isoleucine. As is apparent, arginine 349 is also in proximity to the carborane. The performance of docking studies using carborane derivatives is not facile, as none of the available software packages (including AutoDock, Surflex, FlexX, and Glide) have the parameters necessary to describe the hexavalent boron atoms found in polyhedral boranes or carboranes. However, one group has recently reported efforts in this area.[18]

RESULTS AND DISCUSSION

To ascertain the contribution of the benzoylpiperidine moiety of **1** to drug activity, we have designed and synthesized a number of analogues in which this moiety was absent or replaced by other groups (Figure 2). These compounds

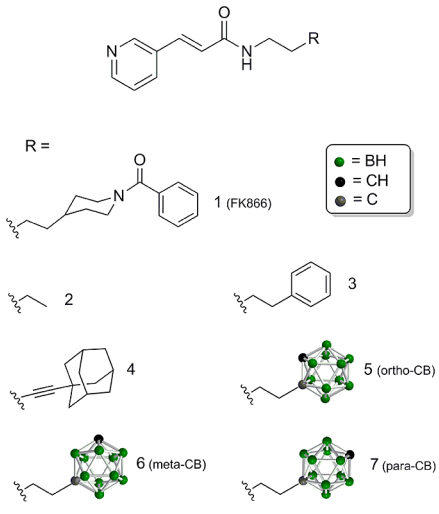

Figure 2. Molecular structures of Nampt inhibitors investigated in this study.

included a structure bearing an unsubstituted butylene moiety **2** and other structures in which benzoylpiperidine was replaced by a phenyl ring **3**, adamantane **4**, ortho-carborane **5**, meta-carborane **6**, or para-carborane **7**. We evaluated the activities of **1**–**7** by performing cell viability assays against three human cancer cell lines: A549 (lung), T47D (breast), and DLD1 (colon). We employed the MTT assay[19] because this test provides a direct measure of mitochondrial activity and enables comparison of our results with those previously reported for **1**.[16] Figure 3 depicts the concentration–response curves

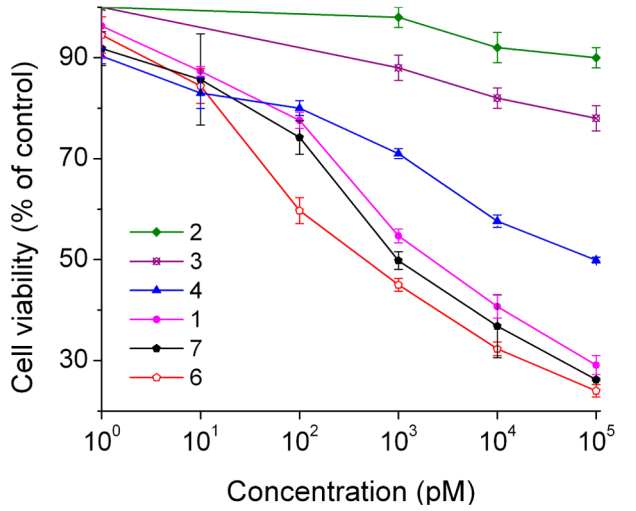

Figure 3. Concentration dependent cell viability exhibited by **1**–**4**, **6**, **7** against the A549 human lung cancer cell line.

measured against the A549 cell line for five of the test compounds and **1**. For clarity, the curve for **5** is omitted, as this agent exhibited activities between those of **1** and **7**.

As evidence for our hypothesis, the absence of benzoylpiperidine in **2** resulted in a near-complete loss of activity, exemplifying the necessity for a bulky, hydrophobic moiety at this position. The addition of a phenyl ring in place of benzoylpiperidine in **3** resulted in an ~100-fold increase in activity over **2**. Previous studies that utilized carborane as a pharmacophoric unit often describe the substitution of a phenyl ring with a carborane in a drug structure.[5−8] Owing to extensive electron delocalization, carboranes may be described as three-dimensional analogues of benzene.[20] Remarkably, analogues **5**–**7**, which bear a carborane moiety at the same position as the phenyl ring in **3**, exhibited activities 3–4 orders of magnitude higher.

As a hydrophobic, polycyclic compound, adamantane is similar in size and bulk to the carboranes (differing in mass by only 10 amu) and it is often employed in drug design studies. An analogue bearing this substituent might be expected to exhibit activities comparable to those of the carborane-based analogues **5**–**7**. The inclusion of an adamantyl group in **4** produced an agent with significant activity, exhibiting cell-line-dependent IC_{50} between 89 and 295 nM, further demonstrating the utility of a bulky, hydrophobic group in this location. However, the carborane-based agents demonstrated activities 100- to 500-fold higher than **4**.

Remarkably, all three carborane-based analogues demonstrated potencies higher than **1** in each of the three cell lines tested. Table 1 lists the IC_{50} values for each of the significantly potent agents in our study.

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Table 1. Measured Half-Maximal Inhibitory Concentrations (IC_{50}) for Agents 1, 4−7 against the Human Tumor Cell Lines A549 (Lung), DLD1 (Colon), and T47D (Breast)

	IC_{50} (nM)		
compd	A549	DLD1	T47D
1 (FK866)	1.62 ± 0.04	3.14 ± 0.11	3.20 ± 0.16
4	88.9 ± 1.1	20.3 ± 0.44	186.4 ± 4.1
5	0.92 ± 0.02	1.69 ± 0.06	1.29 ± 0.13
6	0.41 ± 0.01	0.31 ± 0.01	0.32 ± 0.02
7	0.99 ± 0.03	2.20 ± 0.12	0.58 ± 0.03

The molecule demonstrating the highest potency, **6**, bears a meta-carborane moiety and exhibited subnanomolar IC_{50} concentrations in each of the three cell lines, corresponding to a 10-fold increase in potency over **1** in DLD1 and T47D and a 4-fold increase in A549. While the three isomers of carborane are essentially identical in size, their dipole moments differ because of the relative positions of the C−H vertices. The magnitudes of these dipole moments range between a high of 4.53 D for the ortho isomer and a low of 0 D for the para isomer.[21] If the carborane moieties interacted with the enzyme simply through hydrophobic interactions, then one might expect that the para-isomer **7**, the most hydrophobic of the three, would demonstrate the highest potency. However, the meta-carborane-based analogue **6** exhibited significantly higher activity in each of the three cell lines, indicating that other carborane−enzyme interactions are involved. These data, coupled with the significantly higher potency of the carborane-based agents over those incorporating a phenyl, adamantyl, or benzoylpiperidine moiety, indicate that strong carborane−enzyme interactions are formed, possibly through the formation of dihydrogen bonds.

Each of our new molecules are structurally similar to **1**, with each sharing the pyridinylacrylamide moiety. We presumed that, as in the case for **1**, the basis for the observed antiproliferative activity was the inhibition of Nampt activity.

To confirm this, we performed cell rescue assays using the Nampt enzymatic product NMN against the A549 and DLD1 cell lines. Cells were treated with 10 nM solutions of each test compound, a concentration high enough to kill most of the cells. Increasing concentrations of NMN were added, and cell viability was assayed. Figure 4 depicts the concentration-dependent rescue effects of NMN. At high NMN concentrations, a nearly complete rescue was afforded in each of the three cell lines. The assay results were virtually identical among the three carborane-based agents **5**−**7** and **1**, indicating that Nampt is the common target for each of these agents.

To further confirm Nampt inhibition, we measured the effect of our most potent inhibitor, **6**, on recombinant Nampt activity using a commercially available colorimetric assay. In this assay, **6** exhibited ∼100-fold higher inhibitory activity than **1**.

CONCLUSION

Previous work has demonstrated that carboranes are potentially valuable pharmacophores owing to their hydrophobicity, extensively developed chemistry, and high biostability. In a systematic manner, we have compared the three isomers of carborane with similarly sized organic moieties in a single drug structure, demonstrating that these clusters may be utilized in drug design to produce simple, yet exceptionally potent new molecules. To our knowledge, these new carborane-based agents are now the most potent inhibitors reported to date for

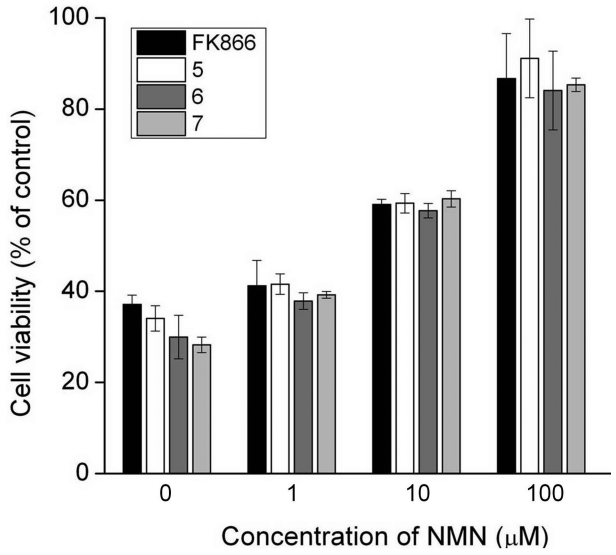


Figure 4. Concentration-dependent cell rescue. A549 cells were treated with 10 nM **1**, **5**, **6**, or **7**. Error bars represent the mean ± SD. Cell viability was measured using the MTT assay; each measurement was repeated four times. A nearly complete rescue of the cells was afforded with increasing concentrations of the Nampt product NMN.

Nampt, an enzyme that has only recently come into focus as a central link connecting metabolism, cancer, and inflammation. Given the tremendous unmet need for more efficacious and affordable chemotherapeutic agents, our findings further demonstrate that these clusters may provide a manner in which to improve the efficacy and specificity of new drugs, one not provided through the use of organic chemistry alone.

EXPERIMENTAL SECTION

General Methods. See Supporting Information for details. Chemical reactions were performed using a combination of Schlenk line and glovebox techniques under an inert atmosphere of dry argon. NMR spectra were recorded on Bruker DRX and Avance spectrometers for ^{1}H at 300, 400, or 500 MHz, for ^{13}C at 100 or 125 MHz, and for ^{11}B at 160 MHz. Mass spectra were obtained using Perseptive Biosystems Mariner API-Tof, Waters Q-Tof, or ABI QSTAR using ESI or APCI. Data are reported as "*m*/*z*, calculated; *m*/*z*, found". The isotopic distribution of each carborane containing ion matched that expected for the normal abundance of boron.

All cell lines were obtained from ATCC (Manassas, VA).

The MTT assay[18] was carried out as follows: A549, DLD1, and T47D cells were plated in 96-well plates at a density of 10 000 cells per well overnight at 37 °C. Cells were then treated with different doses of agents **1**−**7** for 72 h. MTT reagent was added to the cells for 3 h for developing formazan crystals. Solubilization buffer was added to the wells, and the absorbance was measured at 570 nm. **1** was tested simultaneously in each 96-well plate to directly compare with the results of the tested compounds. The experiments were repeated five times, and the standard deviations are reported.

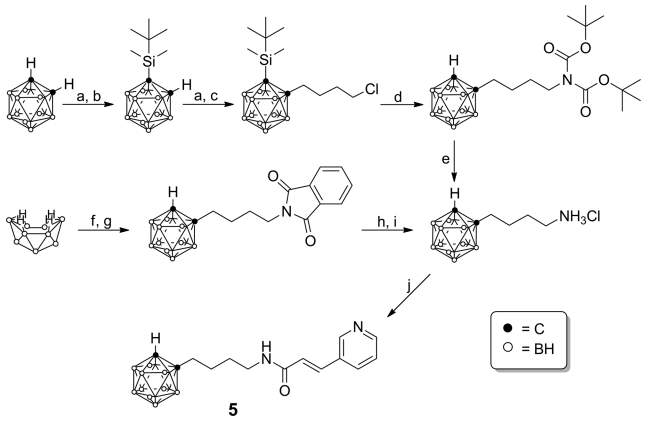
Cell rescue assays were conducted using NMN as described previously.[22] Briefly, A549 and DLD1 cells were plated at a density of 10 000 cells per well in 96-well plates overnight. Cells were then treated with 10 nM concentrations of agents **1**, **5**−**7**. Simultaneously, 1, 10, or 100 μM NMN was added to the cultures. Cultures were then continued for 72 h. After this time, MTT reagent was added to the cells for a period of 3 h and the MTT crystals were solubilized using solubilization buffer. Readings were taken at 570 nm wavelength. Control cells were considered as 100% survival to plot the inhibition and rescue graph. The experiments were repeated four times.

Before testing, each compound was repurified by RP-HPLC using a Beckman System Gold liquid chromatograph equipped with a

Phenomenex Luna C18(2) (250 mm × 4.6 mm) column using a 1 mL/min MeOH/H_2O gradient (0.1% HCOOH) with analyte detection by UV absorbance at 270 nm. After repurification, all compounds were determined to be >95% pure by HPLC using UV analysis.

Synthetic Methods. Compounds **2**–**7** were synthesized by the coupling of *trans*-3-(3′-pyridyl)acrylic acid with the respective amines.[23] Scheme 1 outlines two synthetic routes that we followed

Scheme 1[a]



[a]Reagents and conditions: (a) *n*-BuLi, −78 °C, 4 h; (b) *t*-BuMe_2SiCl, −78 °C, 1 h, rt, 12 h; (c) $Cl(CH_2)_4Br$, rt, 12 h; (d) 5% LiI, Cs_2CO_3, $NHBoc_2$, 2-butanone, 2 days, reflux; (e) 4 M HCl in dioxane, rt, 3.5 h; (f) Et_2S, toluene, reflux, 4 h; (g) $HC_2(CH_2)_4N(CO)_2C_6H_4$, reflux, 12 h; (h) $NaBH_4$, 6:1 2-propanol/H_2O, rt, 12 h; (i) HCL_{conc}, reflux, 12 h; (j) DMF, BOP, Et_3N, $C_5H_4(CH)_2COOH$, rt, 12 h.

to obtain **5**. Derivatives of *o*-carborane may be produced through direct substitution of a carbon atom on *o*-carborane or by inserting an alkyne into decaborane to form the closo icosahedron.[3] A strong base, such as *n*-butyllithium, can extract a weakly acidic proton off one (or both) carbon atom within a carborane. The resulting anion is nucleophilic and will react with electrophiles, such as the 1-bromo-4-chlorobutane used here. The resulting chloride was converted to a protected amine using di-*tert*-butyl dicarbonate; this reaction also removed the carborane silyl protecting group. Deprotection of the amine was afforded by treatment with 4 M HCl in dioxane.

We also prepared **5** using decaborane ($B_{10}H_{14}$) as a starting material. Decaborane reacts with weak Lewis acids, such as dihydrogen sulfide or acetonitrile, to form the adduct $B_{10}H_{12}L_2$, which will react with a substituted alkyne to form the corresponding ortho-carborane. This reaction inserts the carbon–carbon triple bond into the open face of $B_{10}H_{12}L_2$, forming the closo cluster. We chose to use the commercially available *N*-(5-hexynyl)phthalimide for this purpose. Subsequently, the phthalimide was reduced using sodium borohydride, followed by refluxing in concentrated aqueous HCl. In both synthetic routes, the final product **5** was prepared by coupling the amine with *trans*-3-(3′-pyridyl)acrylic acid using Castro's reagent (BOP) as a coupling agent. Similar synthetic strategies were used to prepare the other carborane containing derivatives and are fully described in the Supporting Information. An important safety consideration is to ensure that decaborane does not come in contact with halegenated solvents, such as carbon tetrachloride, which forms a spontaneously explosive mixture. Accidents based on this combination have been reported in the past.

The recombinant Nampt inhibition assay was performed according to the manufacturer's protocol (CycLex NAMPT colorimetric assay kit, MBL International Corp., Woburn, MA). Briefly, 2 μL of recombinant Nampt, dH_2O, and different concentrations of **1** and **6**, ranging from 1 pM to 100 nM (or equal amount of vehicle), was added to each well in a 96-well plate and mixed well. The reaction was initiated by adding 60 μL of one-step assay buffer to each well and mixing thoroughly, followed by incubation at 30 °C for 20 min. After this period, the absorbance at 450 nm was measured and compared with the positive control.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures concerning the synthesis, spectral characterization data, and testing of all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATION USED

amu, atomic mass unit; APCI, atmospheric pressure chemical ionization; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMN, nicotinamide mononucleotide

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