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Complete List of Authors:	Lujan, Berkley; University of California Riverside, Chemistry Zhang, Mingfeng; City of Hope National Medical Center, Diabetes Complications & Metabolism Research Cao, Yujie; University of California Riverside College of Natural & Agricultural Sciences, Chemistry Kacker, Arnav; University of California Riverside College of Natural & Agricultural Sciences, Chemistry Mai, Lina; University of California Riverside College of Natural & Agricultural Sciences, Chemistry Wu, Shunquan; City of Hope National Medical Center, Diabetes Complications & Metabolism Research Alexander, Taylor; University of California Riverside College of Natural & Agricultural Sciences, Chemistry Huang, Wendong; City of Hope National Medical Center, Diabetes Complications & Metabolism Research Kou, Kevin; UC Riverside, Chemistry

ARTICLE

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Berkley Lujan,[a] Mingfeng Zhang,[†b] Yujie Cao,[†a] Arnav Kacker,[a] Lina Mai,[a] Shunquan Wu,[b] Taylor Alexander,[ac] Wendong Huang,*[b] and Kevin G. M. Kou*[a]

Berbamine, a bisbenzylisoquinoline alkaloid (bisBIA), is a promising lead for developing novel therapeutics to treat aggressive cancers such as lymphoma, by targeting the CaMKIIγ:cMyc axis. Herein, we report an aza-Friedel–Crafts method for *ortho*-aminoalkylation of berbamine's phenolic motif, enabling the semisynthesis of the natural product bersavine and analogs that complement current methods focusing on modifying the phenolic oxygen. Several new analogs synthesized by this method exhibit potent cytotoxicity against lymphoma-associated cell line H9 exceeding the naturally occurring berbamine (**1**) and bersavine (**3a**). A molecular docking analysis was used to devise a model that rationalizes the structure-activity relationship between the novel bisBIA analogs and CaMKIIγ inhibition.

c-Myc is a transcription factor that regulates cellular metabolism, cell growth, division, and apoptosis.[1] Dysregulated and overexpressed in many cancers including both B cell and T cell lymphomas, c-Myc is considered an undruggable oncogene.[2] One of our labs (Huang) first identified CaMKIIγ as a key c-Myc regulator that can be targeted by the bisbenzylisoquinoline alkaloid (bisBIA) berbamine (**1**).[3] By inhibiting CaMKIIγ, this natural product destabilizes c-Myc and reduces tumor volume with minimal toxicity in mice, thus establishing berbamine (**1**) as a CaMKIIγ inhibitor and the CaMKIIγ:c-Myc axis as a druggable therapeutic target for aggressive cancers.[4] In contrast, CaMKII inhibitors in development primarily target isoforms involved in cardiac treatment, none of which have advanced to the clinic.[5] Efforts to capitalize on the biomedical properties[6] of berbamine and related bisBIAs have primarily relied on semisynthesis, which has generated novel analogs **2** with enhanced potencies against CaMKIIγ and leukemia cell lines (Figure 1).[3,7] However, all accessible analogs to-date are derived only from modification of the nucleophilic phenolic group of berbamine, which has severely limited structure-activity relationship (SAR) analyses.[3,4,7-12]

The bisBIA bersavine (**3a**) was isolated from the root bark *Berberis vulgaris* in 2019, exhibiting pronounced inhibition against prolyl oligopeptidase[13] and antiproliferative effects against a variety of human cancer cell lines.[14] Inspired by its structure with an aminoalkyl group on C14 and the Kou lab's recent developments in Friedel–Crafts alkylations,[15,16] including *ortho*-alkylation of phenolic derivatives,[17] we hypothesized that selective *ortho*-aminoalkylation of berbamine's phenolic motif would give rise to bersavine (**3a**). The ability to selectively derivatize a site beyond acylation, sulfonylation, or etherification of the phenolic group would effectively generate novel, unexplored berbamine derivatives for pharmacological optimization. Application of this Friedel–Crafts-type aminoalkylation method to berbamine (**1**) led to the semisynthesis of nine bisBIA analogs with unprecedented *C*- and *O*-modifications, including the naturally occurring bersavine (**3a**), all of which interfered with tumor cell viability when tested *in vitro* against the lymphoma cell line H9. This work builds on our labs' interest in developing isoquinoline alkaloids for cancer therapy.[18]

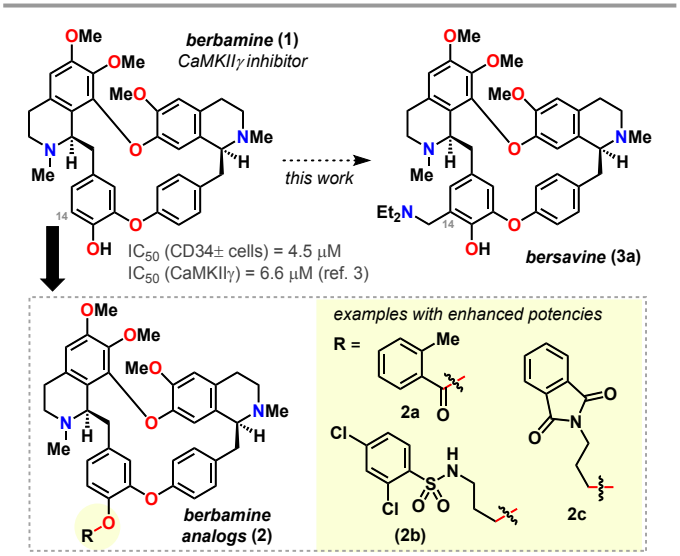

Fig 1 BisBIA natural products and select analogs. CD34± refers to CD34+ and CD34– cells.

[a] Department of Chemistry, University of California, Riverside, California 92507, United States.
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[c] Kyowa Kirin, Inc., 9420 Athena Cir, La Jolla, CA 92037.
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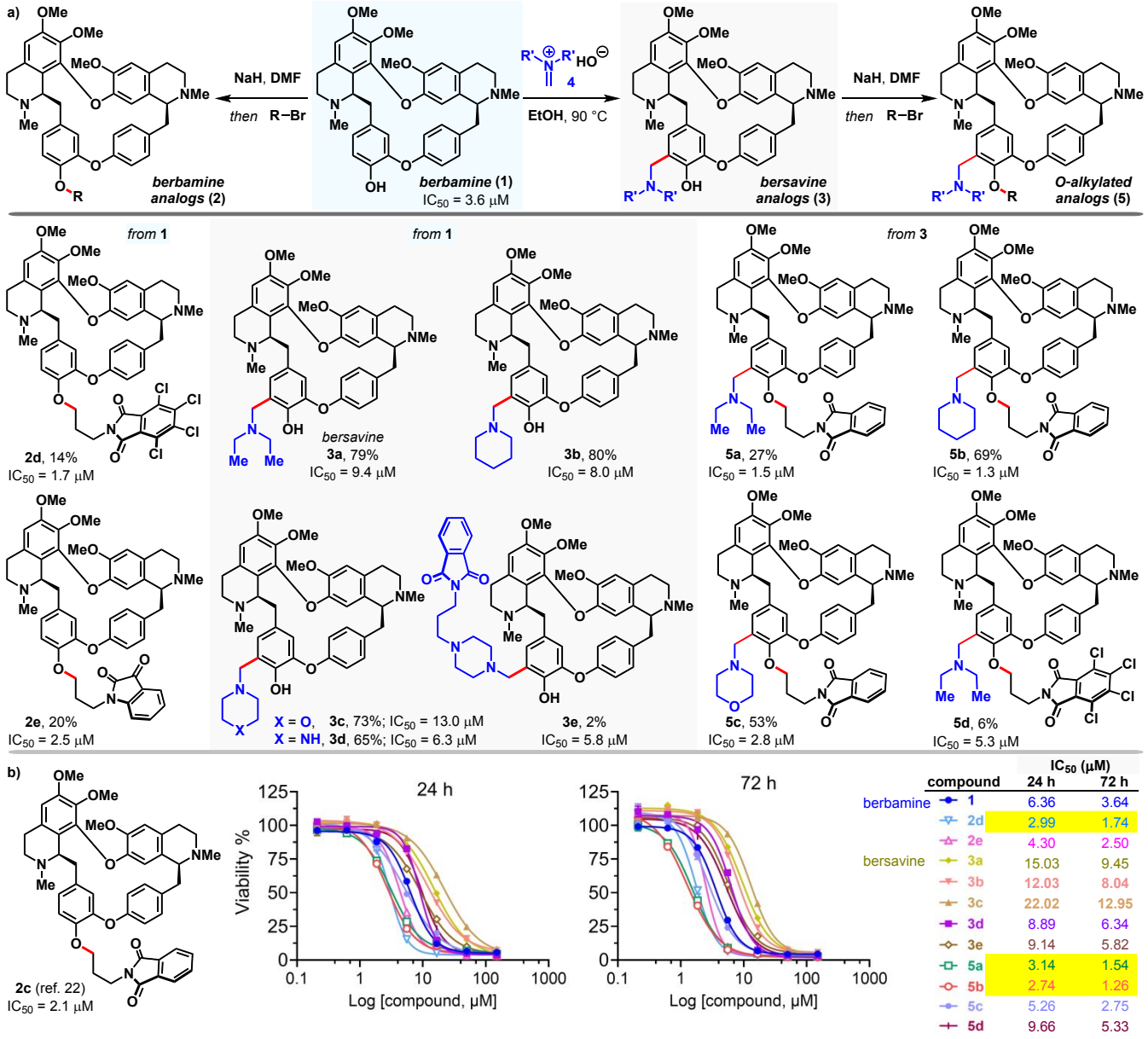

Scheme 1 a) Semisynthesis of bersavine derivatives. b) IC_{50} efficacies of berbamine and bersavine analogs on H9 cells.

Aza-Friedel–Crafts reactions of electron-rich arenes such as indoles, pyrroles, and phenols typically require strong Brønsted or Lewis acids[19–21] that are often incompatible under complex molecular settings. This is especially the case for bisBIA natural products that contain two Lewis basic nitrogen atoms. As a conseqence, attempts to utilize the synergistic Brønsted/Lewis acid approach that one of our labs developed for alkylating phenolic systems[15,16] were unsuccessful when applied to the alkylation and aminoalkylation of berbamine (**1**).

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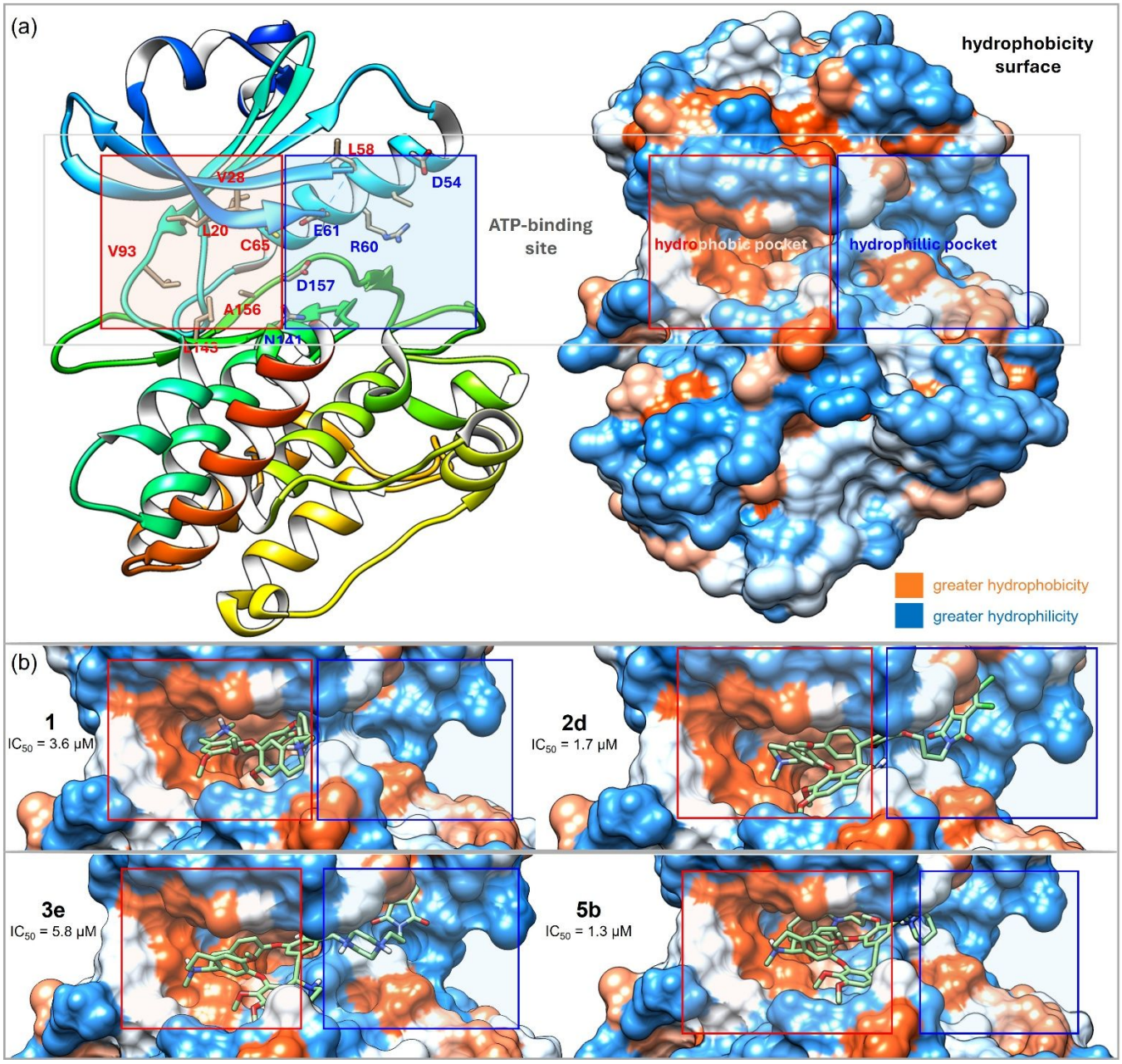
One of our labs previously reported that phenolic *O*-alkylation of berbamine (**1**), especially with the propylphthalimide[10,11,23] (i.e., **2c**) chain, produced derivatives significantly more potent than the parent natural product (Figure 1). As such, we hypothesized that *O*-alkylation of bersavine analogs **3** would confer greater potency. S_N2 etherification of bersavine analogs **3a–3c** furnished **5a–5c** with the propylphthalimide chain in 27–69% yields. Because *O*-alkylation has a drastic effect on potency, we sought to survey



Scheme 2 a) Ribbon and hydrophobicity surface image renderings of CaMKIIγ. b) Molecular docking of berbamine and bersavine derivatives to the ATP-binding site of CaMKIIγ.

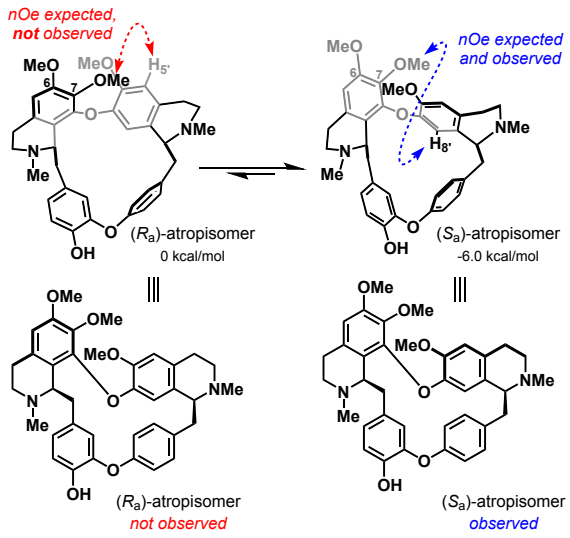
new *O*-alkyl fragments on the phenolic moiety by substituting the phthalimide group for tetrachlorophthalimide and isatin groups. However, the etherification reactions produced mixtures that required several rounds of purification, which resulted in low 14% and 20% yields of the two new berbamine derivatives **2d** and **2e**, respectively. Similarly, bersavine analog **5d** with the propyl(tetrachlorophthalimide) ether was synthesized in 6% yield. The especially low isolated yields of compounds **3e** and **5d** are attributed to instability of the tetrachlorophthalimide group during silica gel and alumina-based chromatography, likely due to S_NAr or hydrolytic decomposition promoted by the basic NH_4OH additive in the eluent. Despite the lower isolated yields with the new *O*-alkylated bisBIAs, sufficient amounts of compounds were obtained for biological testing that contributed to SAR analysis.

The cell viability of human T cell lymphoma cells H9 was assessed following 24 h and 72 h exposures to the new bisBIA derivatives (Scheme 1b). To aid with aqueous solubility, the HCl-salt form of the bisBIAs were prepared by treatment with HCl and dried prior to *in vitro* assays. The results were compared to naturally occurring berbamine (**1**). Consistent with previous studies, *O*-alkylated berbamine derivatives **2d** and **2e** with IC_{50}'s of 1.7–2.5 μM performed better than the parent natural product, which has an IC_{50} = 3.6 μM. Both the *N*-propyl(tetrachlorophthalimide) and *N*-propylisatin appendages imparted similarly effective cell inhibitory potential compared

to the *N*-propylpthalimide chain in berbamine analog **2d** (IC_{50} = 2.1 μM, Scheme 1b) that has been patented to treat T cell lymphoma and other cancers.[23] Bersavine (**3a**) and its piperidinyl (**3b**) and morpholino (**3c**) analogs with IC_{50}'s of 8.0–13.0 μM were not as effective as berbamine (**1**). Interestingly, a better IC_{50} = 5.8 μM was observed when the propylphthalimide chain was moved from the phenolic position to the nitrogen of piperazine in analog **3e**. The most effective compounds arose from the *O*-alkylation of bersavine analogs **3** with the propylphthalimide group. The efficacy trend mirrored that of the bersavine analogs, with piperidinyl **5b** > diethylamino **5a** > morpholino **5c**. Of note, *O*-alkylated bersavine derivatives **5a** and **5b** with IC_{50}'s of 1.54 μM and 1.26 μM, respectively, were found to exert potencies significantly greater than natural products berbamine (**1**) and bersavine (**3a**), as well as efficacies equally or greater than reported *O*-alkylated analogs such as **2d**. We posit that bersavine-derived *C*-,*O*-dialkylated derivatives can give rise to a novel subclass of bisBIA compounds for CaMKIIγ inhibition.

Berbamine competitively inhibits CaMKIIγ by targeting the ATP-binding site.[3] Here, we present a simplified docking model to rationalize the inhibitory potential of bisBIAs. The catalytic lobe that houses the ATP-binding site is outlined in the ribbon and hydrophobicity surface image renderings of PDB structure 2V7O[24] (Scheme 2a). Within this lobe, we define a hydrophobic pocket lined with valine, leucine, cysteine, and alanine residues (red), and a hydrophilic pocket lined with aspartate, glutamate, and arginine residues (blue). Using AutoDock Vina,[25,26] docking studies of berbamine analogs **1** and **2d**, along with bersavine analogs **3e** and **5b**, were performed on the ATP-binding site of CaMKIIγ (Scheme 2b). The protonated forms of the bisBIAs were utilized in these studies. The natural product berbamine (**1**) inhibits CaMKIIγ by preferentially binding to the hydrophobic pocket. The twofold increase in efficacy for *O*-alkylated **2d** arises from its ability to occupy both the hydrophobic and hydrophilic pockets, with the tetrachlorophthalimide cap extending deep into the hydrophilic pocket. For bersavine derivatives **3a–3e**, the added tertiary amino group, when protonated, potentially forms an interaction with glutamate-61 at the edge of the hydrophilic pocket (see SI). This interaction prevents the macrocyclic scaffolds from settling deep into the hydrophobic pocket, reducing their effectiveness as inhibitors. Bersavine analog **3e** is the most effective in this subseries because its extended alkyl chain can reach into the hydrophilic pocket; however, the protonated piperazine presumably prevents the rest of the bisBIA macrocycle from extending into the hydrophobic pocket. This problem is addressed by compounds **5a–5d**. Exemplified by analog **5b**, the *O*-alkyl chain anchors deep into the hydrophobic pocket while the protonated piperidine moiety maintains the ionic interaction with glutamate-61. This model suggests that novel derivatives capable of reaching deep into both the hydrophobic and hydrophilic pockets are likely to exhibit enhanced potency.

The atropisomerism along the sterically encumbered northern diaryl ether axis of bisBIAs has not been addressed in previous isolation reports nor synthetic studies. In our study, all high-scoring docking poses indicated that bisBIAs adopt the (S_a)-atropisomeric form along the northern ether bond (Scheme 3). Analysis by density functional theory (DFT) at the B3LYP/6-31G(d) level of theory found the (S_a) atropisomer to be favored over the (R_a)-atropisomer by 6.0 kcal/mol. Examination of their minimized 3-dimensional conformations revealed that the (R_a)-atropisomer positions the 7-methoxy group in proximity with $H_{5'}$ of the adjacent aryl ring, while the (S_a)-atropisomer places the 7-methoxy group in proximity with $H_{8'}$. Although bersavine's original isolation report did not account for axial chirality,[13] reanalysis of their NOESY data revealed an nOe between the 7-methoxy group and $H_{8'}$, which is consistent with the computed (S_a)-atropisomer (see SI for details). Given the strain imposed by the macrocycle, free rotation of this diaryl ether is unlikely at ambient temperature.



Scheme 3 Atropisomerism in bisBIAs, computed at the B3LYP/6-31G(d) level of theory.

Conclusions

We developed the first semisynthesis of bersavine analogs and identified bisBIA **5b** to be more effective than both naturally occuring berbamine (**1**) and bersavine (**3a**) for inhibiting CaMKIIγ (IC_{50} = 1.26 μM). Docking studies provided a model to rationalize the inhibitory efficacy of bisBIAs, offering a foundation for designing future CaMKIIγ inhibitors to regulate c-Myc in lymphoma therapy. According to this model, potent inhibitors should anchor deeply into the hydrophilic and/or hydrophobic cavities of the ATP-binding site—a role fulfilled by the alkylphthalimide appendage. Additionally, the protonated ammonium appendage present in the bersavine derivatives may engage an ionic interaction with Glu-61, further securing the ligand onto the binding pocket of the receptor. Our work highlights how scaffold derivatization (*i.e.*, C-functionalization) beyond *O*-alkylation can yield novel inhibitors with enhanced potency. As such, we are currently pursuing a total synthesis strategy to enable versatile derivatizations of the macrocyclic core structure.

Author contributions

K. G. M. K. conceptualized the study. K. G. M. K. and W. H. supervised the project. B. L., Y. C., A. K., L. M., and T. A. carried out the chemical investigation, while M. Z. and S. W. conducted the biological investigations. K. G. M. K. and T. A. performed the docking analysis. All authors contributed to the formal analysis. K. G. M. K. wrote the original draft, and all authors reviewed, edited, and approved the final manuscript.

Conflicts of interest

The authors declare that the novel CaMKIIγ inhibitors described in this study is included in a provisional patent that is jointly owned by the authors, the University of California, Riverside (UCR), and The City of Hope National Medical Center (COH). The potential financial interest in the patent does not affect the integrity or objectivity of the research. All research findings and conclusions are independent of any financial and intellectual property considerations.

Data availability

The data supporting this article, including the procedures for chemical synthesis and biological assays, characterization of new compounds, and NMR spectra, are available at https://doi.org/DOI.

Acknowledgements

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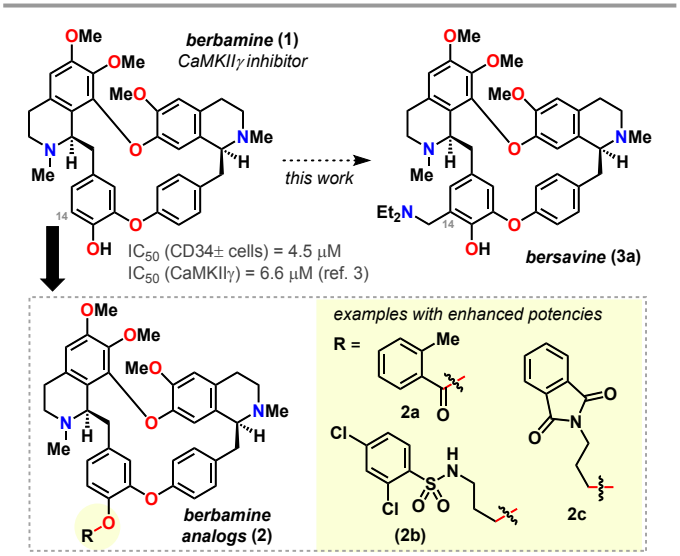

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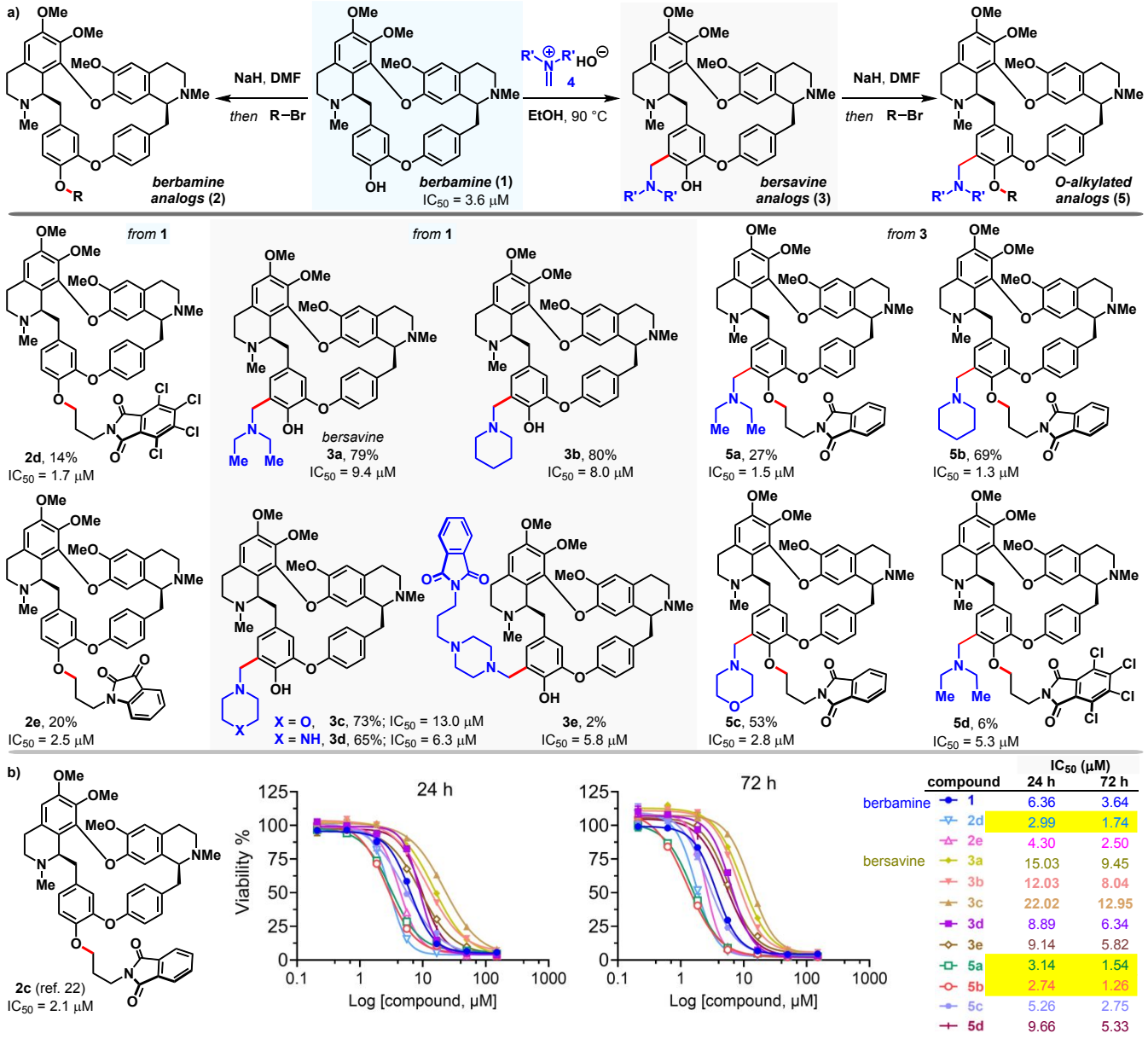

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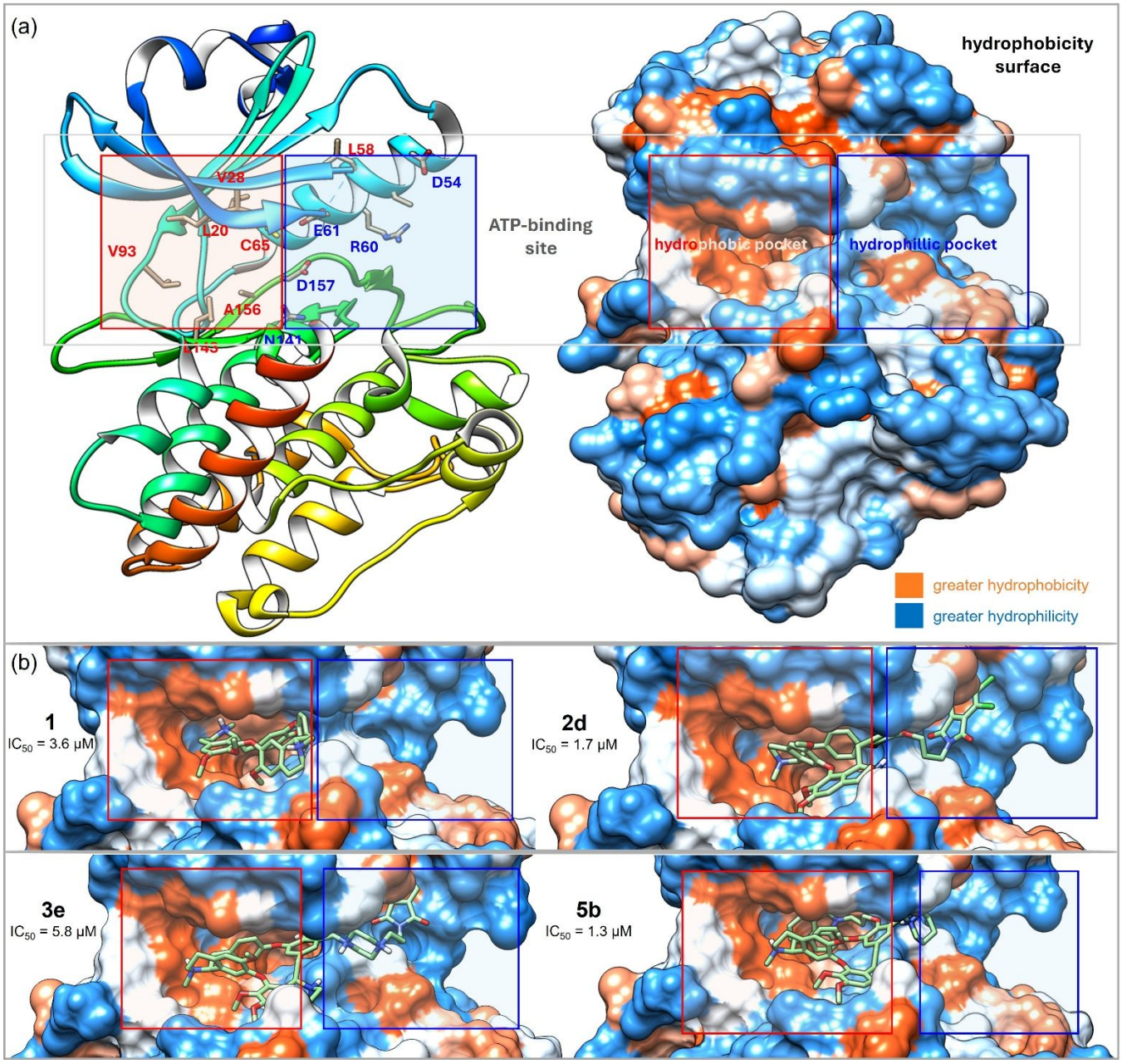
We employed neutral reaction conditions to achieve the desired Mannich-type alkylation.[22] The condensation of a secondary amine with formaldehyde generates the corresponding iminium ion (**4**) and a hydroxide counterion, which could deprotonate the phenolic moiety of berbamine (**1**), thereby activating it towards nucleophilic attack (Scheme 1). This approach was successful in synthesizing bersavine (**3a**) with the diethylaminomethylene appendage in 79% yield. The piperidinyl (**3b**), morpholino (**3c**), and piperazinyl **(3d**) analogues were prepared in 65–80% yields. In contrast, the piperazinyl(propyl)phthalimide derivative (**3e**) was formed and isolated in only a low 2% yield due to challenges associated with chromatographic purification.

One of our labs previously reported that phenolic *O*-alkylation of berbamine (**1**), especially with the propylphthalimide[10,11,23] (i.e., **2c**) chain, produced derivatives significantly more potent than the parent natural product (Figure 1). As such, we hypothesized that *O*-alkylation of bersavine analogs **3** would confer greater potency. S_N2 etherification of bersavine analogs **3a–3c** furnished **5a–5c** with the propylphthalimide chain in 27–69% yields. Because *O*-alkylation has a drastic effect on potency, we sought to survey



Scheme 2 a) Ribbon and hydrophobicity surface image renderings of CaMKIIγ. b) Molecular docking of berbamine and bersavine derivatives to the ATP-binding site of CaMKIIγ.

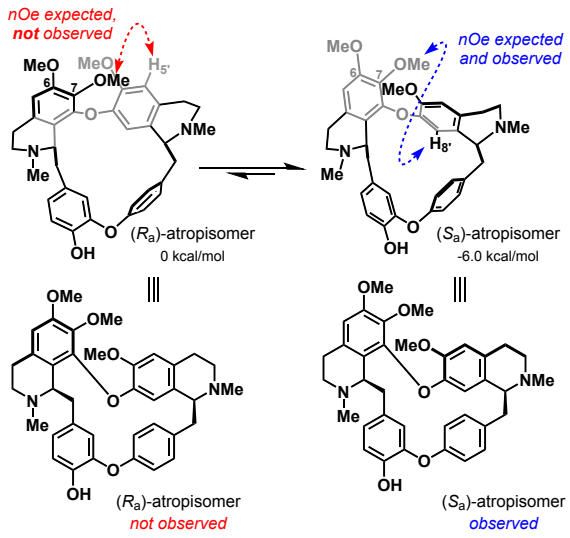
new *O*-alkyl fragments on the phenolic moiety by substituting the phthalimide group for tetrachlorophthalimide and isatin groups. However, the etherification reactions produced mixtures that required several rounds of purification, which resulted in low 14% and 20% yields of the two new berbamine derivatives **2d** and **2e**, respectively. Similarly, bersavine analog **5d** with the propyl(tetrachlorophthalimide) ether was synthesized in 6% yield. The especially low isolated yields of compounds **3e** and **5d** are attributed to instability of the tetrachlorophthalimide group during silica gel and alumina-based chromatography, likely due to S_NAr or hydrolytic decomposition promoted by the basic NH_4OH additive in the eluent. Despite the lower isolated yields with the new *O*-alkylated bisBIAs, sufficient amounts of compounds were obtained for biological testing that contributed to SAR analysis.

The cell viability of human T cell lymphoma cells H9 was assessed following 24 h and 72 h exposures to the new bisBIA derivatives (Scheme 1b). To aid with aqueous solubility, the HCl-salt form of the bisBIAs were prepared by treatment with HCl and dried prior to *in vitro* assays. The results were compared to naturally occurring berbamine (**1**). Consistent with previous studies, *O*-alkylated berbamine derivatives **2d** and **2e** with IC_{50}'s of 1.7–2.5 μM performed better than the parent natural product, which has an IC_{50} = 3.6 μM. Both the *N*-propyl(tetrachlorophthalimide) and *N*-propylisatin appendages imparted similarly effective cell inhibitory potential compared

to the *N*-propylpthalimide chain in berbamine analog **2d** (IC_{50} = 2.1 μM, Scheme 1b) that has been patented to treat T cell lymphoma and other cancers.[23] Bersavine (**3a**) and its piperidinyl (**3b**) and morpholino (**3c**) analogs with IC_{50}'s of 8.0–13.0 μM were not as effective as berbamine (**1**). Interestingly, a better IC_{50} = 5.8 μM was observed when the propylphthalimide chain was moved from the phenolic position to the nitrogen of piperazine in analog **3e**. The most effective compounds arose from the *O*-alkylation of bersavine analogs **3** with the propylphthalimide group. The efficacy trend mirrored that of the bersavine analogs, with piperidinyl **5b** > diethylamino **5a** > morpholino **5c**. Of note, *O*-alkylated bersavine derivatives **5a** and **5b** with IC_{50}'s of 1.54 μM and 1.26 μM, respectively, were found to exert potencies significantly greater than natural products berbamine (**1**) and bersavine (**3a**), as well as efficacies equally or greater than reported *O*-alkylated analogs such as **2d**. We posit that bersavine-derived *C*-,*O*-dialkylated derivatives can give rise to a novel subclass of bisBIA compounds for CaMKIIγ inhibition.

Berbamine competitively inhibits CaMKIIγ by targeting the ATP-binding site.[3] Here, we present a simplified docking model to rationalize the inhibitory potential of bisBIAs. The catalytic lobe that houses the ATP-binding site is outlined in the ribbon and hydrophobicity surface image renderings of PDB structure 2V7O[24] (Scheme 2a). Within this lobe, we define a hydrophobic pocket lined with valine, leucine, cysteine, and alanine residues (red), and a hydrophilic pocket lined with aspartate, glutamate, and arginine residues (blue). Using AutoDock Vina,[25,26] docking studies of berbamine analogs **1** and **2d**, along with bersavine analogs **3e** and **5b**, were performed on the ATP-binding site of CaMKIIγ (Scheme 2b). The protonated forms of the bisBIAs were utilized in these studies. The natural product berbamine (**1**) inhibits CaMKIIγ by preferentially binding to the hydrophobic pocket. The twofold increase in efficacy for *O*-alkylated **2d** arises from its ability to occupy both the hydrophobic and hydrophilic pockets, with the tetrachlorophthalimide cap extending deep into the hydrophilic pocket. For bersavine derivatives **3a–3e**, the added tertiary amino group, when protonated, potentially forms an interaction with glutamate-61 at the edge of the hydrophilic pocket (see SI). This interaction prevents the macrocyclic scaffolds from settling deep into the hydrophobic pocket, reducing their effectiveness as inhibitors. Bersavine analog **3e** is the most effective in this subseries because its extended alkyl chain can reach into the hydrophilic pocket; however, the protonated piperazine presumably prevents the rest of the bisBIA macrocycle from extending into the hydrophobic pocket. This problem is addressed by compounds **5a–5d**. Exemplified by analog **5b**, the *O*-alkyl chain anchors deep into the hydrophobic pocket while the protonated piperidine moiety maintains the ionic interaction with glutamate-61. This model suggests that novel derivatives capable of reaching deep into both the hydrophobic and hydrophilic pockets are likely to exhibit enhanced potency.

The atropisomerism along the sterically encumbered northern diaryl ether axis of bisBIAs has not been addressed in previous isolation reports nor synthetic studies. In our study, all high-scoring docking poses indicated that bisBIAs adopt the (S_a)-atropisomeric form along the northern ether bond (Scheme 3). Analysis by density functional theory (DFT) at the B3LYP/6-31G(d) level of theory found the (S_a) atropisomer to be favored over the (R_a)-atropisomer by 6.0 kcal/mol. Examination of their minimized 3-dimensional conformations revealed that the (R_a)-atropisomer positions the 7-methoxy group in proximity with $H_{5'}$ of the adjacent aryl ring, while the (S_a)-atropisomer places the 7-methoxy group in proximity with $H_{8'}$. Although bersavine's original isolation report did not account for axial chirality,[13] reanalysis of their NOESY data revealed an nOe between the 7-methoxy group and $H_{8'}$, which is consistent with the computed (S_a)-atropisomer (see SI for details). Given the strain imposed by the macrocycle, free rotation of this diaryl ether is unlikely at ambient temperature.



Scheme 3 Atropisomerism in bisBIAs, computed at the B3LYP/6-31G(d) level of theory.

Conclusions

We developed the first semisynthesis of bersavine analogs and identified bisBIA **5b** to be more effective than both naturally occuring berbamine (**1**) and bersavine (**3a**) for inhibiting CaMKIIγ (IC_{50} = 1.26 μM). Docking studies provided a model to rationalize the inhibitory efficacy of bisBIAs, offering a foundation for designing future CaMKIIγ inhibitors to regulate c-Myc in lymphoma therapy. According to this model, potent inhibitors should anchor deeply into the hydrophilic and/or hydrophobic cavities of the ATP-binding site—a role fulfilled by the alkylphthalimide appendage. Additionally, the protonated ammonium appendage present in the bersavine derivatives may engage an ionic interaction with Glu-61, further securing the ligand onto the binding pocket of the receptor. Our work highlights how scaffold derivatization (*i.e.*, C-functionalization) beyond *O*-alkylation can yield novel inhibitors with enhanced potency. As such, we are currently pursuing a total synthesis strategy to enable versatile derivatizations of the macrocyclic core structure.

Author contributions

K. G. M. K. conceptualized the study. K. G. M. K. and W. H. supervised the project. B. L., Y. C., A. K., L. M., and T. A. carried out the chemical investigation, while M. Z. and S. W. conducted the biological investigations. K. G. M. K. and T. A. performed the docking analysis. All authors contributed to the formal analysis. K. G. M. K. wrote the original draft, and all authors reviewed, edited, and approved the final manuscript.

Conflicts of interest

The authors declare that the novel CaMKIIγ inhibitors described in this study is included in a provisional patent that is jointly owned by the authors, the University of California, Riverside (UCR), and The City of Hope National Medical Center (COH). The potential financial interest in the patent does not affect the integrity or objectivity of the research. All research findings and conclusions are independent of any financial and intellectual property considerations.

Data availability

The data supporting this article, including the procedures for chemical synthesis and biological assays, characterization of new compounds, and NMR spectra, are available at https://doi.org/DOI.

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Data Availability Statement

The data supporting this article, including the procedures for chemical synthesis and biological assays, characterization of new compounds, and NMR spectra, have been included as part of the Supporting Information. The raw data (e.g., NMR FID files) are available upon request.