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Vinblastine 20' Amides: Synthetic Analogs That Maintain or Improve Potency and Simultaneously Overcome Pgp-derived Efflux and Resistance

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Abstract

A series of 180 vinblastine 20' amides were prepared in three steps from commercially available starting materials, systematically exploring a typically inaccessible site in the molecule enlisting a powerful functionalization strategy. Clear structure–activity relationships and a structural model were developed in the studies which provided many such 20' amides that exhibit substantial and some even remarkable enhancements in potency, many that exhibit further improvements in activity against a Pgp overexpressing resistant cancer cell line, and an important subset of the vinblastine analogs that display little or no differential in activity against a matched pair of vinblastine sensitive and resistant (Pgp overexpressing) cell lines. The improvements in potency directly correlated with target tubulin binding affinity and the reduction in differential functional activity against the sensitive and Pgp overexpressing resistant cell lines was found to correlate directly with an impact on Pgp-derived efflux.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: Characterization and purity data on all tested compounds detailed herein are provided. (PDF) Molecular formula strings. (CSV) Models of tubulin bound **28**, **121** and **173** (Figure 16). (PDB)

Introduction

The Vinca alkaloids constitute a family of natural products that continue to have a remarkable impact on anticancer drug discovery and treatment.^{1–3} Originally isolated as trace constituents of the Madagascar periwinkle plant (*Catharanthus roseus* (L.) G.Don),^{4,5} vinblastine (**1**) and vincristine are the most prominent members of this class and among the first plant-derived natural products used in the clinic for the treatment of cancer (Figure 1). These two compounds and three recent semi-synthetic analogs are integral oncology drugs employed today in highly successful combination drug therapies. Their mode of action, which involves disruption of microtubulin formation and dynamics through tubulin binding, still remains one of the most successful approaches for the development of oncology drugs.⁶

Vinblastine and vincristine are superb drugs even by today's standards. A major limitation to their continued use is the observation of clinical resistance mediated by overexpression of the drug efflux pump phosphoglycoprotein (Pgp).⁷ The identification of vinblastine analogs that might address such resistance, which also results in multidrug resistance (MDR) and is responsible for the majority of all relapses in oncology, has remained a major focus of the field for over 30 years. Not only would the discovery of a vinblastine analog not susceptible to Pgp efflux serve as a potential effective replacement for vinblastine in its current clinical uses or in instances of Pgp-derived vinblastine resistance, but it could also emerge as a new therapeutic option for other Pgp-derived MDR tumor treatments and constitute a major advance for oncology therapeutics. Despite the efforts focused on vinblastine for the past 40 years that have searched for analogs that effectively overcome Pgp-derived vinblastine resistance, little progress has been made.² Recent advances in the total synthesis of vinblastine, vincristine and related natural products have provided access to analogs of the natural products not previously accessible by semisynthetic modification of the natural products themselves.^{8–19} The latest of these efforts have provided a powerful approach to access a variety of vinblastine analogs that contain systematic deep-seated modifications within either the lower vindoline-derived^{20–27} or upper catharanthine-derived¹¹ subunits.^{28–30} As a result of these developments, we have prepared several series of key analogs, systematically exploring and defining the impact individual structural features and substituents have on tubulin binding affinity and cancer cell growth inhibition.^{10,19} Complementary to the studies detailed herein, we have systematically probed the impact and role of the vindoline C4 acetate,^{31,32} C5 ethyl substituent,³³ C6–C7 double bond,^{34–36} and the vindoline core structure itself,³⁶ and have systematically explored the upper catharanthine-derived subunit C20' ethyl substituent,^{37,38} C16' methyl ester,³⁹ and added C10' or C12' indole substitutions.⁴⁰ In addition and in preceding studies, we have shown that replacement of the C20'-OH with 20' ureas was possible,⁴¹ that substantial⁴² and even remarkable⁴³ potency enhancements were obtainable with such 20' ureas, and that some exhibited further improvements in activity against vinblastine-resistant cancer cell lines.⁴⁴ Herein, we report the important extension of these studies to the evaluation of vinblastine 20' amides conducted with the objective of discovering analogs that match or exceed the potency of vinblastine, but that are not subject to Pgp efflux and its derived vinblastine resistance. Not only did the studies provide vinblastine analogs no longer susceptible to Pgp-derived resistance, but they represent the discovery of a site and functionalization strategy

for the preparation of now readily accessible vinblastine analogs (3 steps) that improve binding affinity to tubulin (on target affinity) and functional potency in cell-based assays while simultaneously disrupting their efflux by Pgp (off target affinity and source of resistance), offering a powerful approach to discover new, improved, and durable oncology drugs.

Results and Discussion

Synthesis of Vinblastine C20' Amides

The extensive series of nearly 200 vinblastine analogs replacing the C20' alcohol with substituted or functionalized C20' amides was obtained through acylation of 20'-aminovinblastine (**6**), derived from reduction of the hydroazidation product **5**, with either an acid chloride (Method 1, 2 equiv RCOCl, 4 equiv *i*-Pr₂NEt, CH₂Cl₂, 23 °C, 2 h) or a carboxylic acid (Method 2, 2 equiv RCO₂H, 4 equiv EDCI, 0.2 equiv DMAP, DMF, 23 °C, 12 h) (Scheme 1). The former procedure that uses an acid chloride generally provided the better yields and avoids the added purification challenges of removing residual coupling reagents from the reaction products.

Notably, the precursor azide **5** is available directly in a single step from commercially available vindoline (**3**) and catharanthine (**2**) by enlisting first their Fe(III)-promoted coupling (5 equiv FeCl₃, 0.1 N aq HCl/CF₃CH₂OH, 25 °C, 2 h),²⁸ which proceeds by single electron oxidative fragmentation of catharanthine and installs exclusively the C16' natural stereochemistry.^{29,30} Subsequent in situ Fe(III)-mediated free radical hydrogen atom transfer hydroazidation of anhydrovinblastine (10 equiv Fe₂(ox)₃, 20 equiv NaBH₄, 30 equiv CsN₃, aq HCl/CF₃CH₂OH, 0 °C, 30 min) provided 20'-azidovinblastine (**5**) directly as a mixture C20' diastereomers, but with exclusive control of the critical C16' stereochemistry.^{19,41} An X-ray structure determination conducted on the major diastereomer of the reaction revealed that it possessed the unnatural vinblastine C20' stereochemistry (leurosidine stereochemistry) and that the minor diastereomer of the reaction possessed the natural C20' vinblastine stereochemistry.^{19,41} This powerful hydrogen atom transfer initiated free radical reaction was developed to provide a general method for functionalization of alkenes with use of a wide range of free radical traps beyond O₂ (air) used for vinblastine itself and was explored explicitly to provide the late-stage, divergent⁴⁵ preparation of vinblastine analogs that bear altered C20' functionality at a site previously inaccessible for systematic exploration (Scheme 1). In addition to other free radical traps that were introduced that included azide, the broad alkene substrate scope was defined, the Markovnikov addition regioselectivity was established, the remarkable functional group tolerance was demonstrated, alternative Fe(III) salts and initiating hydride sources were shown to support the reaction, its underlying free radical reaction mechanism was defined, and mild reaction conditions (0–25 °C, 5–30 min) were developed that are remarkably forgiving to the reaction parameters.^{41,46}

In the course of our examination the 20' amide analogs of vinblastine, we also reexamined a reported Ritter amidation reaction conducted on vinblastine or anhydrovinblastine, which was used to provide a limited series of 20' amides.⁴⁷ Although this reaction was reported to proceed in very modest conversions (5–10%), our interest was in its disclosure as providing

a single diastereomer that possesses the natural 20' vinblastine stereochemistry. By enlisting acetonitrile as the trap of the intermediate carbocation under conditions and in a reaction detailed in this work, we found that the reaction does indeed provide a single 20' diastereomer in low yield (<10%) as described (eq 1).⁴⁷ However, the product **8** of the reaction from vinblastine (**1**) was found to possess the unnatural (leurosidine) 20' stereochemistry and was shown to be identical in all respects with an authentic sample of this unnatural 20' acetamide diastereomer. In retrospect, this is not surprising given the now recognized preference for α -face C20' addition, but was originally conducted at a time this knowledge and the powerful modern day characterization techniques were unavailable. The analogous reaction starting with anhydrovinblastine (**7**) failed to provide any acetamido product. Consequently, the work detailed herein, expanding on the three examples that we disclosed (20' formamide, acetamide and trifluoroacetamide; **9–11**) along with the development of the vinblastine hydroazidation reaction,⁴¹ represent the only authentic 20' amides disclosed and examined to date.

(1)

Biological Activity

As previously highlighted, a major limitation to the clinical use of vinblastine and vincristine is the observation of resistance mediated by overexpression of the drug efflux pump phosphoglycoprotein (Pgp). The identification of analogs that might address such resistance has remained a major focus for over 40 years and would represent a major advance for oncology therapeutics. With this objective in mind, all analogs we have prepared to date, including the 20' amides detailed herein, were screened simultaneously for growth inhibition activity against HCT116 (human colon cancer cell line) and a matched resistant cell line (HCT116/VM46) that is approximately 100-fold resistant by virtue of the overexpression of Pgp. This well-designed set of functional assays simultaneously provides a direct measure of both functional activity (HCT116) and the analog susceptibility to Pgp efflux (resistance, HCT116/VM46). Key members were assessed for tubulin binding affinity for correlation with functional activity and those that emerge as candidates that avoid Pgp efflux were examined in efflux assays to confirm their behavior toward Pgp and related efflux transporters. The cell growth inhibition activity against the L1210 (mouse leukemia) cell line was also measured and the results were qualitatively and quantitatively (IC_{50}) nearly identical to those observed with the HCT116 cell line. Finally, the HCT116 human tumor cell line was found to accurately reflect activity observed against a larger panel of clinically

relevant human tumor-derived cell lines with a class of closely related vinblastine 20' ureas.⁴⁴

The results are presented and discussed below in groups of amides that embody related structural characteristics. Notably and importantly, the examined source of vinblastine resistance is not derived from changes in the drug target and their impact on drug binding (tubulin). Rather it is derived from binding and efflux by an off target protein (Pgp). These display distinguishable structure–activity relationships, one impacting potency (tubulin binding) and a second impacting resistance (Pgp binding and efflux). As important and as interesting as the former are in the discussions below, it is the discovery of a small subset of 20' amides that avoid Pgp efflux and overcome its derived resistance, displaying equal activity against both HCT116 and HCT116/VM46, that became a driving focus of our efforts.

Alkyl 20' Amides

Several important trends were observed with simple aliphatic 20' amides that influenced subsequent studies (Figure 2). First, the small series **9–11**, examined at the time the 20' azidation reaction was generalized,⁴¹ revealed that the simplest of the aliphatic amides (**9** and **10**) reduced activity approximately 10-fold, that both **9** and **10** displayed an approximate 80-fold resistance with HCT116/VM46 similar to vinblastine, that both **9** and **10** were 10-fold more active than the corresponding free amine **6** (see Figure 1), and that the increased electron-withdrawing properties of the acyl group of the trifluoroacetamide **11** was further and significantly detrimental to compound potency. These trends continued to be observed throughout the expanded and systematic series of aliphatic amides summarized in Figure 2 with some important notable exceptions. Only the sterically most bulky aliphatic amides (**14** and **22**) were not tolerated and these led to further reductions in activity. Three of the smaller aliphatic amides (**17**, **18** and **23**) uniquely matched the potency of vinblastine in the vinblastine-sensitive L1210 and HCT116 cell lines. These three compounds displayed activity that was improved over both the small or larger aliphatic amides, and two (**17** and **18**) improved (reduced) the differential activity between the vinblastine sensitive and resistant HCT116 cell lines. Most significantly, a well-defined trend was observed among all the 20' amides against the vinblastine-resistant HCT116/VM46 cell line. The 20' amides with the more hydrophobic substituents displayed a smaller differential in activity between the vinblastine sensitive and resistant cell lines (ratio = HCT116-VM46/HCT116), indicating less effective Pgp efflux in the resistant cell line. Moreover, this differential in activity smoothly and progressively diminishes as the hydrophobic nature of the substituent increased (ratio: R = Me > Et > *i*-Pr, *c*-Pr, *c*-Bu > *c*-pentyl > *c*-hexyl > benzyl > *n*-heptyl). Finally, the acrylamide **23** matched, but did not exceed the activity of vinblastine. It proved to be more active than most, but not all of the simple aliphatic 20' amides and possesses the potential for covalent capture at a tubulin binding site. However, we have found no evidence of such behavior and, as detailed below, have observed improved activity with substituted acrylamides less prone to putative covalent capture.

Benzoyl 20' Amides

The initial exploration of aryl versus aliphatic 20' amides led to analogs displaying activity that merited a detailed and systematic examination of such compounds (Figure 3). The parent unsubstituted 20' benzamide **24** proved to be >5-fold more potent than vinblastine and >50-fold more potent than the saturated cyclohexyl counterpart **20**. But like **20**, compound **24** also displayed a smaller differential in activity between the sensitive and resistant HCT116 cell lines than vinblastine (25-fold vs 88-fold), indicating it also embodied characteristics that make it a less effective substrate for Pgp. Early in the studies detailed herein, we first prepared a small but key series of 4-substituted benzamides to probe the electronic impact of substituents as well as several related analogs to establish sites amenable to substitution. These studies revealed that both 4- and 3-substitution of the phenyl ring were well tolerated and that any given substituent provided nearly equivalent activity when placed at either site (4- (para) > 3- (meta)), but that *o*-substitution reduced the relative potency by 10-fold or more (potency: *p*- > *m*- >> *o*-substitution). In addition, the initial 4-substituted benzamides examined were found to display a well-defined trend in which electron-donating substituents improved potency, whereas electron-withdrawing substituents reduced activity. Plots of the substituent Hammett σ_p constants versus $-\log IC_{50}$ (nM) revealed a linear relationship with slopes (ρ value) of -1.04 (HCT116) and -1.20 (L1210), indicating a large and remarkably well defined electronic contribution to the behavior of the analogs (Figure 4).

Throughout our studies, periodic measurements of relative tubulin binding affinities established that the substituent effects on activity correlated with relative target tubulin binding affinities. In initial studies on the 20' benzamide substituent effects detailed herein, three derivatives (**24**, **54** and **64**; R = H, CN, and NMe₂, respectively) from the Hammett plot set were examined (Figure 5). Consistent with their relative potencies, the three derivatives displayed the same clear trends in their ability to displace tubulin bound BODIPY-vinblastine (**64** > **24** > **54**). This direct correlation of functional cell growth inhibition activity with target tubulin binding affinity and the relative magnitude of the effects indicate that the properties of these C20' amides are derived predominately, if not exclusively, from on target effects on tubulin. Retrospective modeling of key analogs bound to tubulin detailed later herein suggest that this pronounced effect arises from the electronic impact of the substituent on the Lewis basicity of the amide carbonyl, enhancing its ability to serve as a H-bond acceptor for the backbone NH of β -tubulin Tyr224. Notably, this complements the requisite H-bond donor property of the secondary 20' amides (tertiary amides are inactive) in which the secondary amide NH mimics the tertiary alcohol of vinblastine itself and H-bonds to the backbone amide carbonyl of Pro222.

Finally, the early studies revealed that further increasing the hydrophobic character of the benzamide generally reduced the differential in activity between the sensitive and resistant HCT116 cell lines (e.g. compare **24** and **25–29**). This paradox of increasing potency through addition of a typically polar electron-donating substituent, enhancing target tubulin binding, while simultaneously disrupting Pgp transport by further decreasing the polarity of the 20' benzamide is chronicled in the subsequent extensive studies detailed in Figure 3.

Within this series, there are several vinblastine analogs that display stunning enhancements in potencies (e.g. **57**, **60–62**, **64**, **67**, **70**, **71** and **73**), others that display substantially improved potencies (10-fold) and attractive reduced differentials in activity (<10-fold; e.g. **25**, **26**, **30**, **72**, **74**, **97** and **99**), and many that display substantially improved differentials in activity (<10-fold). There are even those that indicate surprisingly large *p*-substituents are well tolerated (e.g. *t*-Bu in **38**). Many of these would be attractive analogs of vinblastine for further study. For us and the prescribed objective of discovering analogs that match or exceed the potency of vinblastine, but which are not subject to Pgp efflux derived resistance (ratio differential <2-fold), it is the analogs **28**, **29**, **32**, **78**, **92**, and **98** that met these defined parameters. Of these, it was **28** that was selected for additional study.

20' Acrylamides

A small series of substituted 20' acrylamides was prepared and examined in part for comparison with the unsubstituted acrylamide **23** (Figure 6). Addition of an aryl group to the terminus of the acrylamide was found to provide vinblastine analogs as much as 10-fold more potent than either **23** or vinblastine itself. Although the number of comparisons is small, the differential in activity between the vinblastine-sensitive HCT116 and vinblastine-resistant HCT116/VM46 cell lines decreased with the increased hydrophobic character of the aryl substitute (Pyr > furanyl, thienyl > Ph). This proved consistent with the observations made with the 20' benzoyl amides (see Figure 3) where increased hydrophobic character reduced the activity differential. In fact, the activity of the benzoyl amide **24** proved essentially indistinguishable from the phenyl substituted acrylamide **108**, both in terms of their potency and this activity differential. As indicated earlier, we found no evidence of covalent capture at a tubulin binding site with **23** or **108–114** and the improved activity with the substituted acrylamides less prone to putative covalent capture is consistent with enhanced tubulin binding affinity derived simply through non-covalent interactions.

Polycyclic Benzoyl-like 20' Amides

An important series of benzoyl-like 20' amides was examined that contained additional rings fused to the aromatic core (Figure 7). In essence, these represent variations on the 1- or 2-naphthyl amides **115** and **116**. Although the 1-naphthyl amide **115** was found to be roughly 10-fold less potent than vinblastine, the 2-naphthyl 20' amide **116** was determined to be approximately 3-fold more potent. Most significantly, the activity of **116** against the resistant HCT116/VM46 cell line was substantially improved such that the differential in activity versus HCT116 was less than 3-fold, indicating that it is no longer effectively subject to Pgp efflux-derived resistance. Although the sensitivity of HCT116 toward **116** was not significantly improved, the improvement in activity against HCT116/VM46 was suggestive that it is no longer a substrate for Pgp efflux. In this series and like the 20' benzamide series, polar electron-donating substituents in conjugation with the amide carbonyl often improved activity (e.g. **117**) but did so at the expense of the differential potency against the sensitive and resistant HCT116 cell lines. The amides in which the carbonyl was attached directly to the aryl ring were more effective than the bicyclic systems attached at an aliphatic site (e.g. **121** and **124** vs **122** and **125**). In general and consistent with expectations, the derivatives with the greater hydrophobic character led to reduced differentials in activity between the

sensitive and resistant HCT116 cell lines. The amides bearing the saturated fused six- or five-membered rings (**121** and **124**) exhibited the unique combination of slightly improved potency relative to vinblastine (ca. 2-fold) and little differential activity (<2-fold) and proved to be essentially indistinguishable from **28** (3,4-dimethylbenzoylamide). These compounds are similar in activity to the parent benzamide **24**, but with an even better improvement in activity against the resistant HCT116/VM116 cell line. As detailed earlier and like **28**, **121** exhibited a profile of activity that we were hoping to discover at the start of our studies and both became key compounds that were further profiled.

Monocyclic Heterocyclic 20' Amides

A systematic series of 20' amides were examined that contain a single heterocyclic ring (Figure 8). In general, the potency of the series against the sensitive cell lines followed trends in which the more hydrophobic and more electron-rich heterocyclic amides displayed the greatest activity (furanyl, thienyl > oxazolyl, thiazoyl, isoxazolyl > imidazolyl, pyrazinyl, pyridazinyl). The exceptions to this generalization are the 4- and 3-pyridyl amides **128** and **129** that proved to be among the most potent analogs in this series despite their polarity and electron-deficient character. Similarly, the differential in activity against the sensitive and resistant HCT116 cell lines also generally increased as the polarity or heteroatom count in the heterocycle increased (furanyl, thienyl < oxazolyl, thiazoyl, isoxazolyl < imidazolyl, pyrazinyl, pyridazinyl, pyridyl). Within this series, impressive potency was observed with the 3-furanyl and 2-thienyl amides (**133** and **136**), displaying activity (IC_{50} = 600–700 pM) 10-fold greater than vinblastine and roughly 2-fold better than the 20' benzamide **24** with additional improved reductions in the differential activity (11-fold vs 88-fold and 25-fold) for the sensitive and resistant HCT116 cell lines. Interestingly, the 4- and 3-pyridyl amides **128** and **129** were among the most potent compounds in the series (IC_{50} = 400–700 pM), whereas the 2-pyridyl amide **130** was among the least potent. However, both **128** and **129** displayed the largest differential in activity against the sensitive and resistant HCT116 cell lines (138-fold and 83-fold, respectively).

The saturated heterocyclic amides **144–148** proved to be much less potent than vinblastine and less potent than most of the aromatic heterocyclic 20' amides. In the small series examined, the compounds appear to follow trends where the more polar substituents not only led to progressive losses in activity, but also increase the differential in activity between the sensitive and resistant HCT116 cell lines. An instructive comparison is the activity of **144** versus **20** in which a polar oxygen atom is introduced into the all carbon six-membered ring. Although the two compounds proved nearly equipotent against the sensitive cell lines, **144** proved to be much less active in the resistant HCT116 cell line, displaying a differential in activity (100-fold) similar to that of vinblastine (88-fold) and much greater than **20** (11-fold).

Polycyclic Heterocyclic 20' Amides

An important series of heterocyclic amides that contain two fused aromatic or non-aromatic rings was examined that also provided important insights into structural features that can enhance potency or disrupt Pgp-derived resistance. These are presented in Figure 9. The trends observed with incorporation of a series of polycyclic heterocycles, which constitute

benzo-fused versions of the monocyclic 20' heteroaromatic amides (Figure 8), are instructive. First and foremost, the differential in activity between the sensitive and resistant HCT116 cell lines for each benzo-fused heterocycle generally improved (diminished) relative to its companion monocyclic heterocycle. This behavior is analogous to the comparison of benzamide **24** and its benzo-fused counterpart **116** (2-naphthyl amide) and likely reflects the increased hydrophobic character of the benzo-fused heterocycle. For the heterocycles containing a basic nitrogen, acyl linkage at the site adjacent to the basic nitrogen with **152**, **156**, **169** and **172** (2-quinolyl, 2-quinoxalyl, 2-benzoxazolyl, 2-benzthiazolyl) led to substantial reductions in activity, an observation analogous to that made with the pyridyl series **128–130**. It is likely that this adjacent heterocyclic nitrogen participates in an intramolecular H-bond to the proximal amide NH, disrupting the stabilizing intermolecular amide N–H H-bond with the backbone carbonyl of Pro222. Otherwise, the heterocycle acyl linkage site proved remarkably flexible tolerating most possibilities. Notably, derivatives were not generally examined that might represent the distinguishing linkage sites of 2- vs 1-naphthyl where the linkage site is adjacent to a ring fusion center and found to be detrimental. However, the productive activity maintained by the 3-benzofuranyl and 3-benzthiophenyl amides **159** and **164** suggest such linkages should not be ruled out. Finally, the potencies of many of the heterocyclic amides were found to be superb with nearly all exceeding the activity of vinblastine. The potency comparisons of each benzo-fused heterocycle with its companion monocyclic heterocyclic amide was more variable although most were found to maintain or even improve on this activity. These generalizations are perhaps best depicted in comparing the quinolyl and isoquinolyl amide series **149–154** with the pyridyl series **128–130**. These maintained the exceptional potency (IC_{50} = 400–700 pM) observed in the pyridyl series (IC_{50} = 400–600 pM), also exhibited a substantial loss in activity when the acyl substitution site was ortho to the basic nitrogen (ca. 100-fold, **152**), and exhibited a substantially improved (diminished) differential in activity between the sensitive and resistance HCT116 cell lines for the potent variants (ca. 10-fold vs 100-fold). Finally and within this series, the 6-benzfuranyl and 6-benzthiophenyl amides (**157** and **162**) displayed activity (IC_{50} = 2–3 nM) 2- to 3-fold greater than vinblastine with additional and superb improved reductions in the differential activity for the sensitive and resistant HCT116 cell lines (3-fold and 2.7-fold vs 88-fold) and proved very comparable to the 2-naphthyl amide **116**.

Even more significant, a series polycyclic heterocyclic 20' amides was examined in which the fused heterocycle was saturated versus aromatic (**161** and **173–176**). In essence, those examined represent benzoyl amides substituted with a para electron-donating substituent incorporated into a fused ring system, some of which introduce more lipophilic character. In general, such amides displayed the superb potency that accompanies the introduction of a para electron-donating substituent (IC_{50} = 300–800 pM). Of these, compound **173** emerged as the most attractive vinblastine analog. It is >10-fold more potent than vinblastine against the sensitive cell lines (IC_{50} = 400–500 pM), >300-fold more active against the resistant HCT116 cell line (IC_{50} = 1.8 nM), and displays a differential in activity of only 3.8-fold. As a result and like **28** and **121**, **173** exhibited a profile of activity that we were hoping to discover at the start of our studies and became a key compound that was further profiled.

This latter set of compounds also provided us with a series of closely related compounds that display good activity and a favorable activity ratio with which the qualitative correlation of the amide lipophilic character and the differential activity could be retrospectively illustrated. A plot of cLogP versus the fold difference in activity (ratio) that includes compounds **28**, **121**, and **173**, along with a series of compounds that progressively exchange in single heteroatoms, qualitatively highlights the reduction in the activity differential (ratio) that accompanies an increase in the amide lipophilicity (Figure 10). This reduction in activity differential that results from the additional relative improvements in activity against the resistant HCT116/VM46 cell line is suggestive such compounds are less effective substrates for Pgp efflux.

C20' Sulfonamides

A small series of C20' sulfonamides was also prepared in a single step from **6** (1.5 equiv RSO₂Cl, 2 equiv *i*-Pr₂NEt, 0.05 M CH₂Cl₂, 23 °C, 16 h; Method 3) and examined (Figure 11). No compound in this series approached the potency of vinblastine. In the cases where a comparison C20' amide was prepared, the corresponding sulfonamide (**179** vs **24**, **180** vs **25**, **181** vs **67** and **185** vs **116**) proved to be approximately 50–100 times less active.

Key 10'-Fluorovinblastine 20' Amides

In recent work, the incorporation of a fluorine atom at the 10' position of vinblastine provided a compound (**187**) with a nearly 10-fold improvement in activity over vinblastine itself.⁴⁰ We were interested in determining whether the incorporation of both the 10'-F substituent and a 20' amide would have the same effect of enhancing the potency of the vinblastine 20' amide analog and also establishing whether a 10'-F substituent would impact the improved differential in activity against the matched sensitive and resistant HCT116 cell line. Three key 20' amides also containing a 10'-F substituent were prepared, each of which constitute 10'-F derivatives of 20' amides that displayed superb or improved reductions in the differential activity (Figure 12). In each case, the potency of 20' amide analog was maintained (**189** vs **121** and **190** vs **70**) or enhanced (**188** vs **78**) and the improvement in the activity differential observed with the parent 10'-H analogs was maintained (**189**) or was further improved (**188** and **190**). The latter compound **190** is notable in that it is exceptionally potent (IC₅₀ = 60–80 pM) against the vinblastine sensitive cell lines and even displays sub-nanomolar activity against the vinblastine-resistant HCT116 cell line (IC₅₀ = 900 pM against HCT116/VM46), representing a >600-fold improvement in this activity.

Additional Assessments of Compounds **28**, **121** and **173**

From the series of 20' amides prepared and examined, three (**28**, **121** and **173**) were chosen for further evaluation. The set consists of a small series of hydrophobic aryl amides that are essentially equipotent with (**28** and **121**) or more potent (**173**) than vinblastine against sensitive cancer cell lines (e.g. HCT116) and that maintain this potency against the matched resistant human cancer cell line (HCT116/VM46). The tubulin binding properties of the three compounds were examined alongside vinblastine for their relative ability to displace tubulin bound BODIPY-vinblastine.⁴³ Like the prior comparisons summarized in Figure 5, the cell-based functional activity of the compounds correlated directly with their relative

tubulin binding affinities (Figure 13). Thus, the affinities of **28** and **121** were essentially indistinguishable from or perhaps slightly better than that of vinblastine, whereas that of **173** was established to be significantly higher.

The observations made with the three compounds also indicated that the derivatives are not subject to resistance derived from Pgp overexpression as found in HCT116/VM46. These results suggested they are no longer effective substrates for Pgp efflux and that this type of modification may disrupt binding and/or transport by Pgp. This was confirmed in two widely used secondary assays (Caco-2 bidirectional permeability and stimulated Pgp ATPase activity in membranes).^{48–50} The results are summarized in Figure 14 where the compounds demonstrate little or no Pgp transport (or efflux) while maintaining the intrinsic permeability of vinblastine. At the start of our studies many years ago now, we viewed this type of result as an initial complete success for the studies – the discovery of vinblastine analogs that matched or exceeded the potency of the clinical drugs, but that would not be subject to clinical resistance derived from Pgp overexpression and efflux.

Finally, we had the three compounds independently examined alongside vinblastine and taxol, which included their independent assessments against HCT116 and HCT116/VM46 and against a representative and slightly larger panel of additional clinically relevant human cancer cell lines, where they displayed trends analogous to those already highlighted (Figure 15).⁵¹ All three compounds exceeded the potency of vinblastine (avg IC₅₀ = 13 nM), matched or exceeded the potency of taxol (avg IC₅₀ = 5.5 nM), and displayed a consistent potency across the slightly larger panel of human cancer cell lines. Compound **173** (avg IC₅₀ = 1.2 nM) proved to be 2–8 fold more potent than **121** and **28**, and **28** (avg IC₅₀ = 4.0 nM) was typically found to be slightly more potent than **121** (avg IC₅₀ = 4.6 nM). Each demonstrated a differential in activity (IC₅₀ for HCT116/VM46 vs HCT116) that was small and consistent with our own assessments (2, 2.9 and 7 vs 1.7, 1.8 and 3.8 for **28**, **121** and **173**), indicating they maintain good activity against the vinblastine-resistant (and taxol-resistant) cell line and are ineffective (**28** and **121**) or poor (**173**) substrates for Pgp efflux.

Models of **28**, **121** and **173** Bound to Tubulin

The binding site for vinblastine lies at the head-to-tail tubulin α/β dimer–dimer interface. As depicted in the X-ray co-crystal structures of tubulin bound complexes,^{52,53} vinblastine is nearly completely buried in the protein binding site. It adopts a T-shaped bound conformation with C3/C4 (bottom of T) lying at the solvent interface and the C20' site (top corner of T) extends deepest into the binding pocket lying at one corner. In contrast to early expectations based on the steric constraints of the tubulin binding site surrounding the vinblastine C20' center, large 20' substituents such as those detailed herein are accommodated. The C20' alcohol extends toward a narrow channel that leads from the buried C20' site to the opposite face of the protein, representing the continuation of the protein–protein interaction defined by the tubulin dimer–dimer interface. Even without adjusting the proteins found in a vinblastine-bound X-ray structure (pdb 5J2T), the modeled 20' amides extend into this narrow channel, continuing along the tubulin α/β dimer–dimer protein–protein interface (Figure 16). The newly introduced vinblastine 20' amide forms two key H-bonds in which the amide N–H serves as a H-bond donor for the backbone carbonyl

of Pro222 (2.0 Å) and the amide carbonyl serves as a H-bond acceptor for the backbone amide N–H of Tyr224 (3.0 Å). These serve to anchor the orientation of the 20' amides such that the attached acyl group extends into the adjacent narrow channel. In this orientation, not only are substituents on the benzamides accommodated at either the 3- or 4-position, but such electron-donating substituents that increase the Lewis basicity of the amide carbonyl would be expected to increase the strength of the H-bond with Tyr224, accounting for the enhanced tubulin binding affinity and the resulting increased activity in cell-based functional assays.

Conclusions

A site and powerful functionalization strategy on vinblastine was exploited that provided access to analogs which simultaneously maintain or improve cell-based functional activity, maintain or improve target tubulin binding affinity, and simultaneously disrupt off target activity (Pgp efflux) responsible for resistance. Thus, an extensive and systematic series of synthetic vinblastine 20' amides were prepared in three steps from commercially available material, targeting a site inaccessible to traditional divergent functionalization.⁵⁴ Many such 20' amides were found to exhibit substantial and some even remarkable potency increases, many exhibited further improvements in activity against a Pgp overexpressing resistant cancer cell line, and an important subset of the vinblastine analogs displayed little or no differential in activity against a matched pair of vinblastine sensitive and resistant (Pgp overexpressing) cell lines. The improvements in potency directly correlated with improvements in target tubulin binding affinity, and the reduction in differential functional activity against the sensitive and resistant cell lines was found to correlate with analogous reductions in Pgp-derived efflux. Well defined structure–activity relationships and a structural model were developed in the studies that confidently account for the structural features that improve functional and target tubulin binding activity and key insights into structural characteristics⁵⁵ that can be used to simultaneously disrupt off target Pgp binding and/or efflux responsible for drug resistance were obtained. Members of this class of vinblastine 20' amides have the potential of not only serving as vinblastine replacements in the clinic, addressing Pgp-derived clinical resistance limiting its continued use, but may also offer opportunities for the development of powerful new frontline treatment options in instances of other multidrug resistant (MDR) tumors (overexpression of Pgp) refractory to most other chemotherapeutic drugs.⁷

EXPERIMENTAL SECTION

General Chemistry Procedures

All commercial reagents were used without further purification unless otherwise noted. All reactions were performed in oven-dried (200 °C) glassware and under an inert atmosphere of Ar unless otherwise noted. Column chromatography was performed with silica gel 60 (43–60 Å). TLC was performed on

Whatman silica gel (250 µm) F₂₅₄ glass plates and spots visualized by UV. PTLC was performed on Whatman silica gel (250 and 500 µm) F₂₅₄ glass plates. Optical rotations were determined on a Rudolph Research Analytical Autopol III automatic polarimeter using the

sodium D line ($\lambda = 589$ nm) at room temperature (23 °C) and are reported as follows: $[\alpha]_D^{23}$, concentration ($c = \text{g}/100 \text{ mL}$), and solvent. FT-IR spectroscopy was conducted on a Nicolet 380 FT-IR instrument. ^1H NMR was recorded on a Bruker 600 MHz spectrometer. Chemical shifts are reported in ppm from an internal standard of residual CHCl_3 (7.26 for ^1H). Proton chemical data are reported as follows: chemical shift (δ), multiplicity (ovlp = overlapping, br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant, and integration. High resolution mass spectra were obtained at The Scripps Research Institute Mass Spectrometry Facility on an Agilent ESI-TOF/MS using Agilent ESI-L low concentration tuning mix as internal high resolution calibration standards. The purity of each tested compound ($>95\%$) was determined on an Agilent 1100 LC/MS instrument using a ZORBAX SB-C18 column (3.5 mm, 4.6 mm $\times$ 50 mm, with a flow rate of 0.75 mL/min and detection at 220 and 254 nm) with a 10–98% acetonitrile/water/0.1% formic acid gradient (two different gradients). A table of the established purity for each tested compound is provided in the Supporting Information.

General Methods for the Synthesis of Vinblastine 20' Amides

Method 1—A solution of 20'-aminovinblastine⁴¹ (**6**, 3.5 mg, 0.004 mmol) in CH_2Cl_2 (0.1 mL) was treated with 4 μL of $i\text{-Pr}_2\text{NEt}$ (0.016 mmol) followed by addition of the acid chloride (0.008 mmol). The reaction mixture was stirred for 2 h at room temperature before being quenched with the addition of saturated aqueous NaHCO_3 (3 mL). The mixture was extracted with 10% MeOH in CH_2Cl_2 (3 mL), and washed with saturated aqueous NaCl (3 mL). The organic layer was dried over Na_2SO_4 , and concentrated under reduced pressure. PTLC (SiO_2 , $\text{EtOAc}:\text{MeOH}:\text{Et}_3\text{N} = 95:5:5$) purification provided the pure products; yields (35–98%).

Method 2—A solution 20'-aminovinblastine⁴¹ (**6**, 4 mg, 0.005 mmol) in DMF (0.1 mL) was treated with EDCI (0.02 mmol), DMAP (20 mol%) and the carboxylic acid (0.01 mmol). The reaction mixture was allowed to stir at room temperature overnight, after which it was diluted with the addition of 10% MeOH in CH_2Cl_2 (3 mL) and aqueous 10% citric acid or 1 M HCl (3 mL). The aqueous layer was further extracted with 10% MeOH in CH_2Cl_2 , and the combined organic phase was washed with saturated aqueous NaHCO_3 (3 mL), and saturated aqueous NaCl (3 mL). The organic layer was dried over Na_2SO_4 , and concentrated under reduced pressure. PTLC (SiO_2 , $\text{EtOAc}:\text{MeOH}:\text{Et}_3\text{N} = 95:5:5$) purification provided the pure products; yields (20–98%).

Method 3—A solution of 20'-aminovinblastine⁴¹ (**6**, 4 mg, 0.005 mmol) in anhydrous CH_2Cl_2 (0.1) was treated with $i\text{-Pr}_2\text{NEt}$ (0.01 mmol) and the sulfonyl chloride (0.008 mmol). The resulting mixture was allowed to stir at room temperature overnight, after which it was diluted with the addition of saturated aqueous NaHCO_3 (2 mL). The mixture was extracted with 10% MeOH in CH_2Cl_2 , and washed with saturated aqueous NaCl (3 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated under reduced pressure. PTLC (SiO_2 , $\text{EtOAc}:\text{MeOH}:\text{Et}_3\text{N} = 97:3:3$) purification provided the pure products; yields (41–54%).

Compound 28—Method 1 was followed using 8.0 mg of 20'-aminovinblastine (**6**, 0.01 mmol) to provide 4.7 mg of **28** as a white solid, yield: 50%. ¹H NMR (600 MHz, CDCl₃) δ 9.83 (br s, 2H), 8.02 (s, 1H), 7.82–7.76 (m, 2H), 7.44 (d, *J* = 7.2 Hz, 1H), 7.24 (d, *J* = 7.7 Hz, 1H), 7.14–7.07 (m, 2H), 6.64 (s, 1H), 6.12 (s, 1H), 6.08 (s, 1H), 5.85 (d, *J* = 5.7 Hz, 1H), 5.48 (s, 1H), 5.30 (d, *J* = 10.3 Hz, 1H), 4.00 (br s, 1H), 3.81 (s, 3H), 3.80 (s, 3H), 3.75 (s, 1H), 3.58 (s, 3H), 3.42–3.36 (m, 2H), 3.30 (td, *J* = 9.5, 4.8 Hz, 1H), 3.22 (t, *J* = 8.7 Hz, 1H), 3.10 (dd, *J* = 14.6, 7.2 Hz, 2H), 2.83 (d, *J* = 16.2 Hz, 1H), 2.72 (s, 3H), 2.67 (s, 1H), 2.45–2.41 (m, 1H), 2.35 (s, 3H), 2.30 (s, 3H), 2.17 (s, 1H), 2.11 (s, 3H), 2.02–1.98 (m, 1H), 1.85–1.78 (m, 2H), 1.62–1.59 (m, 4H), 1.42 (t, *J* = 7.4 Hz, 1H), 1.37–1.31 (m, 2H), 1.26–1.23 (m, 3H), 0.82 (t, *J* = 7.0 Hz, 3H), 0.77 (t, *J* = 6.2 Hz, 3H); HRESI-TOF *m/z* 942.5012 (C₅₅H₆₇N₅O₉ + H⁺, required 942.5011). [α]_D²³–39 (*c* 0.027, CHCl₃).

Compound 121—Method 2 was followed using 8.4 mg of 20'-aminovinblastine (**6**, 0.01 mmol) to provide 4.0 mg of **121** as a white solid, yield: 42%. ¹H NMR (600 MHz, CDCl₃) δ 9.86 (br s, 1H), 8.05 (s, 1H), 7.76 (s, 2H), 7.48–7.46 (m, 1H), 7.19 (d, *J* = 8.2 Hz, 1H), 7.16 (d, *J* = 6.7 Hz, 1H), 7.12–7.10 (m, 2H), 6.66 (s, 1H), 6.14 (s, 1H), 6.10 (s, 1H), 5.88–5.87 (m, 1H), 5.50 (s, 1H), 5.33 (d, *J* = 10.2 Hz, 1H), 4.00 (br s, 1H), 3.84 (s, 3H), 3.82 (s, 3H), 3.78–3.77 (m, 1H), 3.60 (s, 3H), 3.44–3.38 (m, 2H), 3.33 (td, *J* = 9.5, 4.8 Hz, 1H), 3.26–3.23 (m, 1H), 3.14–3.10 (m, 2H), 2.91–2.87 (m, 2H), 2.84–2.83 (m, 1H), 2.82–2.81 (m, 2H), 2.75 (s, 3H), 2.71–2.69 (m, 1H), 2.48–2.43 (m, 1H), 2.39–2.36 (m, 1H), 2.20 (s, 3H), 2.13 (s, 1H), 1.88–1.85 (m, 1H), 1.83–1.82 (m, 4H), 1.61 (s, 3H), 1.54–1.51 (m, 1H), 1.44 (t, *J* = 7.4 Hz, 1H), 1.37–1.33 (m, 2H), 1.28 (s, 2H), 0.92–0.89 (m, 1H), 0.84 (t, *J* = 6.9 Hz, 3H), 0.80–0.78 (m, 3H); HRESI-TOF *m/z* 968.5164 (C₅₇H₆₉N₅O₉ + H⁺, required 968.5168). [α]_D²³–55 (*c* 0.069, CHCl₃).

Compound 173—Method 2 was followed using 8.0 mg of 20'-aminovinblastine (**6**, 0.01 mmol) to provide 3.5 mg of **173** as a pale white solid, yield: 37%. ¹H NMR (600 MHz, CDCl₃) δ 9.84 (br s, 1H), 8.06–8.04 (m, 1H), 7.91 (s, 1H), 7.58–7.50 (m, 2H), 7.30–7.38 (m, 1H), 7.19–7.16 (m, 2H), 6.78–6.77 (m, 2H), 6.53 (s, 1H), 6.11 (s, 1H), 5.88 (s, 1H), 5.47 (s, 1H), 5.32 (d, *J* = 9.8 Hz, 1H), 4.21 (t, *J* = 4.6 Hz, 2H), 3.82–3.82 (m, 6H), 3.76 (br s, 1H), 3.63 (s, 3H), 3.51 (s, 1H), 3.42–3.37 (m, 2H), 3.31–3.25 (m, 2H), 3.15–3.10 (m, 2H), 2.91 (s, 1H), 2.82–2.79 (m, 2H), 2.74 (s, 3H), 2.66–2.62 (m, 1H), 2.45–2.41 (m, 1H), 2.32–2.30 (m, 1H), 2.19 (s, 3H), 2.13–2.12 (m, 2H), 2.11–2.09 (m, 1H), 2.01 (br s, 1H), 1.69–1.66 (m, 4H), 1.55–1.52 (m, 2H), 1.33–1.28 (m, 5H), 0.89–0.84 (m, 6H); HRESI-TOF *m/z* 970.4961 (C₅₆H₆₇N₅O₁₀ + H⁺, required 970.4960). [α]_D²³–76 (*c* 0.059, CHCl₃).

Ritter Reaction used to generate 20'-acetamidoleurosidine (**8**)

A solution containing 22 mg of vinblastine sulfate in 0.6 mL of anhydrous acetonitrile was prepared. 60 μL of 18 M sulfuric acid was added. The resulting solution was stirred at ambient temperature for 7 h and then overnight at 0 °C. Next, 318 mg of Na₂CO₃ and 2 mL of anhydrous MeOH were added. This mixture was stirred for 15 min before 4 mL of a saturated aqueous NaCl was added. The reaction volume was increased to 8 mL by the addition of water. This diluted mixture was stirred for about 15 min, after which time it was extracted four times with an equal volume of benzene. The combined organic layer was dried over Na₂SO₄, and concentrated under reduced pressure. PTLC (SiO₂,

EtOAc:MeOH:Et₃N = 97:3:3) provided three products. The first product was recovered vinblastine (5.5 mg). The second product was 20'-acetamidoylleurosidine (**8**, 1.2 mg) and its structure was confirmed by comparison with a sample prepared from authentic 20'-aminoleurosidine. The third product (1.8 mg) was leurosidine. For 20'-acetamidoleurosidine (**8**): ¹H NMR (600 MHz, CDCl₃) δ 9.81 (s, 1H), 7.98 (s, 1H), 7.52 (d, *J* = 7.8 Hz, 1H), 7.21–7.14 (m, 1H), 7.14–7.08 (m, 3H), 6.50 (s, 1H), 6.16 (s, 1H), 6.08 (s, 1H), 5.88–5.80 (m, 1H), 5.45 (s, 1H), 5.28 (d, *J* = 10.2 Hz, 1H), 3.79 (s, 3H), 3.77 (s, 1H), 3.76 (s, 3H), 3.66–3.61 (m, 1H), 3.59 (s, 3H), 3.40–3.34 (m, 1H), 3.33–3.27 (m, 1H), 3.27–3.21 (m, 2H), 3.15 (t, *J* = 14.4 Hz, 1H), 3.04 (dd, *J* = 14.5, 5.9 Hz, 1H), 2.97 (d, *J* = 10.7 Hz, 1H), 2.91–2.83 (m, 1H), 2.83–2.77 (m, 1H), 2.73 (s, 3H), 2.71–2.64 (m, 2H), 2.63 (s, 1H), 2.47–2.41 (m, 1H), 2.31 (dq, *J* = 14.8, 7.5 Hz, 1H), 2.27–2.22 (m, 1H), 2.22–2.15 (m, 1H), 2.09 (s, 3H), 1.88 (s, 3H), 1.78 (dt, *J* = 14.4, 7.4 Hz, 1H), 1.75–1.68 (m, 1H), 1.43 (dq, *J* = 14.3, 7.2 Hz, 1H), 1.36–1.27 (m, 1H), 1.07–0.99 (m, 1H), 0.96 (d, *J* = 15.1 Hz, 1H), 0.81 (t, *J* = 7.4 Hz, 3H), 0.78 (t, *J* = 7.4 Hz, 3H); ¹³C NMR (151 MHz, CDCl₃) δ 174.5, 171.8, 171.1, 169.8, 158.1, 153.1, 135.0, 130.9, 130.2, 129.4, 124.6, 123.3, 123.0, 122.6, 119.2, 118.3, 117.0, 110.8, 94.5, 83.5, 79.8, 76.6, 65.8, 56.8, 56.0, 54.4, 53.4, 52.6, 52.4, 50.5, 44.7, 43.5, 42.8, 38.6, 37.1, 30.9, 30.7, 30.1, 24.7, 21.3, 8.6, 8.2; IR (film) ν_{max} 3467, 2958, 1738, 1666, 1459, 1229, 1039, 749 cm⁻¹; HRESI-TOF *m/z* 852.4547 (C₄₈H₆₁N₅O₉ + H⁺, required 852.4542). [α]_D²³ +13 (*c* 0.31, CHCl₃). Identical in all respects with reported data and an authentic sample.⁴¹

Cell Growth Inhibition Assay—Compounds were tested for their cell growth inhibition of L1210 (ATCC no. CCL-219, mouse lymphocytic leukemia), HCT116 (ATCC no. CCL-247, human colorectal carcinoma), and HCT116/VM46 (a vinblastine-resistant strain of HCT116) cells in culture. A population of cells (>1 × 10⁶ cells/mL as determined with a hemocytometer) was diluted with Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco) to give a final concentration of 30,000 cells/mL. To each well of a 96-well plate (Corning Costar) was added 100 μL of the cell media solution with a multichannel pipet. The cultures were incubated at 37 °C in an atmosphere of 5% CO₂ and 95% humidified air for 24 h. The test compounds were then added to the plate as follows: test compounds were diluted in DMSO to a concentration of 1 mM. 10-fold serial dilutions in DMSO were next performed on a separate 96-well plate. Fresh culture media (100 μL) was added to each well of cells resulting in 200 μL of medium per well followed by 2 μL of each test agent. Compounds were tested in duplicate (*n* = 2–8 times) at six concentrations between 0–1000 nM or 0–10000 nM. Following the addition of compound, cultures were incubated for an additional 72 h. A phosphatase assay was used to establish IC₅₀ values as follows: the media was removed from each well and treated with 100 μL of phosphatase solution (100 mg phosphatase substrate in 30 mL of 0.1 M NaOAc, pH 5.5, 0.1% Triton X-100 buffer). The plates were incubated at 37 °C for 5 min (L1210) or 15 min (HCT116 and HCT116/VM46). After the given incubation time, 50 μL of 0.1 N NaOH was added to each well and the absorption at 405 nm was determined using a 96 well plate reader. Given the absorption is directly proportional to the number of living cells, IC₅₀ values were calculated and reported values represent the average of 4–16 determinations (SD ±10%).

Tubulin Binding Competition Assay.⁴³—Tubulin (0.1 mg/mL, 0.91 μ M) was incubated with BODIPY-vinblastine (BODIPY-VBL, 1.8 μ M) for 15 min at 37 °C in PEM buffer containing 850 μ M GTP. Subsequently, a competitive ligand (vinblastine, **64**, **24**, or **54**) was added to the solution at a final concentration of 18 μ M. After incubation for 60 min at 37°C, 100 μ L aliquots from each incubation was measured in a fluorescence microplate reader (FI; ex 480 nm, em 514 nm). Control experiments were performed with BODIPY-VBL in the absence of a competitive ligand (control 1, maximum FI enhancement) and in the absence of tubulin (control 2, no FI enhancement). % BODIPY-VBL displacement was calculated by the formula: (control 1 FI – experiment FI)/(Control 1 FI – Control 2 FI) $\times$ 100. Reported values are the average of 5 measurements $\pm$ SD.

Efflux and Permeability Assays—The amount of drug stimulated Pgp ATPase activity generated by either vinblastine or analog **121** was determined using a MDR1 PREDEASY™ ATPase Kit Assay Protocol (SOLVO Biotechnology, Version Number: 1.2) purchased from Sigma–Aldrich (St. Louis, MO) and following the manufacturer’s protocol.^{48,49} The Caco-2 cell permeability assay was conducted comparing vinblastine, **28**, **121**, and **173** by following a previously published procedure⁵⁰ and was conducted by Sekisui XenoTech, LLC (Kansas City, KS). The results are reported in Figure 14 and only the diffusion in the B to A direction in the presence of positive control Pgp inhibitor valspodar (1 μ M) impacted vinblastine transport (efflux ratio 2.7) as expected, but did not substantially impact the rate or efflux ratio of **28**, **121** and **173**.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

DMAP	4-(dimethylamino)pyridine
DMF	dimethylformamide
DMSO	dimethylsulfoxide
EDCI	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
MDR	multidrug resistant
Pgp	P-glycoprotein

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Figure 1.
Natural product structures and 20' amide analogs of vinblastine.

compound	IC ₅₀ (nM) ^a			ratio ^b
	L1210	HCT116	HCT116/VM46	
Vinblastine (1)	6.0	6.8	600	88
R = H (9) ^c	65	85	6500	76
R = methyl (10) ^c	65	90	7500	83
R = CF ₃ (11) ^c	660	690	8100	12
R = ethyl (12)	60	60	2100	35
R = <i>i</i> -propyl (13)	70	90	1400	16
R = <i>t</i> -butyl (14)	2800	3600	4100	1.1
R = <i>n</i> -pentyl (15)	55	40	450	11
R = <i>n</i> -heptyl (16)	50	60	420	7
R = cyclopropyl (17)	5.5	6.1	95	16
R = cyclobutyl (18)	5.4	5.4	85	16
R = cyclopentyl (19)	30	25	380	15
R = cyclohexyl (20)	55	70	760	11
R = benzyl (21)	55	55	550	10
R = CHPh ₂ (22)	760	3800	5100	1.3
R = vinyl (23)	7.0	6.0	520	87

^aIC₅₀ values represent the average of 4–16 determinations (SD ±10%). ^bIC₅₀ HCT116-VM46/HCT116. ^cReported in ref. 41.

Figure 2.
Vinblastine aliphatic 20' amides.

Figure 3.
Vinblastine 20' benzoyl amides.

Figure 4.
Plots of Hammett σ_p constant versus $-\log \text{IC}_{50}$ (nM) for an initial series of 4-substituted benzamides that were examined. A. HCT116. B. L1210.

Figure 5.

Tubulin binding assay measuring the % displacement of tubulin-bound BODIPY-vinblastine under conditions where vinblastine results in 50% displacement. Compounds **64** ($88 \pm 5\%$), **24** ($72 \pm 4\%$), and **54** ($56 \pm 5\%$) are the 20' benzamide derivatives containing a 4-NMe₂, 4-H, and 4-CN substituent, respectively. IC₅₀ (HCT116) = 0.18, 0.8, 3.1 and 6.8 nM for **64**, **24**, **54** and vinblastine, respectively.

compound	IC ₅₀ (nM) ^a			
	L1210	HCT116	HCT116/VM46	ratio ^b
Vinblastine (1)	6.0	6.8	600	88
R = H (23)	7.0	6.0	520	87
R = Ph (108)	0.8	0.9	20	22
R = 4-Pyr (109)	0.6	0.7	50	71
R = 3-Pyr (110)	5.9	5.7	320	60
R = 2-Pyr (111)	0.7	0.7	25	36
R = 3-furanyl (112)	0.7	0.7	20	29
R = 2-furanyl (113)	1.3	1.3	30	23
R = 3-thienyl (114)	0.5	0.6	20	35

^aIC₅₀ values represent the average of 4–16 determinations (SD $\pm 10\%$). ^bIC₅₀ HCT116-VM46/HCT116.

Figure 6.
Vinblastine 20' acrylamides.

Figure 7.
Polycyclic benzoyl-like 20' amides.

compound	IC ₅₀ (nM) ^a			
	L1210	HCT116	HCT116/VM46	ratio ^b
Vinblastine (1)	6.0	6.8	600	88
R = 4-Pyr (128)	0.4	0.4	55	138
R = 3-Pyr (129)	0.7	0.6	50	83
R = 2-Pyr (130)	55	50	460	9
R = 2-pyrazinyl (131)	40	20	550	28
R = 4-pyridazinyl (132)	30	9	700	78
R = 3-furanyl (133)	0.7	0.6	6.3	11
R = 2-furanyl (134)	0.7	0.7	12	17
R = 3-thienyl (135)	0.5	0.7	7.7	11
R = 2-thienyl (136)	0.6	0.6	6.7	11
R = 4-oxazolyl (137)	4.5	4	90	22
R = 5-oxazolyl (138)	3.9	3.5	65	19
R = 4-thiazolyl (139)	5.6	6.3	80	13
R = 5-thiazolyl (140)	1.8	0.6	45	75
R = 5-N ¹ Me-Imid (141)	4	2.2	80	36
R = 4-N ¹ Me-Imid (142)	80	50	>1000	nd
R = 3-isoxazolyl (143)	4.5	7	90	13
144	70	35	3500	100
145	460	450	5200	12
146	4200	>10000	>10000	nd
147	50	45	500	11
148	5300	4400	6600	1.5

^aIC₅₀ values represent the average of 4–16 determinations (SD ±10%). ^bIC₅₀ HCT116-VM46/HCT116.

Figure 8.
Monocyclic heterocyclic 20' amides.

compound	IC ₅₀ (nM) ^a			
	L1210	HCT116	HCT116/VM46	ratio ^b
Vinblastine (1)	6.0	6.8	600	88
R = 7-quinolyl (149)	0.6	0.7	8.2	12
R = 6-quinolyl (150)	0.6	0.6	4.9	8
R = 3-quinolyl (151)	0.7	0.6	5.7	9
R = 2-quinolyl (152)	40	40	270	7
R = 7-isoquinolyl (153)	0.4	0.4	3.7	9
R = 6-isoquinolyl (154)	0.6	0.6	7.3	12
R = 6-quinoxalyl (155)	0.6	0.6	7.5	13
R = 2-quinoxalyl (156)	6.2	5.9	60	10
R = 6-benzfuranyl (157)	3.3	2.2	6.5	3
R = 5-benzfuranyl (158)	5	3.8	25	7
R = 3-benzfuranyl (159)	4	4.6	35	8
R = 2-benzfuranyl (160)	1.8	1.1	15	14
161	0.3	0.25	2.4	10
R = 6-benzthiophenyl (162)	3.1	2.9	7.7	2.7
R = 5-benzthiophenyl (163)	4.2	4.9	16	3.3
R = 3-benzthiophenyl (164)	5.2	5.7	20	3.3
R = 2-benzthiophenyl (165)	3.1	1.9	9.2	4.9
166	0.7	0.6	8	13
R = 6-benzoxazolyl (167)	0.6	0.6	30	50
R = 5-benzoxazolyl (168)	5.4	5.1	70	14
R = 2-benzoxazolyl (169)	60	60	190	3.2
R = 6-benzthiazolyl (170)	0.5	0.6	20	33
R = 5-benzthiazolyl (171)	0.6	0.7	25	36
R = 2-benzthiazolyl (172)	180	80	470	6
173	0.4	0.5	1.8	3.8
174	0.6	0.8	8.7	11
175	0.6	0.5	5.3	11
176	0.3	0.3	4.9	16
R = 5-indolyl (177)	5.1	6.7	60	9
R = N-Boc-5-indolyl (178)	35	15	90	6

^aIC₅₀ values represent the average of 4–16 determinations (SD ±10%). ^bIC₅₀ HCT116-VM46/HCT116.

Figure 9.
Polycyclic heterocyclic 20' amides.

Figure 10.

Plot of 20' amide cLogP vs differential activity (ratio) for **28**, **121**, **173** and closely related compounds that progressively exchange in heteroatoms.

compound	IC ₅₀ (nM) ^a			
	L1210	HCT116	HCT116/VM46	ratio ^b
Vinblastine (1)	6.0	6.8	600	88
R = Ph (179)	45	50	680	14
R = 4-MePh (180)	40	30	330	11
R = 4-MeOPh (181)	35	30	240	8
R = 3-NO ₂ Ph (182)	450	490	430	0.9
R = 2-NO ₂ Ph (183)	360	420	440	1
R = 2,4,6-Me ₃ Ph (184)	500	580	710	1.2
R = 2-naphthyl (185)	50	60	440	8
R = 5-Me ₂ N-1-naphthyl (186)	30	30	60	2

^aIC₅₀ values represent the average of 4–16 determinations (SD ±10%). ^bIC₅₀ HCT116-VM46/HCT116.

Figure 11.
Vinblastine 20' sulfonamides.

compound	IC ₅₀ (nM) ^a			
	L1210	HCT116	HCT116/VM46	ratio ^b
Vinblastine (1)	6.0	6.8	600	88
187	0.6	0.7	30	43
188	0.6	0.7	1.0	1.4
189	5	6.2	19	3
190	0.06	0.08	0.9	11

^aIC₅₀ values represent the average of 4–16 determinations (SD $\pm 10\%$). ^bIC₅₀ HCT116-VM46/HCT116.

Figure 12.
10'-Fluorovinblastine 20' amides.

compd	% displacement	IC ₅₀ (nM, HCT116)
1	61 ± 13%	6.8
28	67 ± 8%	4.5
121	63 ± 5%	4.9
173	98 ± 7%	0.5

Figure 13.

Tubulin binding assay measuring the % displacement of tubulin-bound BODIPY-vinblastine, comparing compounds **28**, **121** and **173** with vinblastine.

Figure 14.

Caco-2 bidirectional permeability and stimulated Pgp ATPase activity in membranes for vinblastine and compounds **173**, **121** and **28**.

Cell Line ^a	IC ₅₀ (nM)				
	1	28	121	173	Taxol
HCT116	6.8	4.6	4.9	0.7	4.1
HCT116/VM46	>110 ^b	9.0 (2)	14 (2.9)	4.9 (7)	>200 ^b
HCT116/VP35	37	5.8	6.2	1.4	6.3
LNCaP	5.1	3.0	3.9	0.8	1.9
LS174T	20	6.1	6.6	2.4	9.5
MDA-MB-468	14	4.8	6.3	1.2	5.4
NCI-H520	4.5	1.4	1.8	0.8	8.2
PA-1	4.6	2.3	2.6	1.0	3.0

^aHCT116 (Colon), HCT116/VM46 (Colon, vinblastine resistant), HCT116/VP35 (Colon, etoposide resistant), LNCaP (Prostate), LS174T (Colon, high levels of MUC2 mRNA), MDA-MB-468 (Breast, triple negative, amplified EGFR), NCI-H520 (Lung), PA-1 (Ovary, overexpression of AIB1).^bHighest concentration tested. Measured fold resistant vs wt HCT116 in parentheses.

Figure 15.

Evaluation of **28**, **121**, and **173** against additional representative human cancer cell lines and an independent assessment HCT116 versus HCT116/VM46 activity.⁵¹

Figure 16.

Models of **28** (panels C), **121** (panels A) and **173** (panels B) bound to tubulin generated from the X-ray structure of a vinblastine-bound complex (pdb 5J2T).⁵³ Left: Stick figures highlighting the key H-bonds of the 20' amide: the amide N–H with the backbone carbonyl of Pro222 (2.0 Å) and the amide carbonyl with the backbone amide N–H of Tyr224 (3.0 Å). Right: Space filling models of tubulin highlighting the extension of the 20' amides into a narrow channel formed along the continuing protein–protein interaction at the tubulin α/β dimer–dimer interface.

Scheme 1.