Published in final edited form as:

Bioorg Med Chem Lett. 2020 January 15; 30(2): 126786. doi:10.1016/j.bmcl.2019.126786.

Upregulation of p53 through Induction of MDM2 Degradation: Amino Acid Prodrugs of Anthraquinone Analogs

Abiodun Anifowose, Zhengnan Yuan, Xiaoxiao Yang*, Zhixiang Pan, Yueqin Zheng, Zhongwei Zhang, Binghe Wang*

Department of Chemistry and Center for Diagnostics and Therapeutics, Georgia State University, Petit Science Center, 100 Piedmont Ave, Atlanta, GA 30303, United States.

Abstract

Previously, we reported a class of MDM2-MDM4 dimerization inhibitors that upregulate p53 and showed potent anticancer activity in animal models. However, water solubility hinders their further development. Herein we describe our effort to develop a prodrug approach that overcomes solubility problem. The prodrug of **BW-AQ-238**, a potent anthraquinone analog, was made by esterification of the hydroxyl group with various natural amino acids. Cytotoxicity of these compounds toward Hela and EU-1 cells, their aqueous solubility, and the release kinetics of these prodrugs in buffer and in the presence of hydrolytic enzymes were studied. The results demonstrate that the amino acid prodrug approach significantly improved the water solubility while maintaining the potency of the parent drug.

Keywords

Anticancer agent; anthraquinone; amino acid prodrug; esterase; kallikrein

Since its discovery in 1979,¹⁻³ the tumor suppressor p53 has been firmly established as a critical transcription factor responsible for the regulation of cell cycle, apoptosis, DNA repair, and senescence.^{4, 5} Dysregulation of p53 functions takes on two forms: deletion or mutation of p53, and overexpression of its inhibitory proteins.^{6, 7} Approximately 50% of human cancer has p53 mutation, resulting in inactivation or loss of p53 protein functions.⁸ Even in cancer with wild-type p53, its function is ultimately inhibited, leading to accelerated cancer development and growth.^{6, 9} The latter scenario is the result of the overexpression of its negative regulator, the murine double minute 2 (MDM2) protein, which masks the p53 transactivation domain, impairing nuclear import of the p53 protein, and catalyzes the ubiquitination, leading to the proteasomal degradation of the p53 protein.^{10, 11} Activating p53 in cancer by either down-regulating MDM2 or intercepting MDM2/p53 interaction has

*Corresponding authors. xyang20@gsu.edu (X. Yang); wang@gsu.edu (B. Wang).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Supplementary Material

Supporting information for this article is given via a link at the end of the document.

proven to be a promising approach for cancer therapy. Therefore, many efforts have been devoted to targeting the MDM2-p53 interaction by small molecules^{12–15} and peptides¹⁶ for the reactivation of p53 for the treatment of cancer that retains wild-type p53.

Along this line, our lab has synthesized a series of anthraquinone-based compounds capable of inducing MDM2 degradation, p53 upregulation, and thus apoptosis.^{17–19} The efficacy of such compounds has been proven in treating acute lymphocytic leukemia (ALL) in a mouse xenograft model using our lead compound, **BW-AQ-101** (Figure 1).¹⁷ Complete remission was achieved after treatment with 20 mg/kg/day three doses per week for two weeks followed by 150 days of observation; whereas all control mice died within 45 days as expected. Preliminary results also showed minimal or no toxicity to normal cells at therapeutic concentration. Studies in the EU-1 ALL cells revealed that **BW-AQ-101** inhibited MDM2 but not MDM4, induced activation of p53, and its target proteins p21 and PUMA in a dose- and time-dependent manner.

The results thus far clearly demonstrated the emerging potential of this class of anthraquinones. However, the poor water solubility of the active anthraquinone analogs posed a serious developability problem. Thus, we were interested in examining the possibility of using a prodrug strategy to solve this solubility problem. Amino acids have been widely used as a prodrug strategy to improve the delivery of those with poor solubility and permeability.^{20–22} Some amino acid prodrugs were also shown to enable transporter-mediated drug transport in intestinal epithelial cells.²³ Herein, we report the development of amino acid-based prodrugs for this class of anthraquinones. These prodrugs were found to have improved water solubility and release the parent drug under near physiologic conditions upon esterase-mediated hydrolysis (porcine liver esterase and porcine pancreas kallikrein). The cytotoxicity of these prodrugs was evaluated by the water-soluble tetrazolium salt (WST) assay in Hela and EU-1 cell lines. The valine prodrug was found to have comparable potency along with significantly increased aqueous solubility.

In order to prepare ester prodrugs of the parent drug, a hydroxyl handle is needed. **BW-AQ-101** has two phenolic hydroxyl groups, which could conceivably be used for prodrug derivatization. However, phenol esters are generally not stable. Therefore we designed and synthesized a new analog by installing a 2-hydroxyl ethoxy group on each of the phenolic hydroxyl positions to afford **BW-AQ-230**. This design was based on our earlier findings that the phenolic hydroxyl groups can be modified with alkyls, alkylalkyl groups without abolishing the activity of **BW-AQ-101**.¹⁹ The two hydroxyl groups are intended as sites for prodrug derivatization.

The synthesis of **BW-AQ-238** started with the methyl ester of rhein (**1**),¹⁸ followed by the installation of the *tert*-butyldimethylsilyl (TBS) protected ethyl alcohol using potassium carbonate as a base and potassium iodide as a catalyst to obtain compound **2**. Hydrolysis of the ester in compound **2** using lithium hydroxide in water afforded the carboxylic acid derivative (**3**) in about 65% yield. The resulting carboxylic acid group was converted to an azido group using a literature procedure to afford compound **4**. Then the azido moiety was subjected to Curtius rearrangement to yield compound **5**, which was converted to the corresponding amide (**6**) through reaction with chloroacetyl chloride. Deprotection of **6** with

tetrabutylammonium fluoride (TBAF) yielded compound **7** (**BW-AQ-238**) as a bright yellowish solid (Scheme 1, see Supporting Information for detail).

HeLa cell line and an in-house established wild-type p53 leukemia cell line (EU-1)¹⁷ were used for cytotoxicity assessment using the WST assay. The IC₅₀ values of **BW-AQ-238** were determined to be 3.76 μ M and 0.89 μ M in HeLa and EU-1 leukemia cells, respectively (Table 3, see supporting information for detail). In comparison, IC₅₀ values of **BW-AQ-101** were determined to be 2.21 μ M and 0.83 μ M in HeLa and EU-1 leukemia cells, respectively. Western blot studies also showed that **BW-AQ-238** induced downregulation of MDM2 in a dose- and time-dependent manner and therefore upregulated p53 level in EU-1 cells (Figure 2). Specifically, at the 15-h time point, **BW-AQ-238** at a dosage of 0.8 μ M significantly down-regulated cytoplasmic MDM2 and increased the p53 level. Down-regulation of MDM2 could be detected after 4-h treatment with 1 μ M **BW-AQ-238** and proceeded to 16 hours; accordingly, the p53 level was elevated after 4 hours of treatment and maintained at a high level after 8 hours. Such results are consistent with that of the lead compound **BW-AQ-101**¹⁷ and the preliminary SAR result we reported.¹⁹

With **BW-AQ-238** in hand, the preparation of water-soluble amino acid was achieved by coupling with twelve amino acids choosing from the simplest glycine to hydrophobic phenylalanine to arginine with an ionizable side chain (Scheme 2, Table 1). First, coupling of compound **7** with the corresponding *N*-protected amino acid using 1-ethyl-3-(3-dimethylaminopropyl)carbo-diimide (EDC) as a coupling reagent and 4-dimethylamino pyridine (DMAP) as a catalyst in acetonitrile afforded compounds **8a-l**. Subsequent deprotection with 2 M hydrogen chloride in diethyl ether using dichloromethane (DCM) as a co-solvent led to the formation of corresponding hydrogen chloride salts of amino acid prodrugs, **9a-l**, in quantitative yield.

One requirement for the success of a prodrug strategy is the ability to release the parent drug under physiologic conditions. Therefore, we examined the chemical and enzyme-mediated hydrolysis of **9a-l** in PBS at pH 7.4 and 37 °C using HPLC. Specifically, porcine liver esterase (PLE) and porcine pancreas kallikrein (PPK) were chosen as model enzymes for the parent drug release studies. PLE has been extensively used by others²⁴ and us^{25–27} in the study of esterase-sensitive prodrugs. PPK was used in this study to resemble human tissue kallikrein (KLKs). KLKs is a class of serine protease with diverse roles in a variety of human tissues and is found to be upregulated in several cancers including prostate, ovarian, and breast. Several KLKs members have been identified as cancer markers including prostate and breast tissues.²⁸ Among them, human glandular kallikrein 2 has been used to develop a peptide-based prodrug targeting prostate cancer.²⁹ Since PPK has been found to be able to hydrolyze the amino acid ester,³⁰ it was included in this study to explore other specific metabolic release of the prodrug besides esterase. The chemical hydrolysis stability and enzyme specific release of the prodrugs were examined in PBS solution by monitoring the formation of **BW-AQ-238** and the disappearance of the respective prodrug. The metabolic profiles are shown in Figure S1–S3 (supporting information), and the *t*-life values are shown in Table 2. In PBS solution the ester bond of the prodrug can be chemically hydrolyzed. The release rate relatively slow except for **9a** with the least steric hindrance with a glycine promoiety. This is consistent with reported ester prodrugs.³¹ The hydrolysis rate

was boosted by either PLE or kallikrein, with half-lives ranging from 0.2 to 118 min. These results clearly showed that the aromatic amino acid promoiety such as Phe and Tyr are more susceptible to PLE with half-lives within 2 minutes. PLE also showed its preference to other hydrophobic amino acids, with the order of decreasing stability being Val $\approx$ Ile > Gly > Ala > Met > Leu. Such results are consistent with literature reports in the hydrophobic substrate specificity of PLE.³² In the presence of kallikrein, due to its trypsin-like nature, hydrolysis showed a strong preference for basic amino acids with the Arg prodrug being hydrolyzed faster than the Lys counterpart. In addition, cleavage of Phe and Tyr prodrug was also found with the treatment, suggesting a trypsin-like and chymotrypsin-like dual substrate specificity.³³ This could be further verified by its preference to the methionine prodrug **9l**.

The key reason for the preparation of these prodrugs was to improve solubility. For this, we assessed the water solubility of these prodrugs. It is important to note that all the amino acid prodrugs could be easily dissolved in pH 7.4 PBS at a concentration greater than 100 mg/ml, indicating a significant improvement in solubility compared with the lead compound **BW-AQ-101** and the parent drug **BW-AQ-238**. In addition, according to the observations from several reported studies that the prodrug with phenylalanine promoiety resulted in the lowest solubility among other amino acid-based prodrugs,²³ we chose the phenylalanine prodrug **9f** as a benchmark to test aqueous solubility and quantitatively compare with its parent drug. Determined by an HPLC method³⁴ (see supplementary materials for detail), the water solubility of phenylalanine prodrug was 703 mg/ml, which was over 11000-fold greater than the solubility of parent drug **BW-AQ-238** (60.0 μ g/ml). Comparing with the solubility result **BW-AQ-101** (8.5 μ g/ml), the improvement was even more striking (Table S2, supporting information). Therefore, it is reasonable to assume that the solubility of the prodrugs was greatly improved.

With success in demonstrating improved solubility and the ability to release the parent drug, we were interested in examining whether the prodrugs would be able to deliver a sufficient quantity of the parent drug to cells for the desired cytotoxicity. Therefore, the cytotoxicity of these prodrugs was tested in Hela and EU-1 leukemia cell lines using the WST-8 assay. The IC₅₀ values of these prodrugs ranged from 2.8 to 13 μ M in Hela cells and 1.08 to 3.5 μ M in EU-1 cells (Table 3). The IC₅₀ of **9d** in Hela cells is marginally lower than that of the parent. However, the small difference makes it hard to interpret whether there is any mechanistic significance.

To further examine the prodrug activation in cell compounds **9a** and **9k** were selected to test the stability in the culture media. Their half-lives turned out to be 19.3 and 156.1 respectively (Figure S4), which are shorter than their chemical hydrolysis in PBS (31.5 min for **9a** and 268.6 min for **9k**). Therefore, can assume that prodrug activation is due to the combined effect of enzymatic and chemical hydrolysis, with enzymatic activation likely playing a dominant role.

In EU-1 leukemia cells, the IC₅₀ values are around 1 μ M for most prodrugs, which are comparable with that of the parent drug. In order to study prodrug activation by cellular components, we used none-denatured cell lysate of EU-1 leukemia cells and the valine prodrug **9c** as an example. It was found that the ester bond was readily cleaved within 15

min to give the parent drug **BW-AQ-238** (Figure S5). This activation was much faster than chemical hydrolysis ($t_{1/2}$ of ~148 min) and PLE-mediated degradation ($t_{1/2}$ ~25.3 min). Such results suggest that the activation of prodrug **9c** can be at least partially attributed to the cytosolic enzymatic degradation.

In conclusion, to address the poor solubility of anthraquinone-based anti-leukemia agents, twelve amino acid prodrugs of **BW-AQ-238** were synthesized with remarkable improvement in aqueous solubility. The chemical and enzymatic hydrolytic release of the parent drug from **9a-l** were determined by HPLC. Efficient release of the parent drug was achieved with both PLE and PPK. For PLE, the aromatic amino acid promoieties such as Phe and Tyr showed the fastest release rate followed by the hydrophobic amino acid such as Leu and Met. On the other hand, PPK favored the basic amino acids such as Arg and Lys. The kallikrein metabolic profile also revealed that this peptidase could also hydrolyze an ester bond. Both valine prodrug **9c** and leucine prodrug **9d** demonstrated comparable cytotoxicity with the parent drug in HeLa and EU-1 cell lines. Given that valine has been successfully utilized as the promoiety of antiviral compound Zanamivir-L-Val³⁵ and a marketed drug Valacyclovir,³⁶ **9c** and **9d** should be considered for further *in-vivo* anti-cancer therapeutic studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support from the National Institutes of Health (CA180519) is gratefully acknowledged. We thank the Center for Diagnostic and Therapeutics for providing a CDT fellowship to Abiodun Anifowose and the Molecular Basis of Disease Program for providing an MBD fellowship to Zhixiang Pan.

References

1. Lane DP, Crawford LV. T antigen is bound to a host protein in SV40-transformed cells. *Nature*. 1979;278: 261–263. [PubMed: 218111]
2. Linzer DI, Levine AJ. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell*. 1979;17(1): 43–52. [PubMed: 222475]
3. DeLeo AB, Jay G, Appella E, Dubois GC, Law LW, Old LJ. Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse. *Proc Natl Acad Sci U S A*. 1979;76(5): 2420–2424. [PubMed: 221923]
4. Fridman JS, Lowe SW. Control of apoptosis by p53. *Oncogene*. 2003;22(56): 9030–9040. [PubMed: 14663481]
5. Vousden KH, Lu X. Live or let die: the cell's response to p53. *Nat Rev Cancer*. 2002;2(8): 594–604. [PubMed: 12154352]
6. Cheok CF, Verma CS, Baselga J, Lane DP. Translating p53 into the clinic. *Nat Rev Clin Oncol*. 2011;8(1): 25–37. [PubMed: 20975744]
7. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. *Nature*. 2000;408(6810): 307–310. [PubMed: 11099028]
8. Hainaut P, Hollstein M. p53 and human cancer: the first ten thousand mutations. *Adv Cancer Res*. 2000;77: 81–137. [PubMed: 10549356]

9. Onel K, Cordon-Cardo C. MDM2 and prognosis. *Mol Cancer Res.* 2004;2(1): 1–8. [PubMed: 14757840]
10. Momand J, Zambetti GP, Olson DC, George D, Levine AJ. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell.* 1992;69: 1237–1245. [PubMed: 1535557]
11. Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. *Nature.* 1997;387(6630): 296–299. [PubMed: 9153395]
12. Vassilev LT, Vu BT, Graves B, et al. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science.* 2004;303(5659): 844–848. [PubMed: 14704432]
13. Ding K, Lu Y, Nikolovska-Coleska Z, et al. Structure-based design of potent non-peptide MDM2 inhibitors. *J Am Chem Soc.* 2005;127(29): 10130–10131. [PubMed: 16028899]
14. Shangary S, Qin D, McEachern D, et al. Temporal activation of p53 by a specific MDM2 inhibitor is selectively toxic to tumors and leads to complete tumor growth inhibition. *Proc Natl Acad Sci U S A.* 2008;105(10): 3933–3938. [PubMed: 18316739]
15. Ding Q, Zhang Z, Liu JJ, et al. Discovery of RG7388, a potent and selective p53-MDM2 inhibitor in clinical development. *J Med Chem.* 2013;56(14): 5979–5983. [PubMed: 23808545]
16. Chang YS, Graves B, Guerlavais V, et al. Stapled alpha-helical peptide drug development: a potent dual inhibitor of MDM2 and MDMX for p53-dependent cancer therapy. *Proc Natl Acad Sci U S A.* 2013;110(36): E3445–3454. [PubMed: 23946421]
17. Gu L, Zhang H, Liu T, et al. Inhibition of MDM2 by a Rhein-Derived Compound AQ-101 Suppresses Cancer Development in SCID Mice. *Mol Cancer Ther.* 2018;17: 497–507. [PubMed: 29282301]
18. Yang X, Sun G, Yang C, Wang B. Novel rhein analogues as potential anticancer agents. *ChemMedChem.* 2011;6: 2294–2301. [PubMed: 21954017]
19. Draganov AB, Yang X, Anifowose A, et al. Upregulation of p53 through induction of MDM2 degradation: Anthraquinone analogs. *Bioorg Med Chem.* 2019; 27(17):3860–3865. [PubMed: 31324563]
20. Anderson A, Belelli D, Bennett DJ, et al. Alpha-amino acid phenolic ester derivatives: novel water-soluble general anesthetic agents which allosterically modulate GABA(A) receptors. *J Med Chem.* 2001;44: 3582–3591. [PubMed: 11606122]
21. Cai EB, Yang LM, Jia CX, et al. The Synthesis and Evaluation of Arctigenin Acid Ester Derivatives. *Chem Pharm Bull.* 2016;64: 1466–1473. [PubMed: 27383415]
22. Cai E, Guo S, Yang L, et al. Synthesis and antitumour activity of arctigenin amino acid ester derivatives against H22 hepatocellular carcinoma. *Nat Prod Res.* 2018;32: 406–411. [PubMed: 28415847]
23. Vig BS, Huttunen KM, Laine K, Rautio J. Amino acids as promoieties in prodrug design and development. *Adv Drug Deliv Rev.* 2013;65: 1370–1385. [PubMed: 23099277]
24. Gollnest T, Dinis de Oliveira T, Rath A, et al. Membrane-permeable Triphosphate Prodrugs of Nucleoside Analogues. *Angew Chem Int Ed.* 2016;55(17): 5255–5258.
25. Yuan Z, Zheng Y, Yu B, Wang S, Yang X, Wang B. Esterase-Sensitive Glutathione Persulfide Donor. *Org Lett.* 2018;20(20): 6364–6367. [PubMed: 30299967]
26. Zheng Y, Yu B, Ji K, Pan Z, Chittavong V, Wang B. Esterase-Sensitive Prodrugs with Tunable Release Rates and Direct Generation of Hydrogen Sulfide. *Angew Chem Int Ed.* 2016;55(14):4514–4518.
27. Ji X, Ji K, Chittavong V, Yu B, Pan Z, esterase-activated click and release approach to metal-free CO-prodrugs. *Chem Commun (Camb).* 2017;53(59): 8296–8299. [PubMed: 28685779]
28. Emami N, Diamandis EP. Utility of kallikrein-related peptidases (KLKs) as cancer biomarkers. *Clin Chem.* 2008;54(10): 1600–1607. [PubMed: 18687738]
29. Janssen S, Jakobsen CM, Rosen DM, et al. Screening a combinatorial peptide library to develop a human glandular kallikrein 2-activated prodrug as targeted therapy for prostate cancer. *Mol Cancer Ther.* 2004;3(11): 1439–1450. [PubMed: 15542783]
30. Fiedler F, Leysath G, Werle E. Hydrolysis of amino-acid esters by pig-pancreatic kallikrein. *Eur J Biochem.* 1973;36(1): 152–159. [PubMed: 4738939]

31. Beauchamp LM, Orr GF, de Miranda P, Bumette T, Krenitsky TA. Amino Acid Ester Prodrugs of Acyclovir. *Antivir Chem Chemother.* 1992;3: 157–164.
32. Toone EJ, Werth MJ, Jones JB. Enzymes in organic synthesis. 47. Active-site model for interpreting and predicting the specificity of pig liver esterase. *J Am Chem Soc.* 1990;112(12): 4946–4952.
33. Kalinska M, Meyer-Hoffert U, Kantyka T, Potempa J. Kallikreins - The melting pot of activity and function. *Biochimie.* 2016;122: 270–282. [PubMed: 26408415]
34. Kerns EH, Di L, Carter GT. In vitro solubility assays in drug discovery. *Curr Drug Metab.* 2008;9(9): 879–885. [PubMed: 18991584]
35. Gupta SV, Gupta D, Sun J, et al. Enhancing the intestinal membrane permeability of zanamivir: a carrier mediated prodrug approach. *Mol Pharm.* 2011;8(6): 2358–2367. [PubMed: 21905667]
36. Beutner KR. Valacyclovir: a review of its antiviral activity, pharmacokinetic properties, and clinical efficacy. *Antiviral Res.* 1995;28(4): 281–290. [PubMed: 8669888]

Rhein **BW-AQ-101**

Figure 1.
Lead compound derived from Rhein.

Figure 2.

Western blot showed the downregulation of MDM2 and upregulation p53 by **BW-AQ-238** in dosage- (left panel) and time-dependent (right panel) manner in EU-1 leukemia cells. GAPDH was blotted as the loading control.

Scheme 1.

Synthesis of **BW-AQ-238**. a) (TBS)OC₂H₄Cl, K₂CO₃, KI, DMF, 120 °C, 4 h; b) LiOH in H₂O, THF, r.t., 2 h; c) DPPA, TEA, DMF, r.t., 2 h; d) (i) dioxane, reflux, 2 h (ii) H₂O, 60 °C, 1 h; e) chloroacetyl chloride, dioxane, r.t., 5 mins; g) TBAF, THF, r.t., 4 h.

Scheme 2.

Synthesis of **9 a-l**. a) *N*-Boc protected amino acids, EDC, DMAP, ACN, $0-21^\circ\text{C}$, 4 – 6 h; b) 2M HCl in diethyl ether, DCM, 0°C -r.t.

Table 1.Prodrug intermediates and the amino acid prodrugs^[a].

	R¹		R²
8a	Boc-Gly-	9a	Gly-
8b	Boc-Ala-	9b	Ala-
8c	Boc-Val-	9c	Val-
8d	Boc-Leu-	9d	Leu-
8e	Boc-Ile-	9e	Ile-
8f	Boc-Phe-	9f	Phe-
8g	Boc-Tyr(OtBu)-	9g	Tyr-
8h	Boc-Lys(Boc)-	9h	Lys-
8i	Boc-Arg(Boc)-	9i	Arg-
8j	Boc-Gln(Trt)-	9j	Gln-
8k	Boc-Glu(tBu)-	9k	Glu-
8l	Boc-Met-	9l	Met-

^[a] All amino acids are L-amino acids; ester bond was formed with carboxylic acid group of the amino acid; detailed structure is listed in supporting information (Table S1).

Table 2.

Prodrug degradation half-life.

No.	Half-life (mins) ^[a]		
	PBS (pH 7.4)	PLE	PPK
9a	31.5 (26.1–39.6)	10.1 (9.2–11.1)	35.9 (35.5–36.3)
9b	100.9 (93.8–109.1)	6.2 (5.9–6.5)	24.7 (24.1–25.2)
9c	147.7 (87.4–123.5)	25.3 (23.3–27.5)	126.3 (120.9–132.3)
9d	127.7 (73.4–111.5)	2.5 (2.4–2.7)	39.6 (34.6–46.2)
9e	154.5 (123.4–206.5)	24.3 (23.1–25.7)	48.4 (44.7–52.8)
9f	73.7 (64.8–85.5)	1.0 (0.9–1.2)	6.5 (6.1–6.8)
9g	70.6 (60.3–85.1)	1.7 (1.6–1.7)	6.5 (6.3–6.8)
9h	140.8 (132.8–149.8)	44.2 (42.9–45.6)	2.4 (2.3–2.5)
9i	187.1 (176.8–198.6)	63.0 (61.1–65.0)	0.2 (0.1–0.3)
9j	137.1 (127.7–148.2)	73.0 (71.5–74.5)	66.9 (63.8–70.4)
9k	268.6 (264.4–272.9)	117.9 (116.2–119.6)	110.0 (104.9–115.6)
9l	44.8 (41.2–48.9)	3.7 (3.3–4.2)	12.0 (11.3–12.8)

^[a]Experiment was done in triplicate and 95% confidence intervals are shown in parentheses.

Table 3.

Cytotoxicity of amino acid prodrugs.

Compound No.	Amino Acid	IC ₅₀ (μM) ^[a]	
		HeLa cell	EU-1 ALL
BW-AQ-238	-	3.76 (3.39–4.49)	0.89 (0.86–0.92)
9a	Gly	3.66 (3.44–3.90)	1.10 (1.04–1.16)
9b	Ala	8.21 (7.44–9.04)	1.66 (1.51–1.83)
9c	Val	4.15 (3.27–5.27)	1.08 (0.96–1.20)
9d	Leu	2.80 (2.51–3.13)	1.17 (1.03–1.31)
9e	Ile	5.75 (5.48–6.02)	1.14 (1.03–1.27)
9f	Phe	5.64 (5.52–5.76)	1.23 (1.16–1.30)
9g	Tyr	5.40 (4.74–6.16)	1.27 (1.00–1.60)
9h	Lys	10.7 (9.60–11.8)	1.28 (1.48–1.90)
9i	Arg	13.0 (11.4–14.6)	2.07 (1.91–2.25)
9j	Gln	12.3 (9.70–15.5)	1.45 (1.29–1.63)
9k	Glu	9.11 (7.89–10.5)	3.21 (2.94–4.10)
9l	Met	7.07 (6.52–7.66)	1.24 (1.16–1.32)

^[a] Experiment was done in triplicate and 95% confidence intervals of IC₅₀ are shown in parentheses.