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ORIGINAL ARTICLE

Synthesis and antiproliferative activity of glutamic acid-based dipeptides

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Abstract A small and focused library of 22 dipeptides derived from *N*,*N*-dibenzylglutamic acid α- and γ-benzyl esters was prepared in a straightforward manner. The evaluation of the antiproliferative activity in the human solid tumor cell lines HBL-100 (breast), HeLa (cervix), SW1573 (non-small cell lung), T-47D (breast), and WiDr (colon) provided γ-glutamyl methionine (GI_{50} = 6.0–41 μM) and α-glutamyl proline (GI_{50} = 7.5–18 μM) as lead compounds. In particular, glutamyl serine and glutamyl proline dipeptides were more active in the resistant cancer cell line WiDr than the conventional anticancer drugs cisplatin and etoposide. Glutamyl tryptophan dipeptides did not affect cell growth of HBL-100, while in T-47D cells, proliferation was inhibited. This result might be attributed to the inhibition of the $ATB^{0,+}$ transporter.

Keywords Antitumor agents · Dipeptide · Glutamic acid · Structure–activity relationships

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Introduction

Naturally occurring amino acids represent a powerful source of relatively inexpensive, widely available and enantiomerically pure building blocks, even on a bulk scale. They have been largely used in organic chemistry as intermediates in peptide synthesis or in the synthesis of more complex structures with diverse applications. Of particular interest to us is the use of amino acids as source for antitumor compounds. In this context, L-glutamic acid is present in the antitumor drug aminopterin (**1a**) (Farber et al. 1948) and its well-known *N*-methylated derivative methotrexate (**1b**) (Skeel 2008). Other anticancer drug's derivatives of L-glutamic acid are thalidomide (**2a**) and its analogs pomalidomide (CC-4047, **2b**) and lenalidomide (CC-5013, **2c**) (Bartlett et al. 2004). In these compounds, L-glutamic acid binds through its amino group to the rest of the molecule forming amide or imide bonds (Fig. 1a). Another use of L-glutamic acid is as poly-glutamic acid conjugates, which act as drug carrier because they increase the efficacy of anticancer drug and decrease its toxicity toward normal cells (Melancon and Li 2011). In this strategy, the drug is mostly linked to the γ-carboxyl group through ester or amide bonds.

The natural amino acid L-glutamine (**3a**) is the γ-amide of L-glutamic acid and it is essential for cell growth and proliferation. In healthy cells, L-glutamine is synthesized from L-glutamic acid by L-glutamine synthetase. However, in neoplastic cells, L-glutamine cannot be produced due to the lower reactivity of L-glutamine synthetase. In addition to glucose, cancer cells utilize L-glutamine as a carbon source for ATP production and biosynthesis. L-Glutamine can be internalized through cell surface transporters such as ASCT2 (Pochini et al. 2014). These considerations have increased the interest of targeting glutamine internalization

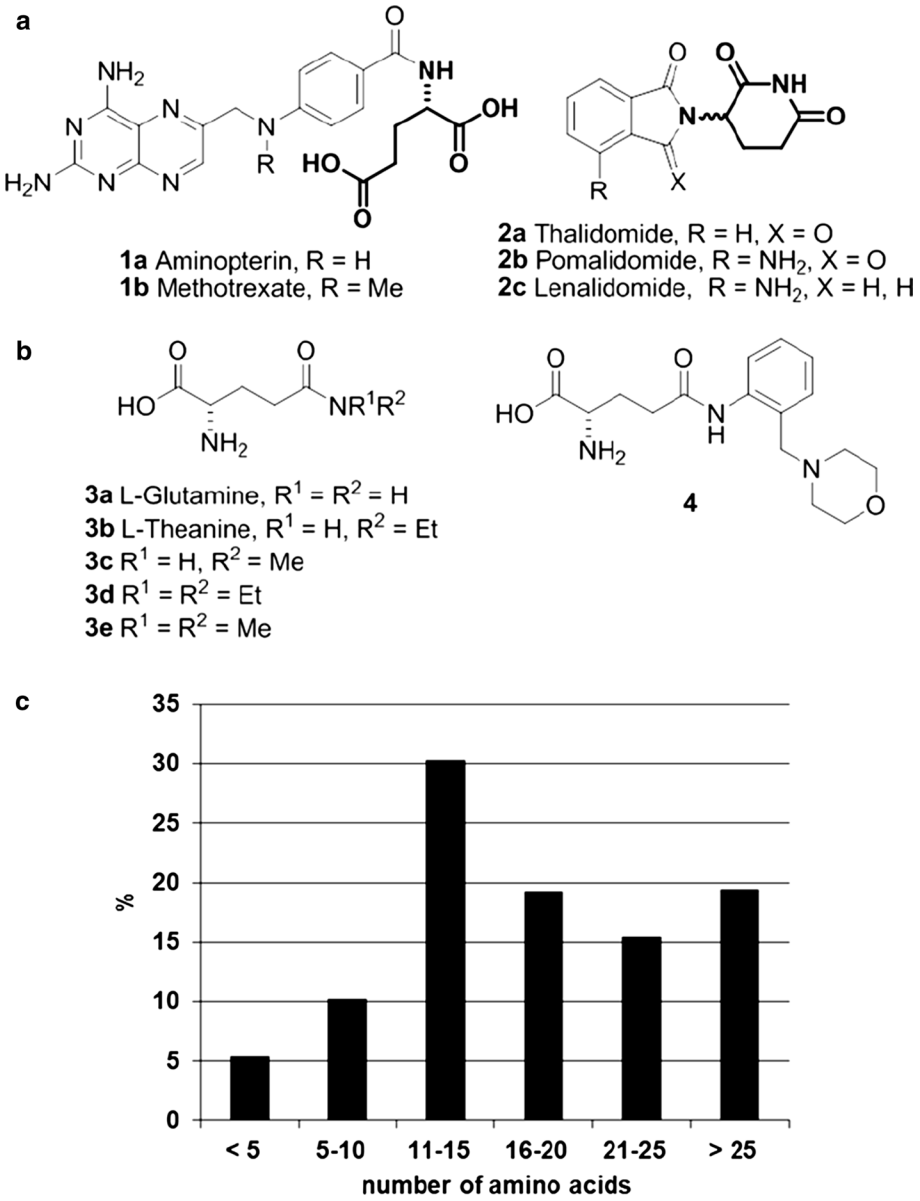

Fig. 1 **a** L-Glutamic acid derivatives with anticancer activity. Bond thickness emphasizes the L-glutamic acid fragment. **b** L-Glutamine and N^{γ}-alkylated derivatives. **c** Statistical analysis of amino acid residues of the ACPs listed in the CancerPPD database

(Wang et al. 2014) and metabolism (Lukey et al. 2013) in cancer therapy. L-Glutamine derivatives (Fig. 1b) investigated along these lines include the natural product theanine (**3b**) (Liu et al. 2009), the glutamine-utilizing enzyme inhibitors **3c**–**3e**, or the ASCT2 inhibitor **4** (Schulte et al. 2015).

In addition to enzyme inhibitors or drug carriers, amino acids constitute peptides that are being used for the therapy of a significant number of diseases (Sato et al. 2006; Mustata and Dinh 2006). In particular, anticancer peptides (ACPs) have shown relevant since they exhibit cancer-selective toxicity while avoiding the shortcomings of the conventional chemotherapy (Thundimadathil 2012). ACPs are small peptides with less than 50 amino acids. The CancerPPD (http://www.crdd.osdd.net/raghava/cancerppd/) database contains over 3400 experimentally verified ACPs. The statistical analysis in terms of amino acid residues shows that small peptides (<5 amino acids) have not being explored exhaustively as potential anticancer agents (Fig. 1c). When it comes to the study of anticancer dipeptides, the literature is scarce in examples. Although tyrosine- and cystine-based dipeptides have been reported to exhibit antiproliferative activity in human solid tumor cell lines (Horvat et al. 2006; Banerji et al. 2013), to the best of our knowledge, there are no reports on the antiproliferative activity of glutamic acid-based dipeptides.

L-Glutamic and L-aspartic acid are potentially valuable molecules providing that the two carboxylic groups could be synthetically differentiated. Recently, we have explored the regioselective esterification of L-glutamic acid (Silveira-Dorta et al. 2014). Our methodology allows to obtain

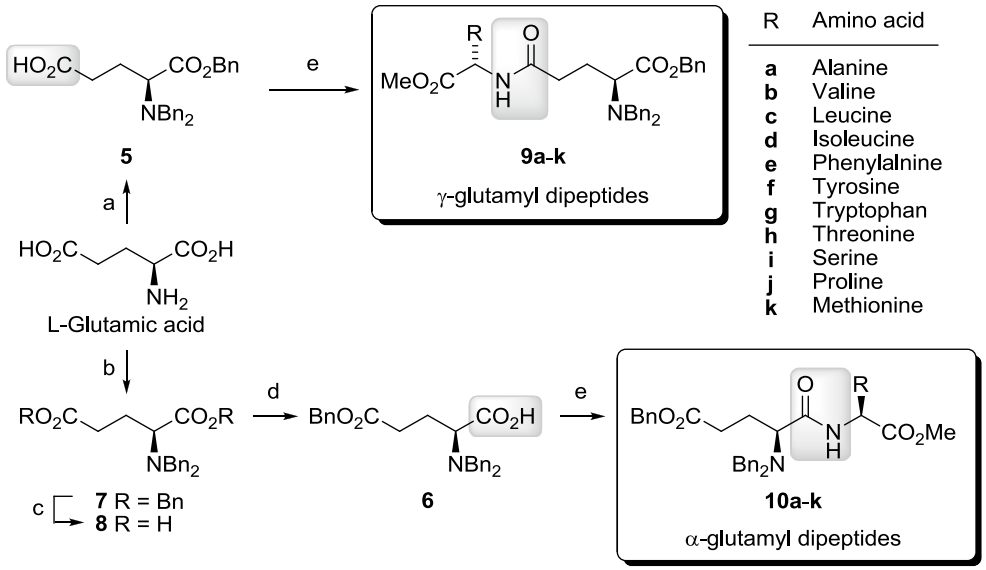

Fig. 2 Synthesis of glutamic acid-based dipeptides **9**–**10**. Reagents and conditions: **a** BnBr, K_2CO_3-KOH, MeOH-H_2O (75:25), Δ, 8 h, 73 %; **b** BnBr, K_2CO_3, MeOH-H_2O (1:1), Δ, 20 h, 90 %; **c** NaOH, MeOH (1:1), Δ, 20 h, 98 %; **d** CH_3SO_3H, BnOH, toluene, Δ, 5 h, 70 %; **e** TBTU, DIPEA, DMF, rt, overnight, 50–70 %

unambiguously both *N*,*N*-dibenzylglutamic acid α- (**5**) and γ-benzyl esters (**6**) from commercially available L-glutamic acid (Fig. 2). As a part of our screening program directed at the discovery of new biologically active molecules with antiproliferative activity, we found that both **5** and **6** were active against human solid tumor cells. These results together with the lack of antiproliferative activity studies on glutamic acid-based dipeptides encouraged us to explore further this type of molecules as scaffolds for new anticancer drugs. Herein, we report on the synthesis of glutamic acid-based dipeptides and their antiproliferative activity against human solid tumor cells.

Experimental section

All reagents were used as purchased from commercial suppliers without further purification. Solvents were dried and purified by conventional methods prior to use. The human solid tumor cell lines HBL-100 (breast), HeLa (cervix), SW1573 (lung), T-47D (breast), and WiDr (colon) were used in this study. These cell lines were a kind gift from Prof. G. J. Peters (VU Medical Center, Amsterdam, The Netherlands).

General procedure for the preparation of glutamic acid-based dipeptides

Monobenzyl ester **5** or **6** (150 mg, 0.4 mmol), *N*-*N*-diisopropyl ethyl amine (94.2 mg, 0.12 mL, 0.8 mmol), and TBTU (176.6 mg, 1.5 mmol) were dissolved in DMF (5 mL). The reaction mixture was stirred at room temperature for 30 min. Amino acid methyl ester (71.6 mg, 0.4 mmol) was added and stirring was continued overnight. The reaction mixture was diluted with water (25 mL) and extracted with Et_2O (3 × 25 mL). The combined organic phases were dried over magnesium sulfate, filtered, and the solvent was evaporated. The crude product was purified by column chromatography (silica gel, Hexane:AcOEt 8:2 or 7:3). The title compound **9** or **10** was obtained in 70–80 % yield.

Chemosensitivity testing

Cells were inoculated onto 96-well microtiter plates in a volume of 100 μL per well at densities of 10,000 (HBL-100, HeLa and SW1573), 15,000 (T-47D), and 20,000 (WiDr) cells per well, based on their doubling times. Compounds **9**–**10** were initially dissolved in DMSO at 400 times the desired final maximum test concentration. Control cells were exposed to an equivalent concentration of DMSO (0.25 % v/v, negative control). Each agent was tested in triplicate at different dilutions in the range of 1–100 μM. The drug treatment started on day 1 after plating. Drug incubation times were 48 h, after which cells were precipitated with 25 μL ice-cold TCA (50 % w/v) and fixed for 60 min at 4 °C. Then, the SRB assay was performed. The optical density (OD) of each well was measured at 492 nm, using BioTek's PowerWave XS Absorbance Microplate Reader. Values were corrected for background OD from

wells only containing medium. The antiproliferative activity for each compound, expressed as GI_{50} values, was calculated according to NCI formulas (Monks et al. 1991).

Results and discussion

During the course of our investigation on the regioselective benzylation of L-glutamic acid, we analyzed the antiproliferative activity of compounds **5**–**8**. As a model to study the antiproliferative activity, we selected the panel of representative human solid tumor cell lines HBL-100 (breast), HeLa (cervix), SW1573 (non-small cell lung), T-47D (breast), and WiDr (colon). The in vitro antiproliferative activity was evaluated using the National Cancer Institute (NCI) protocol (Monks et al. 1991). The standard anticancer drugs cisplatin (Dasari and Tchounwou 2014) and etoposide (Najar and Johri 2014) were used as reference agents. The results expressed as GI_{50} are shown in Table 1. We found that monobenzyl esters **5**–**6** were able to induce cell growth inhibition in all cell lines with GI_{50} values in the range of 22–46 μM, whereas the perbenzylated analog **7** and the diacid **8** were inactive. In addition, compound **5** showed slightly more active than compound **6**. This result encouraged us to synthesize a small library of α- and γ-glutamyl dipeptides and test their antiproliferative activity.

Synthesis of glutamic acid-based dipeptides

The general synthetic pathway for the preparation of the glutamic acid-based dipeptides is outlined in Fig. 2. The direct regioselective benzylation of commercially available L-glutamic acid afforded *N*,*N*-dibenzylglutamic acid α-benzyl ester (**5**) in 73 % yield. We have reported recently that the reaction conditions play a critical role in the outcome of the products (Silveira-Dorta et al. 2014). However, *N*,*N*-dibenzylglutamic acid γ-benzyl ester (**6**) cannot be obtained directly from L-glutamic acid. Instead, a three-step sequence is needed. Briefly, conventional perbenzylation of L-glutamic acid afforded compound **7** in high yields as reported earlier (Rodriquez and Taddei 2005). Then, hydrolysis of the benzyl ester groups of **7** under standard basic conditions led to diacid **8** in almost quantitative yield. Finally, esterification under acid conditions (Albert et al. 1987) allowed us to obtain exclusively the γ-benzyl ester **6** in 70 % yield. Intermediates **5** and **6** were coupled successfully with the commercially available methyl ester hydrochlorides of the amino acids alanine (**a**), valine (**b**), leucine (**c**), isoleucine (**d**), phenylalanine (**e**), tyrosine (**f**), tryptophan (**g**), threonine (**h**), serine (**i**), proline (**j**), and methionine (**k**) to provide dipeptides **9a**–**k** and **10a**–**k**, respectively. In this process, 2-(1H-benzotriazole-1yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) was selected as coupling agent. The reactions were carried out in DMF and *N*,*N*-diisopropylethyl amine was used as based. Yields were in the range of 50–70 %. With this methodology, we obtained a small library of dipeptides

Table 1 Antiproliferative activity (GI_{50}) against human solid tumor cells of compounds **5**–**8**

Compound	Cell line				
	HBL-100	HeLa	SW1573	T-47D	WiDr
5	39 (±2.6)	22 (±6.6)	38 (±4.1)	29 (±5.6)	29 (±7.8)
6	46 (±9.0)	30 (±6.8)	40 (±4.4)	34 (±7.2)	44 (±9.4)
7	>100	>100	>100	>100	>100
8	>100	>100	>100	>100	>100
Cisplatin	1.9 (±0.2)	2.0 (±0.3)	3.0 (±0.4)	15 (±2.3)	26 (±5.3)
Etoposide	2.3 (±0.9)	3.0 (±0.9)	15 (±1.5)	22 (±5.5)	23 (±3.1)

Values are given in μM and are means of two to five experiments; standard deviation is given in parentheses

Table 2 Antiproliferative activity (GI_{50}) against human solid tumor cells of compounds **9**–**10**

Compound	Cell line				
	HBL-100	HeLa	SW1573	T-47D	WiDr
9a	24 (±2.2)	22 (±4.4)	36 (±2.6)	22 (±2.9)	24 (±1.2)
9b	22 (±8.5)	19 (±2.7)	65 (±26)	38 (±10)	40 (±4.8)
9c	35 (±2.8)	25 (±8.2)	30 (±9.8)	33 (±2.6)	40 (±3.1)
9d	22 (±0.8)	23 (±5.5)	26 (±4.5)	21 (±1.9)	26 (±2.8)
9e	36 (±6.2)	27 (±7.0)	14 (±4.1)	21 (±0.1)	36 (±9.0)
9f	20 (±7.2)	14 (±1.6)	18 (±6.1)	22 (±7.7)	20 (±4.9)
9g	>100	18 (±4.0)	20 (±7.1)	33 (±2.5)	40 (±11)
9h	37 (±7.1)	31 (±2.0)	44 (±7.2)	39 (±8.4)	33 (±8.2)
9i	17 (±2.2)	15 (±2.2)	17 (±4.4)	18 (±2.5)	18 (±5.6)
9j	20 (±4.6)	17 (±1.3)	24 (±4.2)	19 (±1.1)	21 (±1.1)
9k	6.0 (±1.1)	7.9 (±1.2)	41 (±9.6)	11 (±2.3)	13 (±5.3)
10a	17 (±1.2)	10 (±6.5)	31 (±0.1)	27 (±0.4)	32 (±0.3)
10b	9.1 (±1.2)	9.0 (±6.5)	23 (±7.8)	23 (±3.3)	17 (±2.4)
10c	16 (±6.0)	12 (±5.1)	25 (±4.1)	24 (±6.5)	33 (±2.1)
10d	16 (±0.1)	19 (±2.8)	41 (±21)	33 (±4.9)	36 (±5.2)
10e	24 (±9.6)	35 (±14)	7.4 (±0.9)	48 (±3.5)	37 (±15)
10f	18 (±2.9)	19 (±2.4)	26 (±2.0)	30 (±1.4)	37 (±5.6)
10g	>100	21 (±6.3)	31 (±6.8)	36 (±7.8)	57 (±2.8)
10h	19 (±4.0)	18 (±2.1)	30 (±4.6)	31 (±5.9)	24 (±2.7)
10i	16 (±1.3)	16 (±0.3)	18 (±2.1)	19 (±2.3)	18 (±1.7)
10j	7.5 (±0.6)	9.3 (±2.9)	13 (±4.9)	18 (±4.4)	10 (±4.0)
10k	14 (±5.9)	17 (±1.8)	25 (±0.5)	31 (±2.7)	39 (±8.6)

Values are given in μM and are means of two to three experiments; standard deviation is given in parentheses

comprising two sets of regioisomers, the γ- (**9a**–**k**) and the α-glutamyl series (**10a**–**k**).

Biological evaluation of glutamic acid-based dipeptides

The antiproliferative activity of γ-glutamyl (**9a**–**k**) and α-glutamyl dipeptides (**10a**–**k**) was evaluated and the results are shown in Table 2 and Fig. 3. Although the set of compounds is not large, some structure–activity relationships could be inferred from the antiproliferative data. The data showed a clear difference in potency between both set of compounds. Based on average GI_{50} values, α-glutamyl dipeptides (**10a**–**k**) were more active than the γ-glutamyl derivatives (**9a**–**k**) in HBL-100, HeLa, and SW1573 cells. In the more resistant cell lines T-47D and WiDr, the overall effect favored the γ-glutamyl derivatives. The most potent compound of both series was the α-glutamyl dipeptide **10j** and exhibited GI_{50} values in the range of 7.5–18 μM. In the γ-glutamyl set, the lead in terms of GI_{50} values was **9k** with GI_{50} values in the range of 6.0–41 μM. However, in SW1573 cells, none of the designated lead was the most potent compound. Instead, the phenylalanine dipeptide (**e**) was the most active. It is noteworthy that when compared to the standard anticancer drugs etoposide and cisplatin, dipeptides **9i**, **9j**, **10i**, and **10j** were more active in the resistant cell line WiDr.

All compounds were able to inhibit cell growth in all cell lines with GI_{50} values in the range of 6–65 μM with the exception of compounds **9g** and **10g** (tryptophan dipeptides) against HBL-100 cells. We speculate that the difference in activity observed against the mammary epithelial cancer cell lines HBL-100 and T-47D might relate to the $ATB^{0,+}$ (SLC6A14, solute carrier family 6 member 14) transporter. $ATB^{0,+}$ accepts tryptophan as a substrate with high affinity and is up-regulated markedly in some types of cancer (Gupta et al. 2005, 2006). Diverse tryptophan derivatives inhibit $ATB^{0,+}$ causing antiproliferative effects in cell lines overexpressing the transporter such as MCF-7, ZR-75.1, and T-47D (Karunakaran et al. 2008). However, in HBL-100, HMEC, and MCF10A cells, the expression of $ATB^{0,+}$ is undetectable and the tryptophan derivatives do not affect cell growth. Our findings for **9g** and **10g** are consistent with these results.

When considering the aliphatic side chain in the α-glutamyl series **10a**–**d**, valine dipeptide **10b** stands out against all cell lines. However, in the γ-glutamyl set **9a**–**d**, this trend was not observed. The antiproliferative activity of the compounds with an aromatic side chain (**9e**–**g** and **10e**–**g**) did not show clear tendency. In peptides containing a hydroxyl group, serine dipeptides (**i**) resulted more active than the corresponding threonine derivative (**h**) in all cell lines tested. Finally, proline dipeptide (**j**) in terms of activity was significant in the γ-glutamyl set, while methionine dipeptide (**k**) was relevant in the γ-glutamyl series.

Conclusion

In summary, we have reported the synthesis of a novel class of *N*,*N*-dibenzylglutamic acid-based dipeptides. Commercially available amino acids were coupled to the appropriate carboxylic acid of glutamic acid to generate the α- and

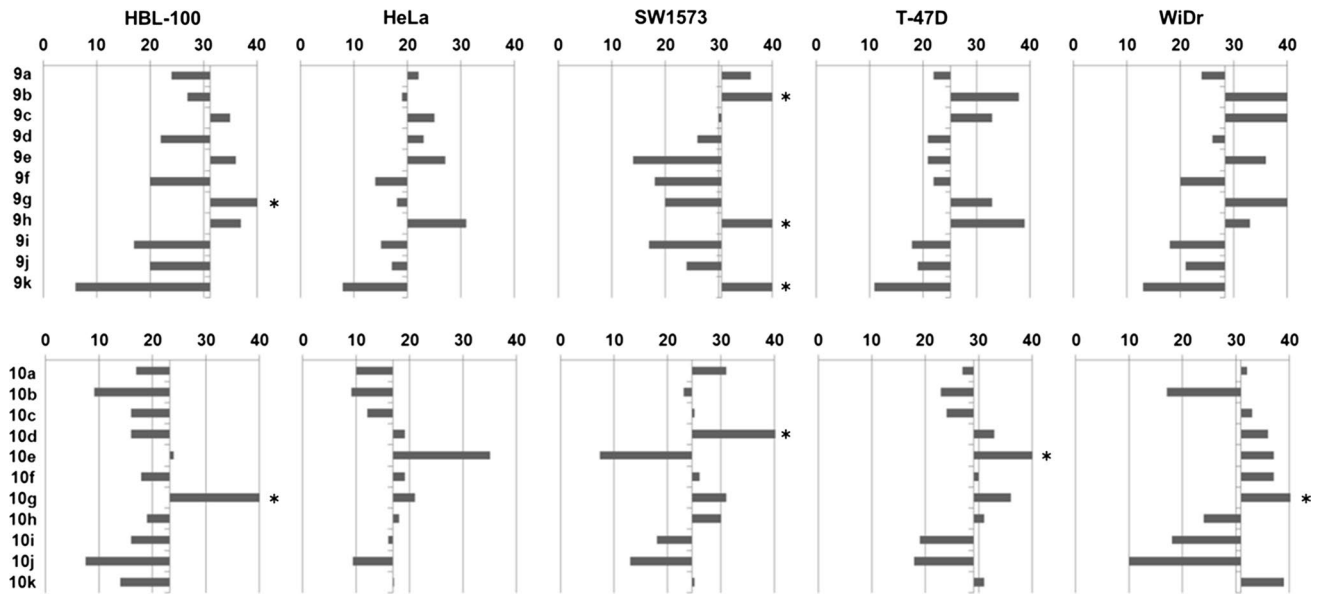

Fig. 3 Mean graph plots for the antiproliferative activity of glutamic acid-based dipeptides **9a**–**k** (*top*) and **10a**–**k** (*bottom*). The *middle line* represents the median GI_{50} (μM) value of each set of compounds against each individual cell line. GI_{50} values >40 μM are indicated with *asterisk*

the γ-glutamyl series of dipeptides. The dipeptides were tested for their antiproliferative activity against five human solid tumor cell lines. Overall, the compounds show active against all cancer cell lines tested. Remarkably, some dipeptides were more active in the resistant cancer cell line WiDr than conventional anticancer drugs. From the data on growth inhibition, γ-glutamyl methionine **9k** and α-glutamyl proline **10j** were identified as lead compounds. More experiments are needed to establish the scope and limitations of *N*,*N*-dibenzylglutamic acid derivatives as novel anticancer agents, as well as to identify their mechanism of antiproliferative activity. Further research involving novel derivatives of *N*,*N*-dibenzylglutamic acid is in progress and will be reported elsewhere. Finally, the results obtained for the tryptophan dipeptides **9g** and **10g** in breast cancer cells merit further investigation, which remain beyond the scope of our study. In particular, the ability of the tryptophan dipeptides to inhibit the $ATB^{0,+}$ transporter.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standard This article does not contain any studies with human participants or animals performed by any of the authors.

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