Different Inhibitory Effects of 5-*S*-Glutathionyl-*β*-alanyl-L-dopa (5-*S*-GA-L-D) Analogues on Autophosphorylation and Substrate Phosphorylation of Src Protein Tyrosine Kinase

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Starting with 5-*S*-glutathionyl-*β*-alanyl-L-dopa (1) and 5-*S*-glutathionyl-*β*-alanyl-dopamine (2), a series of analogues with truncated glutathionyl and *β*-alanyl-dopa moieties were synthesized, and their inhibitory effects on autophosphorylation and substrate phosphorylation reaction by c-Src and by epidermal growth factor receptor (EGFR) were evaluated. When the glutamyl residue was removed, the inhibitory effects on v-Src autophosphorylation decreased about 4- to 5-fold, and concomitant removal of the glutamyl and *β*-alanyl residues resulted in a 40- to 60-fold decrease in the inhibition of v-Src autophosphorylation. On the other hand, these modifications had little effect on the inhibitory activity of substrate (Raytide™) phosphorylation by c-Src. Interestingly, 5-*S*-cysteinyl dopamine inhibited the Src substrate phosphorylation reaction with comparable potency to that of genistein. Nonpeptide lipophilic derivatives had a similar inhibition on v-Src autophosphorylation but decreased inhibitory effects on substrate phosphorylation when compared to the lead compounds. Most compounds showed little effect on substrate phosphorylation by EGFR.

Key words protein tyrosine kinase; c-Src; v-Src; EGFR; autophosphorylation; substrate phosphorylation

c-Src, a member of the nonreceptor-type protein tyrosine kinase (PTK) Src family, has been reported to play an important role in signal transduction in normal cells and its elevated activation has been detected in several cancer cell lines, implicating c-Src as a therapeutic target in cancer treatment.[1] In response to growth factors such as platelet-derived growth factor and epidermal growth factor, c-Src is released from the autoinhibited state and undergoes autophosphorylation on a tyrosine residue (Tyr 416 in human c-Src) in the activation loop, resulting in full activation, although its mechanism and physiological relevance have yet to be clearly understood.[2] Therefore a selective inhibitor of Src, or better yet, an inhibitor with different potency toward autophosphorylation and substrate phosphorylation of Src, should prove to be a useful probe in research on Src and signal transduction. We therefore investigated the inhibitory effects of 5-*S*-GA-L-D (**1**) and its structural analogues on v-Src autophosphorylation, substrate phosphorylation of c-Src, and substrate phosphorylation of epidermal growth factor receptor (EGFR). It has previously been demonstrated that 5-*S*-GA-L-D (**1**) and its analogues selectively inhibit nonreceptor PTK (Src), but neither receptor-type PTK EGFR[3] nor serine/threonine protein kinases.[3,4]

Herein we report that some 5-*S*-GA-L-D (**1**) analogues exhibit different inhibitory effects on autophosphorylation and substrate phosphorylation of Src. Starting with the lead compounds 5-*S*-GA-L-D (**1**) and 5-*S*-GADA (**2**), a series of analogues (**3**—**13**) with truncated glutathionyl and *β*-alanyl-dopa moieties were synthesized using mushroom tyrosinase as previously described (Table 1).[5] The results presented in Table 2 seemed to suggest the importance of the glutamyl residue in the glutathionyl moiety in the inhibitory effects of the lead compounds on v-Src autophosphorylation. Upon removal of the glutamyl residue of **1** and **2**, inhibitory effects against the v-Src autophosphorylation decreased about 4- to 5-fold, whereas the removal of the glycine residue yielded little effect on the inhibitory activity. For example, the IC_{50} val-

Table 1. 5-*S*-GA-L-D and Its Synthetic Analogues

Compound	Abbreviation	Compound
1	5-*S*-GA-L-D	5-*S*-Glutathionyl-*β*-alanyl-L-dopa
2	5-*S*-GADA	5-*S*-Glutathionyl-*β*-alanyldopamine
3	5-*S*-GluCysA-L-D	5-*S*-Glutamylcysteinyl-*β*-alanyl-L-dopa
4	5-*S*-GluCys-L-D	5-*S*-Glutamylcysteinyl-L-dopa
5	5-*S*-GlyCysA-L-D	5-*S*-Glycylcysteinyl-*β*-alanyl-L-dopa
6	5-*S*-GlyCys-L-D	5-*S*-Glycylcysteinyl-L-dopa
7	5-*S*-Cys-L-D	5-*S*-Cysteinyl-L-dopa
8	5-*S*-GluCysADA	5-*S*-Glutamylcysteinyl-*β*-alanyldopamine
9	5-*S*-GluCysDA	5-*S*-Glutamylcysteinyldopamine
10	5-*S*-GlyCysADA	5-*S*-Glycylcysteinyl-*β*-alanyldopamine
11	5-*S*-GlyCysDA	5-*S*-Glycylcysteinyldopamine
12	5-*S*-CysADA	5-*S*-Cysteinyl-*β*-alanyldopamine
13	5-*S*-CysDA	5-*S*-Cysteinyldopamine
14	5-*S*-Bzim-L-D	5-*S*-(2-Mercaptobenzimidazol)-L-dopa
15	5-*S*-Triazo-L-D	5-*S*-(3-Mercapto-1,2,4-triazol)-L-dopa
16	5-*S*-BzimDA	5-*S*-(2-Mercaptobenzimidazol)-dopamine
17	5-*S*-TriazoDA	5-*S*-(3-Mercapto-1,2,4-triazol)-dopamine

Table 2. Inhibitory Activities of 5-*S*-GA-L-D Analogues[a)]

Compd.	v-Src Autophosphorylation	c-Src Phosphorylation of Raytide	EGFR Phosphorylation of Angiotensin II
	IC_{50} (μM)[b)]	IC_{50} (μM)[b)]	Inhibition (%)[c)]
1	25	223	2.2
2	17	293	4.9
3	25	283	≒0
4	35	485	≒0
5	107	396	≒0
6	1023	315	2.5
7	66	>1 mM	2.9
8	34	379	52.1
9	44	296	9.3
10	100	367	7.9
11	1076	392	19.6
12	68	327	12.1
13	101	52	16.3
14	14	720	11.7
15	38	894	17.0
16	39	563	15.1
17	31	646	15.9
Genistein	>100	44	98.0

a) The three assay methods[8—10] were modified to measure ^{32}P-labelled phosphorylated proteins or peptides by Fuji Film Bio-image Analyzer BAS 2000. *b*) IC_{50} values obtained from repeated experiments varied within <15% from experiment to experiment. *c*) Inhibition % at a concentration of 500 μM.

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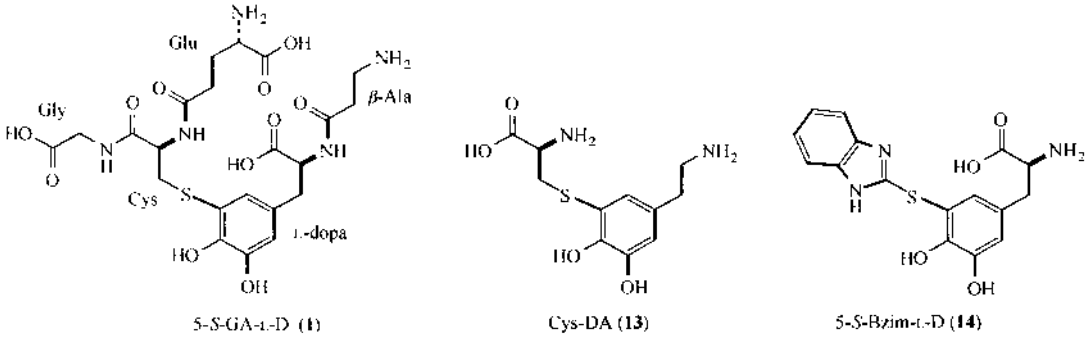

ues of glutamyl-trancated 5-*S*-GlyCysA-L-D (**5**) decreased to 107 μM, as compared to 25 μM of **1** and glycine-truncated 5-*S*-GluCysA-L-D (**3**). When the β-alanyl residue was further deleted from 5-*S*-GlyCysA-L-D (**5**) and 5-*S*-GlyCysADA (**10**) to yield 5-*S*-GlyCys-L-D (**6**) and 5-*S*-GlyCysDA (**11**), a further 10-fold decrease in inhibitory potency was observed, rendering **6** and **11** weak inhibitors with an $IC_{50}>1$ mM. However, this drastic decrease in inhibitory effects upon removal of the β-alanyl residue was not observed with analogues containing the glutamyl residue, as evidenced by the similar IC_{50} values of 5-*S*-GluCysA-L-D (**3**) and 5-*S*-GluCys-L-D (**4**) as well as those of 5-*S*-GluCysADA (**8**) and 5-*S*-GluCysDA (**9**).

Most of these analogues showed similar inhibitory effects on substrate phosphorylation of c-Src. Notable exceptions were compounds 5-*S*-Cys-L-D (**7**) and 5-*S*-CysDA (**13**), in which a single cysteinyl residue is connected with dopa and dopamine, respectively. Compound **7** had virtually no inhibitory effect on substrate phosphorylation ($IC_{50}>1$ mM), while its inhibitory effect on autophosphorylation remaind as potent as that of the other analogues. On the other hand, **13**, which showed decreased inhibitory effect on autophosphoryration, showed a significantly more potent inhibitory effect on substrate phosphorylation with an IC_{50} of 52 μM, comparable to that of genestein, a known nonspecific PTK inhibitor.[6] Meanwhile, nonpeptide, lipophilic derivatives of dopa and dopamine (**14**—**17**) showed similar inhibitory effects on autophosphorylation but decreased effects on substrate phosphorylation when compared to the lead compounds. It should also be noted that, as described earlier,[3] most compounds did not significantly inhibit the phosphorylation reaction catalyzed by EGFR, a receptor-type PTK, with the distinct exception of 5-*S*-GluCysADA (**8**) which showed 52% inhibition at 500 μM. Analogues containing D-dopa were also studied and they showed similar inhibitory effects as their counterparts containing L-dopa (data not shown).

Autophosphorylation at the tyrosine in the activation loop of Lck, another member of the Src family, is accompanied by a structural rearrangement involving several active site residues leading to an "open" active site.[7] A similar conformational rearrangement at the active site of Src upon autophosphorylation has also been suggested.[2] Even though detailed kinetic studies on all the analogues are still incomplete, a preliminary study indicated that the mode of inhibition of **2** toward substrate phosphorylation of Src is substrate competitive, not ATP competitive.[3] Taken together, it is conceivable that the different inhibitory effects of **7** and **13** observed in this study are due to the different local structures of Src involved in autophosphorylation and substrate phosphorylation. To our knowledge, this is the first report of a series of analogues with different inhibitory effects on autophosphorylation and substrate phosphorylation of Src. These results should be useful in elucidating the mechanisms and physiological roles of two phosphorylation reactions catalyzed by Src and other Src family member PTKs.

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