Pergamon

Bioorganic & Medicinal Chemistry Letters 10 (2000) 669–672

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Inhibition of the Ras-Dependent Mitogenic Pathway by Phosphopeptide Prodrugs with Antiproliferative Properties

Wang-Qing Liu,[a] Michel Vidal,[a] Christophe Mathé,[b] Christian Périgaud[b] and Christiane Garbay[a,*]

[a]*Laboratoire de Pharmacochimie Moléculaire et Structurale, U266 INSERM, UMR 8600 CNRS, Faculté de Pharmacie, 4 Avenue de l'Observatoire, 75270 Paris cedex 06, France*

[b]*Laboratoire de Chimie Bioorganique, cc008, Université Montpellier II, 34095 Montpellier cedex 5, France*

Received 7 September 1999; accepted 3 February 2000

Abstract—Phosphopeptide prodrugs bearing two *S*-acyl-2-thioethyl (SATE) biolabile phosphate protections were developed. They are capable to inhibit the Shc/Grb2 interaction and MAP kinases (ERK1 and ERK2) phosphorylation in cellular assay. The *S*-acetyl-2-thioethyl (MeSATE) analogue showed an IC_{50} of 1 μM in the inhibition of the colony formation of tumor cell line NIH3T3/HER2.

Introduction

The deregulation of receptor or intracellular tyrosine kinase activity coupled to Ras activation via Grb2[1,2] has been involved in a number of carcinoma development, such as breast and ovarian cancer and leukemia.[3–5] Many chemical inhibitors have been designed to prevent tyrosine kinases activation and consequently intracellular transduction pathways.[6] Another effort has been focused on interrupting the signaling cascade at the level of protein–protein interactions, such as those involving SH2 or SH3 domains.[7] Numerous synthetic phosphopeptides corresponding to the SH2 domain binding sites able to specifically block in vitro the interaction of phosphorylated proteins with SH2-containing protein targets have been reported.[8–10] Moreover, several protein tyrosine phosphatases resistant phosphotyrosine mimics have been developed with promising consequences,[7] especially 4-phosphonomethylphenylalanine (Pmp)[11] and 4-phosphonodifluoromethylphenylalanine (F_2Pmp).[12] However, these phospho- and phosphono peptides are unable to penetrate the bilayer cell membrane due to the negative charge of phosphate or phosphonate groups at physiological pH. This problem has been overcome in biological studies by microinjection[13] or electroporation[14] of the phosphopeptides into cells or by coupling the phosphopeptides with cell-permeable peptide sequences.[15,16] However, these approaches provide little encouragement for ultimate therapeutical usefulness.

We have shown previously that mononucleoside phosphotriester derivatives, bearing *S*-acyl-2-thioethyl (SATE) groups were able to deliver the corresponding monophosphate inside cells through an esterase-mediated activation process.[17,18] This result prompted us to circumvent the poor cellular permeability of phosphopeptides by the use of this mononucleotide prodrug approach.

In the present work, we describe the design of a phosphopeptide prodrug, with *O*-phosphate protected by two SATE groups, namely *S*-pivaloyl-2-thioethyl groups (*t*BuSATE) and *S*-acetyl-2-thioethyl groups (MeSATE). The phosphopeptide, derived from Shc317, was optimized to have minimum side chain functionalized residues still retaining high binding affinity for the SH2 domain of Grb2. The in vivo capacities of phosphopeptide prodrugs in inhibiting the interaction between Shc and Grb2, and the phosphorylation of MAP kinases (ERK1 and ERK2), in ER22 cells stimulated by EGF are reported. We present also the effects of these two phosphopeptide prodrugs in inhibiting the colony formation in tumor cell line NIH3T3/HER2.

Chemistry

Synthesis of peptides listed in Table 1 was carried out by solid phase peptide synthesis using Fmoc strategy. The

*Corresponding author. Tel.: +33-1-53-73-95-60; fax: +33-1-43-26-69-18; e-mail: garbay@pharmacie.univ-paris5.fr

0960-894X/00/$ - see front matter
PII: S0960-894X(00)00077-9

Table 1. Affinity of phospho- and phosphonopeptides for Grb2 measured by fluorescence

No.	Sequence	K_d (nM)[a]
1	Pro-Ser-*p*Tyr-Val-Asn-Val-Gln-Asn (Shc317)	18 ± 2
2	Pro-Ser-Pmp-Val-Asn-Val-Gln-Asn	260 ± 10
3	Pro-Ser-F_2Pmp-Val-Asn-Val-Gln-Asn	200 ± 15
4	Ac-Pro-Phe-*p*Tyr-Val-Asn-Val-Pro-NH_2	30 ± 5
5	Ac-Pro-Phe-*p*Tyr(tBuSATE)$_2$-Val-Asn-Val-Pro-NH_2	Non-detectable
6	Ac-Pro-Phe-*p*Tyr(MeSATE)$_2$-Val-Asn-Val-Pro-NH_2	Non-detectable

[a]Fluorescence variations of Grb2 after binding with peptides were recorded on a Perkin–Elmer fluorimeter at 25 °C as described in Cussac et al.[27] The excitation was at 292 nm (bandwidth 5 nm) and emission was recorded at 345 nm (bandwith 5 nm). The dissociation constants K_d were determined by the Michaelis–Menten type curve fitting equation described in Cussac et al.[27] using 'enzfit' software.

Fmoc protected Pmp and F_2Pmp were prepared according to our published methods.[19,20] Fmoc-*p*Tyr-(*t*BuSATE)$_2$-OH and Fmoc-pTyr(MeSATE)$_2$-OH were obtained following the method developed by Mathé et al.[21] Except for the prodrugs **5** and **6,** peptides were synthesized by classical DCC/HOBt coupling method or using BOP/HOBt/DIEA coupling.[22,23]

The SATE groups appear to be mono-deprotected by 20% piperidine, conditions for Fmoc deprotection in solid phase peptide synthesis, but they remained in 2% 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU) in CH_2Cl_2, conditions used to remove Fmoc group. Moreover, these biolabile phosphate protecting groups are also sensitive to solutions containing more than 50% TFA, which are absolutely necessary for removing certain amino acid side-chain protecting groups such as trityl group for amide protection. Therefore, the Asn residue was coupled by active ester method using Fmoc-Asn-OPfp to minimize its side-chain dehydration.[24] Phosphotyrosine protected by *t*BuSATE or MeSATE has been introduced through the efficient coupling agent *O*-(7-azobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphate (HATU),[25] in the presence of DIEA due to the very small quantity available for the synthesis. In fact, in place of 10 equiv of amino acid generally used, only 2 equiv of Fmoc-pTyr(RSATE)$_2$-OH were used.

Starting from the acid sensitive Siber amide resin,[26] following stepwise coupling/deprotection cycles, the expected phosphopeptide prodrugs were obtained after cleaving the peptidyl resin with 2% TFA. About 20% of peptide with amide side chain of Asn dehydrated to nitrile were isolated.

Results and Discussion

Design of the phosphopeptide prodrugs

As we mentioned above, phosphonopeptides are phosphatases resistant analogues of phosphopeptides. Nevertheless, the phosphonopeptides of Shc Y317 sequence containing Pmp or F_2Pmp showed about 15-fold lower affinities (peptide **2**, $K_d = 260$ nM; peptide **3**, $K_d = 200$ nM) than the phosphopeptide (peptide **1**, $K_d = 18$ nM), although in some cases the molecules containing F_2Pmp and even Pmp have similar affinities for SH2 domains as the corresponding *p*Tyr-containing molecule.[28,29]

Thus, a phosphopeptide was then selected as peptide model to apply a prodrug strategy. For attaining best cellular uptake and to facilitate the peptide synthesis, it is clear that the peptide prodrug should contain less possible polar functional groups. Therefore, we have optimized the residues surrounding *p*Tyr (results not shown) to avoid side-chain functional groups. The optimized peptide **4** ($K_d = 30$ nM), with modified N- and C-terminal functions and containing only one side chain functionalized residue Asn (which is essential for the recognition of the SH2 domain of Grb2), was taken as parent peptide of prodrug.

The *O*-tyrosinyl phosphate function of this peptide was protected by two different SATE groups, namely *t*Bu-SATE and MeSATE, in order to evaluate the influence of their lipophilicity, their kinetics of decomposition and the biological activity of the corresponding phosphopeptide prodrugs **5** and **6** respectively. As expected, phosphopeptide prodrugs **5** and **6** showed no affinity for Grb2 by fluorescence measurement with no visible increase in Grb2 fluorescence intensity.

Cellular test of phosphopeptide prodrugs 5 and 6

The new phosphopeptide prodrugs **5** and **6** were tested on ER22 cells (Hamster fibroblasts overexpressing human EGF receptor) for their capacity to inhibit both Shc/Grb2 interaction and MAP kinases phosphorylation (Fig. 1).

Cells were grown to confluence, blocked in G0 phase and incubated with the tested peptide overnight. Cells were then EGF-stimulated for 5 min and lysed. Grb2 complexes were pulled-down using sepharose beads coupled to VPPPVPPRRR peptide, a ligand of Grb2-SH3 domains, and the precipitated proteins were then analysed through western blot.[30] While Grb2 amounts were constant (data not shown), the three bands corresponding to the different isoforms of Shc decreased in a dose dependent manner in presence of the phosphopeptide prodrugs **5** and **6** in the cellular medium as shown in Figure 1.

The two prodrugs were also able to inhibit the MAP kinases ERK1 and ERK2 phosphorylation consequent to the EGF stimulation (Fig. 2).

Differences were found in the inhibitory activities of the tested compounds. Under the assays conditions, the MeSATE phosphopeptide prodrug **6** emerged as the most potent inhibitor of both Shc/Grb2 association and MAP kinases activation. Moreover, in contrast to *t*BuSATE derivative **5**, the prodrug **6** inhibits efficiently the cloning on soft agar of NIH3T3 cells transfected by the oncogene HER2 (Fig. 3), with an IC_{50} around 1 μM.

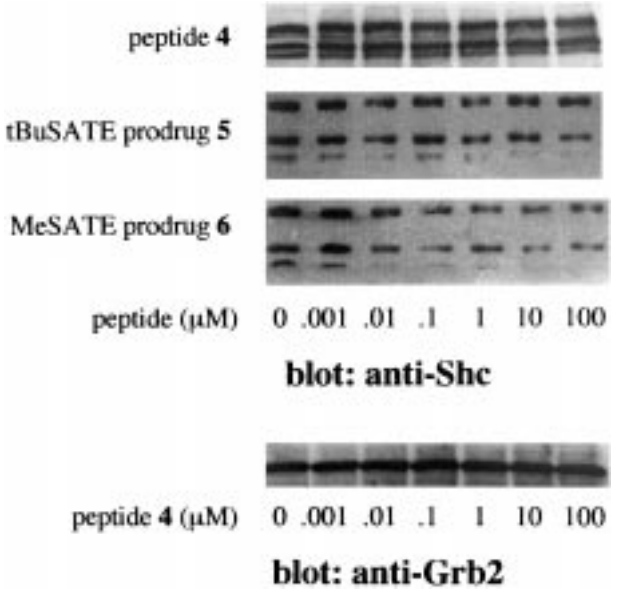

Figure 1. Inhibition of Grb2/Shc interaction by Ac-PFpYVNVP-NH2 alkyl-SATE prodrugs on ER22 cells. Confluent cells in G0 phase were incubated with the drugs overnight, then stimulated with EGF (30 ng/mL) 5 min and lysed. Grb2 was precipitated with VPPPVPPRRR sepharose beads and, after degradation, electrophoresis and transfer, western blot was made using Shc antibody. While MeSATE prodrug **6** is clearly more efficient than tBuSATE derivative **5,** peptide **4** does not inhibit Grb2/Shc interactions. Only the anti-Grb2 blot with peptide **4** is shown. Prodrugs **5** and **6** gave the same result and clearly showed a constant rate of Grb2 (data not shown).

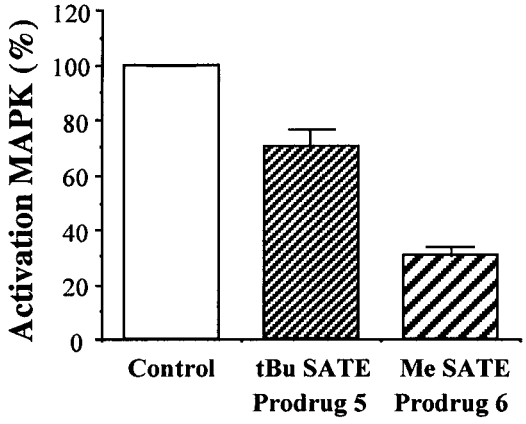

Figure 2. Inhibition of the MAPKinases ERK1 and ERK2 activation on ER22 cells treated by Ac-PFpYVNVP-NH2 alkyl-SATE prodrugs. Confluent cells in G0 phase were incubated with 10 μM of each peptide (tBuSATE and MeSATE) or buffer alone (control) overnight, then stimulated with EGF (30 ng/mL) 5 min and lysed. Western blot using anti-active MAP kinase antibody was made on 10 μg cellular extracts treated or not with prodrugs (histogram shows mean values of each prodrug effect on ERK1 and ERK2 activation). MeSATE (prodrug **6**) is clearly more efficient than tBuSATE (prodrug **5**) for inhibiting ERK1 and ERK2 phosphorylation. Peptide **4** gave same result as the control (data not shown).

The present results demonstrate that the SATE phosphopeptide prodrugs **5** and **6** allow the efficient intracellular delivery of biologically active phosphopeptide **4**, circumventing its low membrane permeability. According to the proposed decomposition pathways of SATE phosphopeptide prodrugs,[21] differences observed

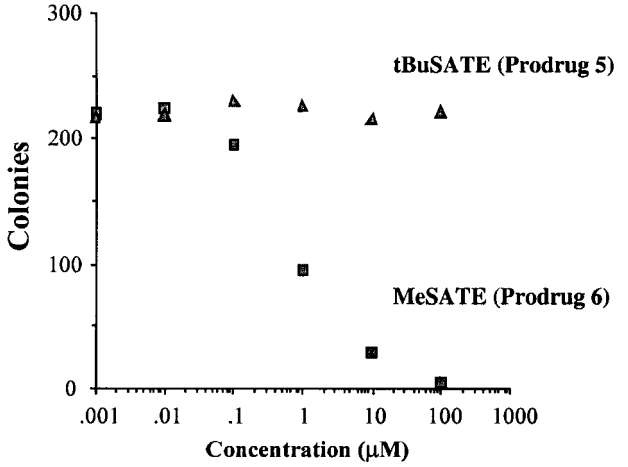

Figure 3. Inhibition of NIH3T3/HER2 cells cloning efficiency by Ac-PFpYVNVP-NH2 alkyl-SATE prodrugs. NIH3T3/HER2 cells were grown on soft agar medium with peptide prodrugs and colonies were counted after 3–4 weeks. While tBuSATE (prodrug **5**) was not really efficient, MeSATE (prodrug **6**) was able to inhibit the cells cloning efficiency with an $IC_{50} < 1$ μM.

between the cellular evaluation of the two studied SATE prodrugs, which differ by a variation of the alkyl chain in the immediate vicinity of the thiol ester functionality, may be explained by their decomposition kinetics. Indeed, we have previously demonstrated, through the study of mononucleoside bis(SATE)phosphotriester derivatives,[17] that a sterically hindered environment around the carboxyl group of the SATE protection increases the enzymatic stability (esterase-depending) of the corresponding prodrugs. Compared to the MeSATE analogue **6**, the *t*BuSATE phosphopeptide prodrug **5** could lead to a slow delivery of the parent phosphopeptide **4** inside cells and, due to the simultaneous activity of cellular phosphatases and peptidases, 'sufficient' intracellular concentrations of the drug could not be reached in order to inhibit the growing of NIH3T3/HER2 tumor cells. Furthermore, an efficient inhibition of Ras signaling pathway may need a fast intracellular accumulation of the phosphopeptide in the early events of cell proliferation. Thus, a close correlation between the kinetic release of the phosphorylated derivative from its prodrug and the expression of targeted cellular process may be a critical factor in the design of such phosphopeptide prodrugs. In this respect, it was already suggested that once bound to the SH2 domain, the phosphopeptide is less susceptible to dephosphorylation by phosphatases.[31]

It is interesting to note, that a F_2Pmp-containing phosphonopeptide prodrug with one pivaloxymethyl (POM) protecting group has been reported and demonstrates good cellular delivery and reconversion rate.[32] Therefore, the prodrug strategy can be applied both for phospho and phosphonopeptides.

In conclusion, we clearly show herein the possibility to target an SH2 domain using phosphopeptide prodrugs which incorporate SATE biolabile phosphate protections. The *t*BuSATE and MeSATE phosphopeptide

derivatives **5** and **6** are able to enter cells and are hydrolyzed to the parent phosphopeptide **4**. The MeSATE protection appears to be more efficient, particularly on NIH3T3/HER2 cloning inhibition.

Acknowledgements

We thank B. P. Roques and J.-L. Imbach for their scientific advice, D. Cussac for his help in fluorescence measurement, C. Dupuis for expert drafting of the manuscript and the Ligue National contre le Cancer (Comité de Paris) for financial support.

References

1. Chardin, P.; Cussac, D.; Maignan, S.; Ducruix, A. *FEBS Lett*. **1995**, *369*, 47.
2. Lowenstein, E. J.; Daly, R. J.; Bazer, A. G.; Li, W.; Margolis, B.; Lammers, R.; Ullrich, A.; Skolnik, E. Y.; Bar-Sagi, D.; Schlessinger, J. *Cell* **1992**, *70*, 431.
3. Pandey, P.; Kharbanda, S.; Kufe, D. *Cancer Res*. **1995**, *55*, 4000.
4. Gishizky, M. *Ann. Reports Med. Chem*. **1995**, *30*, 247.
5. Tauchi, T.; Boswell, H. S.; Broxmeyer, H. E. *J. Exp. Med*. **1994**, *179*, 167.
6. Boutin, J. A. *Int. J. Biochem*. **1994**, *26*, 1203.
7. Bottfield, M. C.; Green, J. *Ann. Reports Med. Chem*. **1995**, *30*, 227.
8. Escobedo, J. A.; Kaplan, D. R.; Kavanaugh, W. M.; Turck, C. W.; Williams, L. T. *Mol. Cell. Biol*. **1991**, *11*, 1125.
9. Klippel, A.; Escobedo, J. A.; Fantl, W. J.; Williams, L. T. *Mol. Cell. Biol*. **1992**, *12*, 1451.
10. Fantl, W. J.; Escobedo, J. A.; Martin, G. A.; Turck, C. W.; del Rosario, M.; McCormick, L. T.; Williams, L. T. *Cell* **1992**, *69*, 413.
11. Marseigne, I.; Roques, B. P. *J. Org. Chem*. **1988**, *53*, 3621.
12. Burke, T. R., Jr.; Smyth, M. S.; Nomizu, M.; Otaka, A.; Roller, P. P. *J. Org. Chem*. **1993**, *58*, 1336.
13. Xiao, S.; Rose, D. W.; Sasaoka, T.; Maegawa, H.; Burke, T. R., Jr.; Roller, P. P.; Shoelson, S. E.; Olefsky, J. M. *J. Biol. Chem*. **1994**, *269*, 21244.
14. Giorgetti-Peraldi, S.; Ottinger, E.; Wolf, G.; Ye, B.; Burke, T. R., Jr.; Shoelson, S. E. *Mol. Cell. Biol*. **1997**, *17*, 1180.
15. Mauricio, R.; Yao, S.; Lin, Y.-Z. *J. Biol. Chem*. **1996**, *271*, 27456.
16. Hall, H.; Williams, E. J.; Moore, S. E.; Walsh, F. S.; Prochiantz, A.; Doherty, P. *Curr. Biol*. **1996**, *6*, 580.
17. Lefebvre, I.; Périgaud, C.; Pompon, A.; Aubertin, A.-M.; Girardet, J.-L.; Kirn, A.; Gosselin, G.; Imbach, J.-L. *J. Med. Chem*. **1995**, *38*, 3941.
18. Périgaud, C.; Gosselin, G.; Imbach, J.-L. In *Current Topics in Medicinal Chemistry*, Alexander, J. C., Ed.; 1997, Vol. 2, pp 15–29.
19. Liu, W.-Q.; Roques, B. P.; Garbay-Jaureguiberry, C. *Tetrahedron: Asymmetry* **1995**, *6*, 647.
20. Liu, W.-Q.; Roques, B. P.; Garbay, C. *Tetrahedron Lett*. **1997**, *38*, 1389.
21. Mathé, C.; Périgaud, C.; Gosselin, G.; Imbach, J.-L. *J. Org. Chem*. **1998**, *63*, 8547.
22. Ottinger, C. A.; Shekel, L. L.; Bernlohr, D. A.; Barany, G. *Biochemistry* **1993**, *32*, 4354.
23. Gordeev, M. F.; Patel, D. V.; Barker, P. L.; Gordon, E. M. *Tetrahedron Lett*. **1994**, *35*, 7585.
24. Gausepohl, H.; Kraft, M.; Frank, R. Peptides 1988, In *Proc. 20th European Peptide Symposium*, Jung, G.; Bayor, E., Eds; 4–9 September 1988; University of Tübingen, Germany, 1989, pp 241–243.
25. Carpino, L. A. *J. Am. Chem. Soc*. **1993**, *115*, 4397.
26. Siber, P. *Tetrahedron Lett*. **1987**, *28*, 2107.
27. Cussac, D.; Frech, M.; Chardin, P. *EMBO J*. **1994**, *13*, 4011.
28. Burke, Jr., T. R.; Smyth, M. S.; Otaka, A.; Nomizu, M.; Roller, P. P.; Wolf, G.; Case, R.; Shoelson, S. E. *Biochemistry* **1994**, *33*, 6490.
29. Chatterjee, S.; Goldstein, B. J.; Csermely, P.; Shoelson, S. E. In Peptides: Chemistry and Biology. Proc. 12th American Peptide Symposium, Rivers, J. E., Ed.; Cambridge, MA, USA, 16–21 June 1991, pp 553–555.
30. Vidal, M.; Montiel, J. L.; Cussac, D.; Cornille, F.; Duchesne, M.; Parker, F.; Tocqué, B.; Roques, B. P.; Garbay, C. *J. Biol. Chem*. **1998**, *268*, 5343.
31. Brunati, A. M.; Pinna, L. A.; Bergantino, E.; Ruzzene, M.; Cirri, P.; Ramponi, G.; Donella-Deana, A. *Biochem. Biophys. Res. Commun*. **1998**, *243*, 700.
32. Stankovic, C. J.; Surendran, N.; Lunney, E. A.; Plummer, M. S.; Para, K. S.; Shahripour, A.; Fergus, J. H.; Marks, J. S.; Herrera, R.; Hubbell, S. E.; Humblet, C.; Saltiel, A. R.; Stewart, B. H.; Sawyer, T. K. *Bioorg. Med. Chem. Lett*. **1997**, *7*, 1909.