SYNTHESIS OF PROTEIN COMPLEXES OF BIS-INDOLE ALKALOIDS FROM *CATHARANTHUS ROSEUS*

Mauri Lounasmaa* and Reija Jokela

Department of Chemistry, Technical University of Helsinki,
SF-02150 Espoo, Finland

Aarre Huhtikangas, Seppo Lapinjoki, Jukka Heiskanen, Timo Lehtola,
and Toivo Naaranlahti

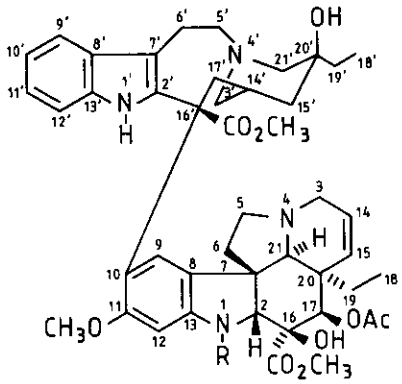
Department of Pharmaceutical Chemistry, University of Kuopio,
POB 6, SF-70211 Kuopio, Finland

Abstract - Conjugation of vincristine (VCR) 1 and vinblastine (VBL) 2 to proteins through the C-10' position of the "catharanthine moiety" is described. The procedure was applied to the production of immunogenic complexes of the drugs and bovine serum albumin (BSA), and to the synthesis of an enzyme-linked tracer from VCR 1 and alkaline phosphatase.

INTRODUCTION

Vincristine (VCR) 1 and vinblastine (VBL) 2 are highly effective antineoplastic alkaloids from the Madagascan plant Catharanthus roseus (L.) G. Don (Apocynaceae).[1-4] These bis-indole alkaloids, containing a "catharanthine moiety" and a "vindoline moiety",[5] are currently used in clinical practice in more or less standard dosages, with no account taken of possible variations in drug plasma levels of individual patients. The extremely low serum concentrations associated with these cytotoxic substances in cancer treatment make it difficult to monitore the serum levels by conventional methods of clinical analysis. Currently, immunoassay methods (RIA, IRMA, ELISA, etc.), which can boast high sensitivity and selectivity in combination with procedural simplicity, are offering a powerful means to accurate and reliable monitoring of samples at femtomole level of drug molecules.

Successful immunochemical assay of VCR 1 and VBL 2 rests upon the production of antibodies against them. Three methods of synthesizing the immunogenic protein complexes needed to produce such antibodies have been described.[6-8] One of the methods has also been applied to produce an enzyme-linked immunotracer,[9] and another to synthesize immunotherapeutic complexes by coupling



"catharanthine moiety"

"vindoline moiety"

1 R = CHO
2 R = CH_3

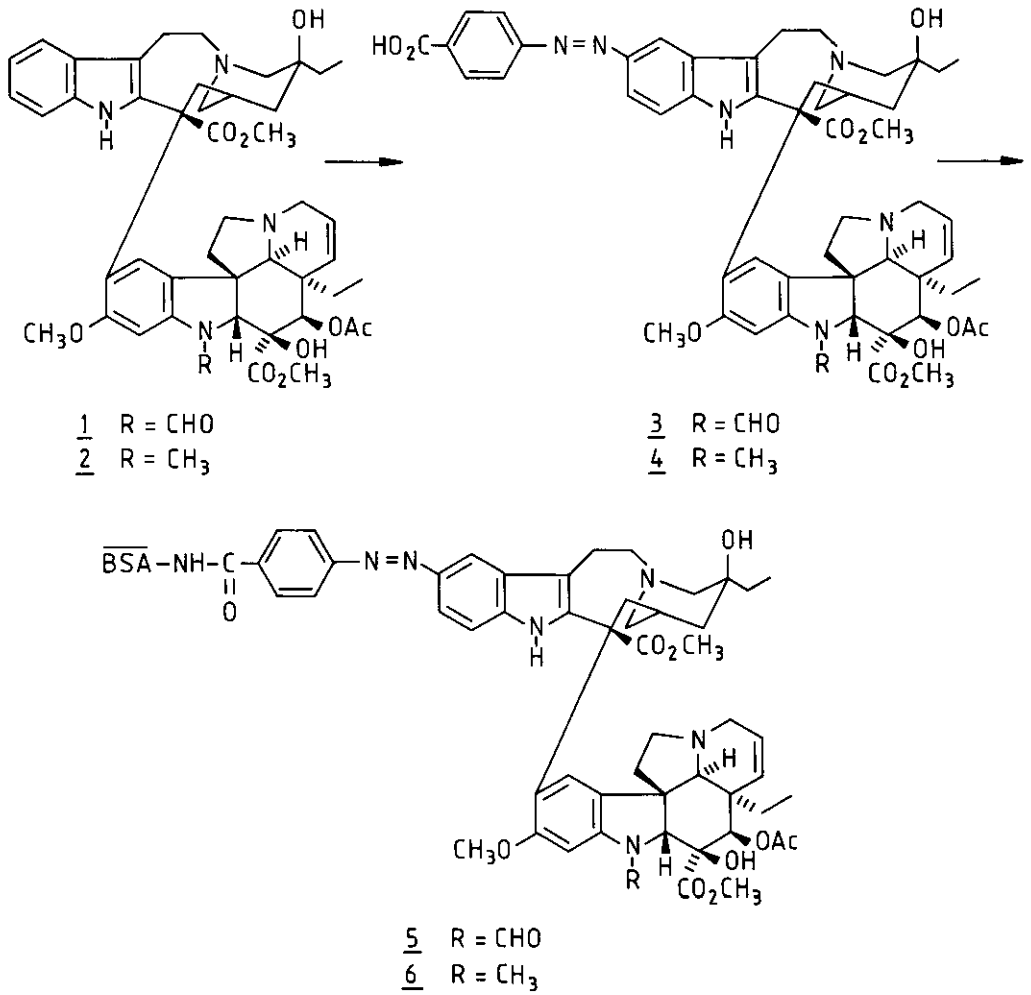
with antibodies produced against cancer cells.[10] However, all three methods have yielded antibodies with only limited specificity against the compound involved in the immunogen synthesis. Evidently, the protein in the final complex partially "covers" the "vindoline moiety", which contains the distinctive features of the molecules.

RESULTS AND DISCUSSION

We present a new method for synthesizing immunogenic protein complexes, based on the diazo-coupling between an aromatic ring of the bis-indole alkaloid and diazotized p-aminobenzoic acid and reaction of the formed azo compound with bovine serum albumin (BSA) (Scheme 1).[11]

Both the "catharanthine moiety" and "vindoline moiety" of VCR 1 and 2 contain an aromatic ring. To take advantage of the activating effect of the indolic nitrogen, the coupling preferentially should take place at the C-10' and/or C-10 position,[12] which is para to the indolic nitrogen. At the C-10' position of the "catharanthine moieties" there is little or no steric hindrance, whereas in the "vindoline moieties" the C-10 position is substituted. Moreover, in the case of VCR 1 the presence of the formyl group strongly diminishes the activating effect at the C-10 position. Therefore, a highly uniform reaction can be expected at the C-10' position of the "catharanthine moieties" when the coupling is performed under the controlled reaction conditions used in the diazo-coupling of aromatic amines (vide infra).[13-15]

For the synthesis of immunogenic protein complexes described here, the pH value of the diazo-coupling solution must be maintained between 4.0 and 7.0 in order to produce uniform intermediates (3 and 4),[16] essential for the specificity of the immunoanalytical methods envisaged.[17]



Scheme 1
$\overline{BSA}$ stands for "BSA less an amino group".

Comparison of the absorption spectra of the synthesized BSA conjugates with those of VCR 1 sulfate, VBL 2 sulfate, and free BSA (Fig. 1), are in good agreement (λ_{max} at ~ 350 nm) with the presumed reaction products 5 and 6 outlined in Scheme 1.

For binding, our method uses the C-10' position of the "catharanthine moiety", far from the groups of the "vindoline moiety", that are functionally important in physiological interactions.[10] Moreover, the p-aminobenzoic acid acts as a spacer, separating the alkaloidal part from the macromolecule. These features resulted in the case of the VCR-BSA conjugate 5 in the production of an antiserum about 1500-fold more sensitive to VCR than to VBL.[18] Previous methods[6-8] have led to antisera of only moderate specificity: the best sensitivity difference achieved between VCR 1 and VBL 2 was 200-fold,[8] while in the other two methods only a slight

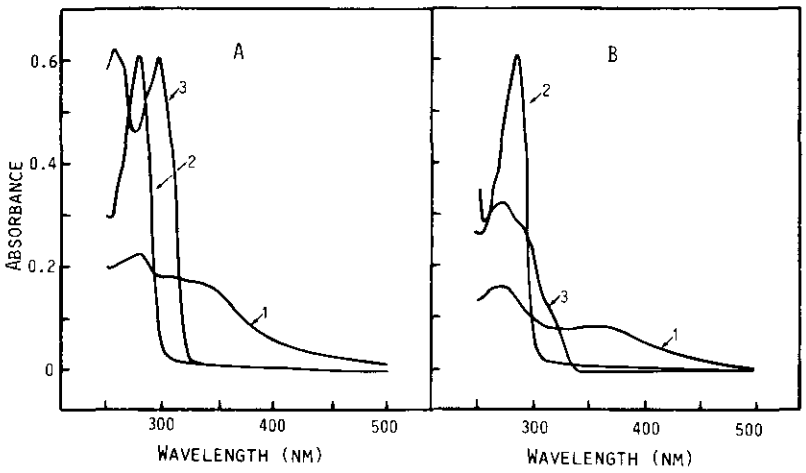


Fig. 1. Absorption spectra of the VCR-BSA conjugate 5 and the VBL-BSA conjugate 6 compared with those of free BSA, VCR 1 sulfate and VBL 2 sulfate.
Part A: 1 VCR-BSA conjugate 5 (~150 mg/l), 2 free BSA (1 g/l), 3 VCR 1 sulfate (40 mg/l).
Part B: 1 VBL-BSA conjugate 6 (~50 mg/l), 2 free BSA (1 g/l), 3 VBL 2 sulfate (40 mg/l).
All the spectra were recorded in H_2O.

difference and practically no difference could be observed between the binding of these closely related ligands. We also applied the present method to the preparation of an enzyme-linked tracer from VCR 1 and alkaline phosphatase with practically no loss of enzyme activity.[18] Thus the reaction conditions used are not denaturing to protein and can be widely used for preparing conjugates for different purposes. The present procedure with its new features may be particularly suitable for the synthesis of immunotherapeutic molecules from Catharanthus alkaloids and of antibodies against cancer cells, in the manner described recently[10] using one of the older conjugation methods.

EXPERIMENTAL

The absorption spectra were recorded with a Hitachi 220 spectrophotometer and the HPLC analysis was executed with a Beckman 420 HPLC apparatus ($MeOH/H_2O$; 72/25; 0.25 % Et_3N; 0.2 ml/min; C_{18} Altex Ultrasphere ODS).

Vincristine (VCR) sulfate, vinblastine (VBL) sulfate and BSA were from Sigma Chemical Co., St. Louis, USA, and the alkaline phosphatase from Boehringer Mannheim GmbH, Mannheim, FRG.

Conjugation of VCR 1 (or VBL 2) to proteins

p-Aminobenzoic acid (1 mg) was dissolved in 0.5 ml of 0.2 M HCl, into which sodium nitrite (1 mg in 0.5 ml of water) was added dropwise. The resulting solution was kept at $0^{o}C$ for 45 min with occasional gentle stirring, after which sulfamic acid solution (1 mg in 0.5 ml of water) was added dropwise. VCR 1 sulfate (or VBL 2 sulfate) (3 mg) was dissolved in a minimum amount of a mixture of DMF and 0.1 M sodium borate buffer (1/1, vol/vol), and the diazotized p-aminobenzoic acid solution was added dropwise. The pH was maintained between 4.0 and 7.0 with additions of 0.1 M sodium borate. Formation of the azo bond was indicated by the appearance of yellow colour. The reaction was allowed to proceed for 3 to 4 h at $4^{o}C$ in darkness. (HPLC-analysis of the reaction mixture of VCR 1 indicated that about 90% of VCR 1 was converted to a uniform, acidic derivative). Thereafter the protein (2 mg of BSA) was added to the solution where the pH was about 6.0. After addition of EDC [1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide] (4 mg) dissolved in a minimum amount of water, the reaction was allowed to proceed overnight at $4^{o}C$. The final product was separated from low molecular weight reactants by ultrafiltration (Amicon PM 30 membrane) and lyophilized.

Preparation of an enzyme-linked tracer from VCR 1 and alkaline phosphatase

The procedure was identical with that described above except that, instead of BSA (2 mg), alkaline phosphatase (3 mg) was added.

ACKNOWLEDGEMENTS

The financial support from the Technology Development Centre (Helsinki, Finland) and from Huhtamäki Oy Leiras Pharmaceuticals (Turku, Finland) is gratefully acknowledged.

REFERENCES AND NOTES

1. E.g., W.I. Taylor and N.R. Farnsworth, Eds., The Catharanthus Alkaloids: Botany, Chemistry, Pharmacology and Clinical Uses, Marcel Dekker, New York, 1975.
2. G.A. Cordell and J.E. Saxton, in The Alkaloids Chemistry and Physiology, Ed. R.G.A. Rodrigo, Vol. 20, Academic Press, New York, 1981, p. 192.
3. G.A. Cordell, in The Monoterpenoid Indole Alkaloids, Ed. J.E. Saxton, John Wiley, New York, 1983, p. 539.
4. F. Guéritte, A. Pouilhès, P. Mangeney, R.Z. Andriamialisoa, N. Langlois, Y. Langlois and P. Potier, Eur. J. Med. Chem. - Chim. Thér., 1983, 18, 419.

5. Since catharanthine and vindoline[19] are direct biogenetic precursors of VCR 1 and VBL 2,[20-22] we call the two indolic parts present simply the "catharanthine moiety" and the "vindoline moiety", although the vindoline part as such is present only in VBL 2 and the catharanthine part as such is not present in either VCR 1 or VBL 2.

6. M.A. Root, K. Gerzon and R.W. Dyke, Proceedings of the 2nd National Meeting, Federation of Analytical Chemistry and Spectroscopy Societies, Indianapolis, 1975, p. 125. See also Ref. 17.

7. J.D. Teale, J.M. Clough and V. Marks, Br. J. Clin. Pharmac., 1977, 4, 169.

8. J.J. Langone, M.R. D'Onofrio and H. van Vunakis, Anal. Biochem., 1979, 95, 214.

9. M.P. Hacker, J.R. Dank, W.B. Ershler, Cancer Res., 1984, 44, 478.

10. G.F. Rowland, C.A. Axton, R.W. Baldwin, J.P. Brown, J.R.F. Corvalan, M.J. Embleton, V.A. Gore, I. Hellström, K.E. Hellström, E. Jacobs, C.H. Marsden, M.V. Pimm, R.G. Simmonds and W. Smith, Cancer Immunol. Immunother., 1985, 19, 1.

11. Although Scheme 1 presents the reaction between one VCR (or VBL) intermediate 3 (or 4) and one BSA unit, it is, of course, quite evident, that several VCR (or VBL) intermediates are attached to the same BSA unit.

12. The biogenetic numbering, J. LeMen and W.I. Taylor, Experientia, 1965, 18, 173.

13. H. Zollinger, Azo and Diazo Chemistry, Interscience, New York, 1961.

14. J. March, Advanced Organic Chemistry: Reactions, Mechanisms, and Structures, 2nd ed., McGraw-Hill, New York, 1977.

15. C. Ferri, Reaktionen der organischen Synthese, G. Thieme Verlag, Stuttgart, 1978.

16. The slightly acidic conditions diminish the tendency to react of the indolic amino group, which is formally a secondary aromatic amino group. On the other hand, the protonation of the indolic amino group decreases the reactivity at the C-10' position.

17. C.J. Barnett, G.J. Cullinan, K. Gerzon, R.C. Hoying, W.E. Jones, W.M. Newton, G.A. Poore, R.L. Robinson, M.J Sweeney and G.C. Todd, J. Med. Chem., 1978, 21, 88.

18. S. Lapinjoki, H. Veräjänkorva, A. Huhtikangas, T. Lehtola and M. Lounasmaa, J. Immunoassay (in press); A. Huhtikangas and S. Lapinjoki, Finnish Pat. Appl. no. 852785.

19. M. Lounasmaa and A. Nemes, Tetrahedron, 1982, 38, 223.

20. A.I. Scott, F. Guéritte and S.L. Lee, J. Am. Chem. Soc., 1978, 100, 6523.

21. S.B. Hassam and C.R. Hutchinson, Tetrahedron Lett., 1978, 1681.

22. F. Guéritte, N.V. Bac, Y. Langlois and P. Potier, J. Chem. Soc., Chem. Commun., 1980, 452.

Received, 23rd December, 1985