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The binding of 5-fluorouracil to native and modified human serum albumin: UV, CD, and ^{1}H and ^{19}F NMR investigation

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Abstract

5-Fluorouracil (FU) is an important and widely used antineoplastic drug that is carried in the serum by plasma proteins. Protein binding studies of this drug to human serum albumin (HSA) have been carried out by several spectroscopic techniques. Difference circular dichroism and UV studies provided information on the class of binding sites involved in the interaction. In particular, displacement experiments showed that FU has at least one secondary binding site in the coumarin binding area, but does not interact with the benzodiazepine binding area. Binding was also investigated by difference ^{1}H NMR and by measuring the increase in the ^{19}F NMR signal of FU when bound to HSA. Finally, evidence was obtained that chemical acetylation of Lys_{199} results in a decreased apparent binding affinity constant (nK) for FU. Such a modification is induced under physiological conditions by aspirin.

Keywords: Human serum albumin; Fluorouracil; Protein binding; Protein modification; Circular dichroism; Nuclear magnetic resonance

1. Introduction

5-Fluorouracil (FU) is a pyrimidine analogue with high anti-tumour activity and is one of the most widely used antimetabolites in cancer chemotherapy [1]. Although FU is a relatively successful antineoplastic drug, its major drawback is the extremely rapid liver catabolism. FU is inactivated by pyrimidine ring reduction carried out by dihydrouracil dehydrogenase; the high turnover ($t_{1/2} = 10$–20 min) markedly impairs drug efficiency, thus forcing a high dosage which leads to serious cytotoxical effects [2]. A major direction in the design of new fluorouracil analogues as chemotherapeutic agents is intended to overcome the problem of rapid catabolism [3]. It is generally accepted that plasma protein binding of drugs is a transport mechanism that also acts as a protection against metabolic agents, thus prolonging the duration of drug activity [4]. FU is complexed by human serum albumin (HSA), the most abundant and important carrier in human plasma. Several attempts have been made to increase the HSA affinity of FU, either by FU derivatization [5] or by combined therapy with other drugs known to interact with HSA [6]. However, HSA complex formation of FU is a poorly understood reaction and

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very few details have been reported [7,8]. The study of FU binding to HSA is, therefore, a necessary preliminary step in solving such an important problem.

HSA has at least six well characterized binding sites [9] but only two of these sites, namely coumarin (area I) and benzodiazepine (area II), are responsible for complex formation of most endogenous and exogenous molecules [10,11]. Moreover, owing to protein conformational flexibility, a single binding area can interact with very different structures, whereas a single compound has usually more than one binding site on the protein [12].

In this report, results of a spectroscopic investigation of FU binding to HSA are presented. This study was carried out by means of absorption (UV) and circular dichroism (CD) spectroscopy as well as by ^{1}H and ^{19}F NMR, in parallel. All the experiments involved native and chemically modified human serum albumin (HSA and AcHSA, respectively) in order to obtain better molecular insight into the interaction. HSA modification comprised the acetylation of a single amino acid residue (Lys_{199}, known to be close to area I) selectively induced by acetylsalicylic acid [13].

2. Materials and methods

HSA (essentially fatty acid free) was supplied by Sigma (Milan, Italy) and was used without further purification. Solutions of the protein were prepared in potassium phosphate buffer (pH 7.4; 50 mM) and actual concentrations were determined from the absorbance at 279 nm ($A_{0.1\%,\,1\,cm} = 0.531$) [14].

Fluorouracil (5-fluoro-2,4-(1*H*, 3*H*)-pyrimidinedione) and deuterium oxide (D_2O) were supplied by Fluka (Milan, Italy). Phenylbutazone (4-butyl-1,2-diphenyl-3,5-pyrazolidinedione, PHE) was supplied by Sigma (Milan, Italy). Diazepam (7-chloro-*N*1-methyl-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one, DZP) was kindly provided by Professor A. Lucacchini, (Istituto Policattedra, Facoltà di Farmacia, Università di Pisa, Italy). Acetylsalicylic acid (2-acetyloxybenzoic acid, aspirin) and all inorganic salts were supplied by Carlo Erba (Milan, Italy).

Chemical acetylation of HSA, induced by aspirin, was carried out as reported in the literature [13]. HSA (1 g) was dissolved in sodium phosphate buffer (100 ml, pH 7.4; 100 mM) and a five-fold molar excess of aspirin was added. The solution was stirred at 37 °C for 24 h and then dialyzed successively against sodium phosphate buffer (pH 7.4; 10 mM), ammonium acetate buffer (pH 7.4; 10 mM) and water. The product was lyophilized, and analyzed by electrophoresis and ^{13}C NMR, and UV and CD spectroscopy [15].

UV and CD spectra were measured using a 1 cm quartz cell on a Varian Cary 4E spectrophotometer and a Jasco J600 spectropolarimeter, respectively, both equipped with personal computers for data analysis.

NMR spectra were measured on a Varian VXR300 spectrometer, operating at 300 and 282.2 MHz for ^{1}H and ^{19}F analysis, respectively, at a constant temperature of 25 °C. To obtain difference spectra, preparation of the solutions was as similar as possible with respect to sample volume and concentration. An equal number of transients was collected for each sample (1024), with a relaxation delay of 1 s. For a spectral width of 5000 Hz, 32 K data points were acquired which were zero-filled to 64 K. No weighting functions were applied.

UV and CD titration curves were obtained from a 30 μM HSA solution by adding an increasing amount of FU, and then subtracting HSA (for UV and CD) and FU (for UV) spectra [16].

Displacement studies were carried out by adding FU to a 30 μM 1:1 complex of HSA and the marker (PHE or DZP for area I or II, respectively), and by subtracting the FU complex spectra recorded under the same conditions (see, for example, Ref. [17]).

In all the NMR measurements, D_2O was used instead of H_2O for the preparation of buffers. In difference ^{1}H NMR studies, a 0.3 mM 1:1 complex of HSA and FU was used, and spectra of free FU and either native or acetylated albumin were subtracted from the corresponding complex spectra. Chemical shifts (δ) are reported in parts per million (ppm) with reference to tetramethylsilane (TMS) as external standard.

^{19}F NMR spectra were recorded for several sets of complex solutions. Each set consisted of a fixed concentration of FU (0.3–0.7 mM) and a variable molar ratio of HSA (1:1 to 1:50 with respect to FU). The same procedure was used for AcHSA. A broadening of the ^{19}F NMR signal was measured by increasing the concentration of the protein. Since the linewidth

greatly exceeded the broadening owing to the magnetic field inhomogeneity, no further precautions were taken to assess the transverse relaxation rates.

3. Results and discussion

The UV and CD spectra of a ligand may be drastically altered by binding to a protein, owing to the environmental change with respect to the solvent medium. A very marked effect is often observed in the CD spectrum since proteins are chiral complexing agents and are able to recognize enantiomers or enantiomeric conformations. HSA binding areas I and II show both kinds of stereoselectivity; in several cases they preferentially bind one enantiomer of chiral ligands and induce a specific enantiomeric conformation for achiral molecules, thus contributing to their CD spectrum [12]. Typical examples of these effects are the preferential binding of (S) enantiomers of the chiral drugs warfarin (area I) and oxazepam (area II), and the selective enantiomeric conformation induced for the achiral drugs phenylbutazone (PHE, area I) and diazepam (DZP, area II) [9]. As a result, the CD spectra of bound ligands may be remarkably altered compared to the free ligand CD spectra, or even induced in the case of achiral molecules. Over the last 30 years, CD analysis has become a widely applied method used to investigate HSA/ligand interactions. Usually, a difference CD (ΔCD) technique is convenient; the free protein and free ligand spectra are subtracted from the complex spectrum. The resulting (difference) signal is due to protein/ligand interactions [14–16]. If the tested ligand absorbs at low energies but the protein does not absorb (HSA CD spectrum has no absorption over 280 nm), that part of the difference spectrum may give information about the conformation or configuration of the bound ligand. Several studies have been reported for phenylbutazone [18–20] and diazepam [16,21,22], that show intense induced Cotton effects over 280 nm.

In the last few years, a method has been developed to study protein binding of ligands by parallel difference CD and UV techniques [16]. A difference between the UV absorption of bound and free ligand may arise from a variation in electronic transition energy, i.e. an absorption wavelength shift, due to a different medium polarity. The two coupled techniques are complementary since ΔCD reveals only the stereospecific interactions whereas ΔUV can detect any interaction between protein and ligand.

In Fig. 1, titration curves are reported for FU/HSA complexes; i.e. the maximum ΔUV and ΔCD signals are plotted versus the ligand:protein concentration ratio. It is important to note that FU does not exhibit any absorption peak over 280 nm and even in difference spectra all the peaks (275 nm for ΔCD, 275 nm positive and 250 nm negative for ΔUV) may be due either to FU or to HSA. FU/AcHSA titration did not exhibit any remarkable difference.

The observed difference signals are low compared to PHE or DZP data; for a 30 μM 1:1 HSA/ligand complex, with cell pathlength 1 cm, FU shows a ΔCD maximum value 50 times smaller than that of PHE [18] and 15 times smaller than that of DZP [16], whereas the ΔUV signals are comparable for FU and DZP [16] although one order of magnitude larger for PHE (data not shown). Moreover, both ΔCD and ΔUV titration curves show a non-saturable trend: the slopes begin to decrease at a molar ratio lower than 1:1. This behaviour is different from ΔCD data on ligands known to stereospecifically bind to HSA, such as PHE [18,19] or DZP [16,21], but still resembles the ΔUV titration trend for non-specific diazepam secondary binding sites [16]. However, the interpretation of these results is difficult owing to the low observed signal and to the wavelength analyzed, where albumin could contribute to ΔCD and ΔUV. Nevertheless, these characteristics make fluorouracil a suitable competitor for displacement experiments, using PHE and DZP as ΔCD markers

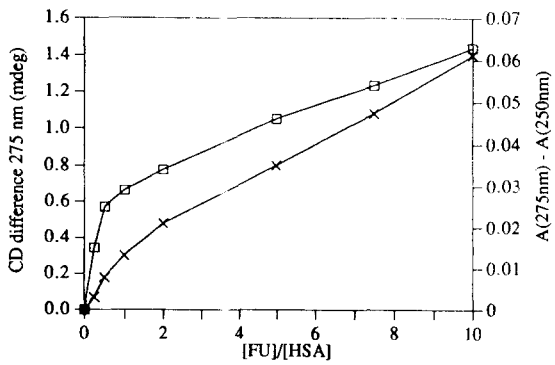

Fig. 1. ΔUV (□) and ΔCD (×) titration of FU/HSA complex. [HSA] = 30 μM in potassium phosphate buffer (pH 7.4; 50 mM), 1-cm pathlength.

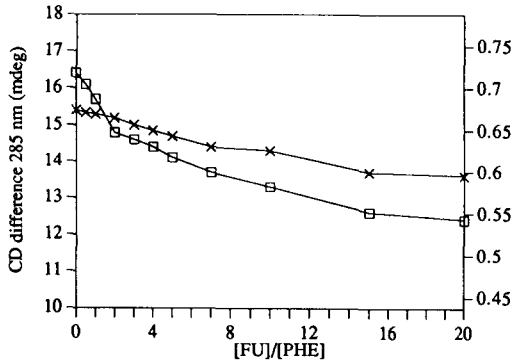

Fig. 2. Displacement of 1/1 HSA/PHE (□) or AcHSA/PHE (×) complexes by FU. [PHE] = 30 μM in potassium phosphate buffer (pH 7.4; 50 mM), 1-cm pathlength.

for areas I and II, respectively. Increasing amounts of FU were added to 1:1 HSA/PHE and AcHSA/PHE complexes (Fig. 2) or to 1:1 HSA/DZP and AcHSA/DZP complexes (Fig. 3), and the protein/FU spectrum at the same concentration was subtracted from the corresponding ternary system.

It appears evident that FU interacts with the stereospecific binding of the albumin/PHE complex, without affecting the albumin/DZP complex. The displacement of PHE is only partial even at a high molar ratio FU:PHE, a typical pattern of indirect competition [23], i.e. the FU binding site does not coincide with the PHE site, but FU interacts with it in an allosteric way or by partial overlapping. In contrast, FU and DZP cobinding to both HSA and AcHSA may be classified as independent [12].

AcHSA/PHE and AcHSA/DZP complexes have a less intense induced Cotton effect than the corresponding HSA complexes [15], but the PHE displacement curve also shows a qualitative difference between native and acetylated

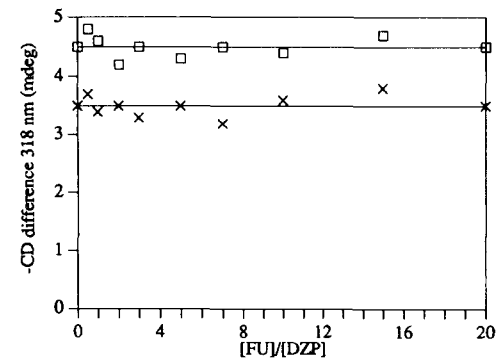

Fig. 3. Displacement of 1:1 HSA/DZP (□) or AcHSA/DZP (×) complexes by FU. [DZP] = 30 μM in potassium phosphate buffer (pH 7.4; 50 mM), 1-cm pathlength.

albumin. It seems likely that FU/PHE competition for AcHSA is much weaker than for native HSA, almost resembling independent binding. The reduced ability of FU to displace PHE following albumin acetylation could be caused by either an enhanced affinity of phenylbutazone [24] or a reduced affinity of fluorouracil. It must be stressed that all the conclusions drawn from CD experiments are confined only to stereospecific binding. Therefore, the interaction between FU and HSA binding area I actually involves only the stereospecific binding site of phenylbutazone, known to be in binding area I.

Another difference spectroscopic method developed some years ago involves proton nuclear magnetic resonance (Δ^1H NMR) [25]. A difference spectrum is obtained by subtracting the free protein and the free ligand ^{1}H NMR spectra from the spectrum of their mixture at the same concentration. Several drugs known to bind to HSA were screened by Oida [25], who recognized three characteristic peak groups related to area I, area II, and other areas of ligand binding, respectively. Oida suggested that specific binding of drugs to HSA brings about a conformational change in the protein which is specifically correlated to the binding site. An analysis using Δ^1H NMR is shown in Fig. 4 for both HSA/FU and AcHSA/FU 1:1 complexes.

None of Oida's Δ^1H NMR spectral patterns (each consisting of 15–20 characteristic peaks) is reproduced by the FU/HSA complex. Nevertheless, it is possible to note several resonances (marked with an asterisk in Fig. 4) in the aromatic (positive at 7.49 and negative at 5.80 ppm), argininic (negative at 2.92 and positive at 2.69 ppm) and aliphatic (positive at 1.21, 1.05 and 0.61 ppm and negative at 1.19 and 0.82 ppm) spectral regions. Many of these resonances are characteristic of binding areas other than I and II, although some of these (2.91, 0.82) may indicate an interaction with area I. From these data, HSA/FU complex formation seems to be a dynamic multiple-site, low-specificity binding rather than a rigid and defined interaction. Acetylated albumin, however, shows less intense peaks, thus suggesting an even less specific interaction. It is interesting that the complex of FU with AcHSA does not induce some of the characteristic peaks for area I observed for HSA, which is in good agreement with the results obtained with competition studies on PHE carried out by CD.

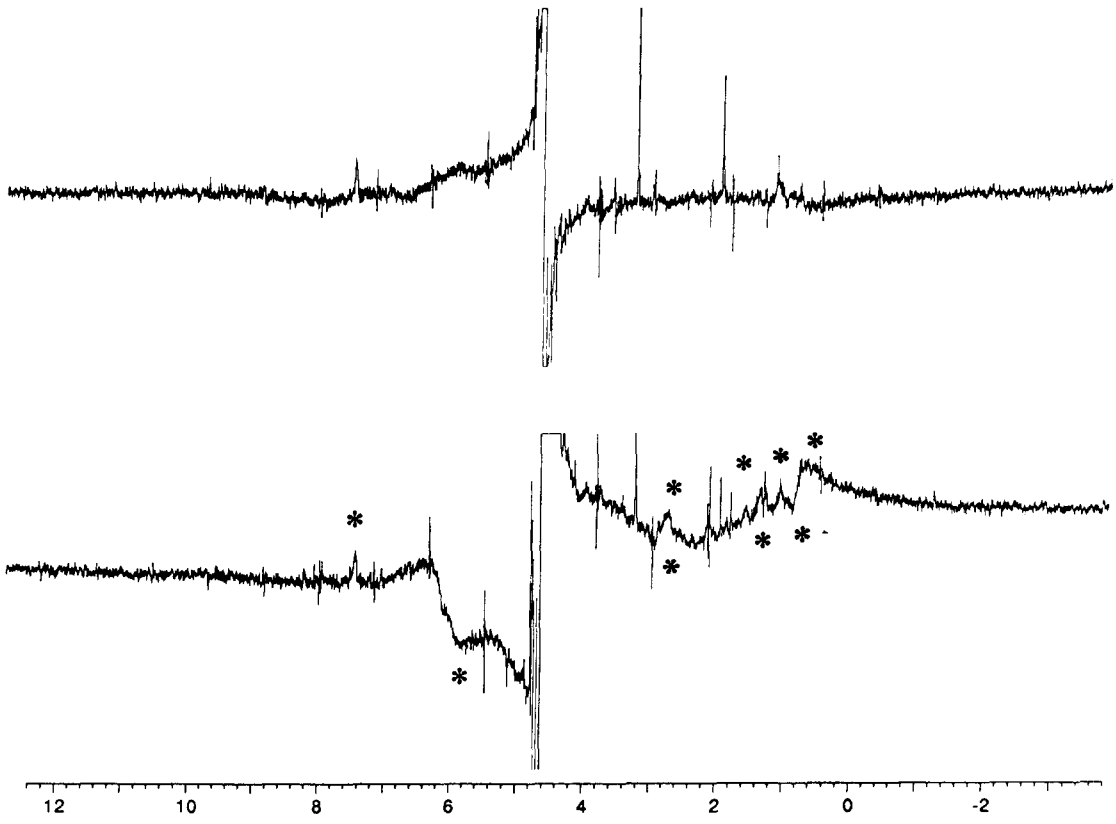

Fig. 4. ^{1}H NMR difference spectra of 1:1 HSA/FU (lower) or AcHSA/FU (upper) complexes, [FU] = 0.3 mM in D_2O/potassium phosphate buffer (pD 7.4; 50 mM), chemical shifts (ppm) with reference to TMS as external standard. Some of the Oida [25] peaks are marked by asterisks.

The interactions of fluorouracil with proteins may also be studied by ^{19}F NMR. This analysis is particularly convenient, since biopolymers do not naturally contain fluorine atoms and thus exhibit no ^{19}F NMR signal. Several NMR parameters of small ligand signals, such as chemical shift and longitudinal and transverse relaxation times, could in principle be related to the binding to a macromolecule. Inspection of ^{19}F NMR spectra of FU/albumin solution at different molar ratios revealed a remarkable broadening of the resonance with increasing protein concentration (Fig. 5).

This effect can be attributed to the exchange of the FU between a free and bound state. In the latter case the drug shares the long correlation time of the protein, behaving as a slow tumbling molecule. Assuming fast exchange between free and bound FU, the observed transverse relaxation rate, R_0, is the average of the parameters for free FU (R_f) and bound FU (R_b), weighted with the molar fraction of the bound FU, x_b:

$$R_0 = R_f(1 - x_b) + R_b x_b$$

The association constant K for n independent and equivalent binding sites can easily be calculated from x_b as

$$K = F(x_b - 1)nH\frac{x_b - 1}{x_b}$$

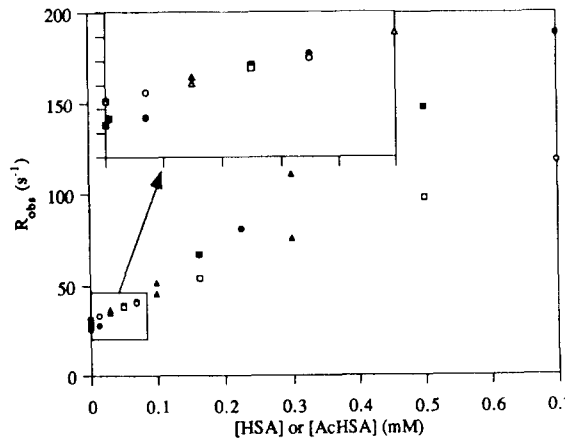

Fig. 5. Observed transverse relaxation rate (R_{obs}) from ^{19}F NMR of albumin/FU complexes vs. total albumin concentration: FU 0.7 mM/HSA (●); FU 0.5 mM/HSA (■); FU 0.3 mM/HSA (▲); FU 0.7 mM/AcHSA (○); FU 0.5 mM/AcHSA (□); FU 0.3 mM/AcHSA (△).

where F and H are the total concentrations of fluorouracil and albumin, respectively. While R_f can be measured from a protein-free solution of FU, R_b, K and n may be obtained by fitting the experimental linewidth values with the two above equations. Unfortunately, K is rather small and, as a consequence, the three parameters are strongly correlated within the range of concentrations that has been examined (and also accessible in the NMR experiments). In such a case, only the product $n(R_b - R_f)K$ can be determined with an acceptable accuracy. If each set of data (R_0 vs. H) at a fixed concentration of FU is fitted by a straight line [26], its gradient m is given by: $m = n(R_b - R_f)K(1 - FK) \approx n(R_b - R_f)K$. Such a product may be thus obtained for each protein as an average at different FU concentrations.

The results for native and acetylated albumins are different and well outside the error limit, being respectively

$$n(R_b - R_f)K = 400 \pm 20\ \mathrm{mM^{-1}\ s^{-1}} \text{ for HSA}$$

$$n(R_b - R_f)K = 200 \pm 15\ \mathrm{mM^{-1}\ s^{-1}} \text{ for AcHSA}$$

This result lends itself to further speculation. If it is assumed arbitrarily that R_b is equal for the native and the acetylated protein, it can be deduced that the apparent affinity constants nK (which determine the pharmacological consequence of protein binding) are exactly in the ratio of 2:1. This assumption implies that the dynamic behaviour of the bound drug is similar for both protein complexes, which differ by only one amino acid residue.

4. Conclusions

The results obtained by different spectroscopic techniques showed that 5-fluorouracil binds to human serum albumin with low affinity and low specificity compared to other common drugs (e.g. diazepam, phenylbutazone). However, evidence was obtained for the localization of binding sites for FU on HSA. In particular, no interaction was observed with HSA binding area II (benzodiazepine), whereas a weak complex formation close to area I (coumarin) was detected by both ΔCD and Δ^1H NMR analysis. Another remarkable interaction site was revealed on a HSA area of the third type following Oida's definition. It is interesting that chemical modification of a single amino acid residue, namely the acetylation of Lys_{199}, clearly reduced FU binding to HSA as assayed by both CD and NMR methods. Qualitative evaluation by ^{19}F NMR suggests that aspirin-induced acetylation of HSA halves the apparent affinity constant of FU. To further speculate on this result, it is possible to propose the existence of two main FU binding sites on HSA, one of which (close to the coumarin binding area) is inaccessible in the acetylated protein. These considerations seem particularly remarkable since aspirin induces such a modification with commonly administered dosages [13].

This report underlines that FU binding to HSA (that could influence FU pharmacokinetics and pharmacodynamics) can be greatly altered by simultaneous HSA binding of other common drugs.

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References

[1] J.L. Grem, D.F. Hoth, J.M, Hamilton, S.A. King and B. Leyland-Jones, Cancer Treat. Rep., 71 (1987) 1249–1264.
[2] W. Hryniuk, Semin. Oncol., 14 (1987) 65–74.
[3] A. Duc-Mauger, J.P. Benoit and F. Puisieux, Pharm. Acta Helv., 61 (1986) 119–124.
[4] T. Peters, Jr., Adv. Protein Chem., 37 (1985) 161–245.
[5] S. Yamashita, Y. Suda, M. Masada, T. Nadai and M. Sumi, Chem. Pharm. Bull., 37 (1989) 2861–2863.
[6] J.A. Laurie, J. Clin. Oncol., 7 (1989) 1447–1456.
[7] R.J. Fraile, L.H. Baker, T.R. Buroker, J. Horowitz and V.K. Vaitercius, Cancer Res., 40 (1980) 2223–2227.
[8] E.O. Deaspuru and J. Zantonaml, Biochem. Biophys. Methods, 27(N2), (1993) 87–89.
[9] U. Kragh-Hansen, Pharmacol. Rev., 33 (1981) 17–53.
[10] K.J. Fehske, W.E. Mueller and U. Wollert, Biochem. Pharmacol., 30 (1981) 687–692.
[11] U. Kragh-Hansen, Dan. Med. Bull., 37 (1990) 57–84.
[12] B. Honoré, Pharmacol. Toxicol., 66, (1990) 7–26.
[13] J.E. Walker, FEBS Lett., 66 (1976) 173–175.
[14] K. Maruyama, H. Nishigori and M. Iwatzuru, Chem. Pharm. Bull., 33 (1985) 5002–5012.
[15] C. Bertucci, A. Viegi, G. Ascoli and P. Salvadori, Chirality, 7 (1995) 57–61.
[16] C. Bertucci, E. Domenici and P. Salvadori, Chirality, 2 (1990) 167–174.
[17] J.H. Perrin, J.J. Vallner and D.A. Nelson, Biochem. Pharmacol., 24 (1975) 769–774.

[18] C.F. Chignell, Mol. Pharmacol., 5 (1969) 244–252.
[19] A. Rosen, Biochem. Pharmacol., 19 (1970) 2075–2081.
[20] K. Marukami, Y. Fusjisaki and T. Sano, Bull. Chem. Soc. Jpn., 60 (1987) 3385–3390.
[21] T. Sjoedin, N. Roodsdorp and J. Sjoeholm, Biochem. Pharmacol., 25 (1976) 2131–2140.
[22] A. Konowal, G. Snatzke, T. Alebic-Kolbah, F. Kajfez, S. Rendic and V. Sunjic, Biochem. Pharmacol., 28 (1979) 3109–3113.
[23] G. Ascoli, C. Bertucci and P. Salvadori, J. Pharm. Sci., in press.
[24] C.F. Chignell and D.K. Starkweather, Mol. Pharmacol., 7, (1971) 229–237.
[25] T. Oida, J. Biochem., 100 (1986) 99–113.
[26] J.J. Fischer and O. Jardezky, J. Am. Chem. Soc., 87 (1965) 3237–3244.