Production and Characterization of 22 Monoclonal Antibodies Directed Against S 20499, a New Potent 5-HT_{1A} Chiral Agonist: Influence of the Hapten Structure on Specificity and Stereorecognition

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Received December 30, 1998; accepted February 13, 1999

Purpose. An immunoconjugate model was proposed to produce stereoselective monoclonal antibodies (MAbs) for the quantitation of a 5-HT_{1A} agonist, S 20499. MAbs produced were characterized in terms of stereoselectivity and specificity towards the opposite enantiomer and structural analogs.

Methods. The immunogen was formed following the effective addition of a butanoic acid spacer arm between the parent S 20499 structure and bovine serum albumin (BSA). After fusion (modified Köhler and Milstein's procedure), specificity of MAbs was obtained using the Abraham's criteria. Experimental and calculated partition coefficients were determined.

Results. Twenty-two hybridoma cell lines were established secreting MAbs (apparent association constants ranging from 1.1×10^8 to 2.8×10^9 M^{-1}). Several MAbs showed cross-reactivity levels of less than 5% with S 20500 (optical antipode), which could allow a stereospecific assay to be set up. Both chroman and azaspiro moieties were part of the epitopic site. Dealkylation and hydroxylation(s) led to various crossreactivity levels. Four antibody families were described in terms of specificity.

Conclusions. This study highlighted the influence of the immunoconjugate construction (coupling site and type of spacer arm) in the immuno-stereospecificity of Abs. The results obtained for two monohydroxylated metabolites suggest that the lipophilicity behavior could be a valuable tool for predicting Ab-crossreactivity.

KEY WORDS: monoclonal antibodies (MAbs); stereoselectivity; specificity; 5-HT^{1A} agonist; molecular lipophilicity potential (MLP); virtual log P.

INTRODUCTION

A significant proportion of organic compounds marketed as drugs possess asymmetric centers: some of these molecules are administered as racemates and others as single enantiomers. Chiral drugs are believed to interact stereoselectively with biological systems (enzymes, receptors, proteins . . .). Consequently, pharmacological activity, or toxicity, may be induced following the administration of opposite configurations (1). Chiral assay techniques are therefore essential in bioanalytical studies to allow drug enantiomers to be monitored after administration of the racemate form or to detect chiral inversion after administration of single enantiomers. Sensitive immunoassay methods represent an effective alternative to the popular and widely used chromatographic techniques which have been previously applied in such studies (2,3). The main limitation of drug immunoassay methods lies with the specificity of antibodies: this includes their stereoselectivity towards chiral drugs. The structural characteristics of the drug—including the steric bulk around the asymmetric center, the flexibility of the molecule in the vicinity of asymmetric center and the ability of the molecule to racemize (4)—are some of the many intrinsic factors which can determine the immuno-stereospecificity. Other parameters, which may be modified by the investigator can enhance the stereorecognition of molecules: an appropriate hapten may be synthesized; the spacer arm between hapten and carrier protein may be selected as a function of the topography of the molecule and a suitably adapted immunization and screening assay procedure may be chosen.

In the present study we report the production and the binding characteristics of 22 MAbs raised against S 20499, an analogue of buspirone with a chiral carbon atom at the 3-position of the chroman moiety (Fig. 1) which has recently been shown to act as a potent 5-HT_{1A} receptor agonist (5). The construction of the immunogen is described as are the advantages of developing an immunoconjugate bearing a close resemblance to the native drug to assay. This was achieved by incorporating a short, non-immunogenic lipophilic spacer arm between S 20499 and the protein carrier. Finally, the lipophilicity behavior of ligands was studied, to help explain the cross-reactivity results: this may also serve as a valuable tool to predict cross-reactivities as a function of the lipophilicity behavior of molecules and their 3D structures.

MATERIALS AND METHODS

Reagents

[3H]-S 20499 (specific activity: 49.6 Ci/mmol) was obtained from the CEA (Gif-sur-Yvette, France). S 20499 and all related compounds, including compounds used for the hapten synthesis, were supplied by Technologie Servier (Orléans, France). Freund's complete and incomplete adjuvants and Bovine Serum Albumin (BSA) were purchased from Sigma Chemicals (USA). Ammonium sulfate, sodium phosphate (monobasic and dibasic) and citric acid were purchased from Merck (Darmstadt, Germany). The myeloma cell line NS1 was kindly donated by Dr. A. Bensussan (Inserm U 93, Paris, France). RPMI 1640 medium, L-glutamine (200 mM), sodium pyruvate (100 mM), penicillin-streptomycin solution (10000 IU/ml and 10000 μg/ml respectively), hypoxanthine (100x), thymidine (100x), aminopterin (100x), NCTC 135 medium and Dulbecco's Mod Eagle Medium were purchased from Gibco (Paisley, Scotland). Polyethyleneglycol 1500 in 75 mM HEPES, fetal calf serum and the Isostrip™ Mouse Monoclonal Antibody

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0724-8741/99/0500-0725$16.00/0

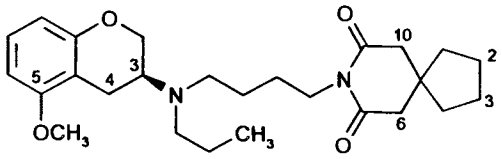
Isotyping kit were purchased from Boehringer Mannheim (Mannheim, Germany). Specific reagents used for the determination of partition coefficients: Titrants: 0.5 M HCl, prepared by dilution of a normadose (Carlo Erba) for 1000 ml of 1M HCl, was used. 0.5 M KOH, prepared by dilution of a normadose (Prolabo) for 1000 ml of 1M KOH, was used. Partition solvents: water-saturated extra-pure octan-1-ol (Merck) and water-saturated HPLC-grade n-heptane (SDS) were used.

Preparation of S 20499 Conjugates

S 20499 was modified to introduce a carboxylic group to permit coupling with BSA (Fig. 1). Two haptens were used in this study. For the first one, a structure similar to that of S 20499 was chosen with the overall introduction of a carboxylic function at the free end of the propyl chain (Fig. 1, A4 structure). For the second one, a heptanoic acid group was added in the place of the propyl chain to study the influence of distancing the coupling site from the asymmetric center (Fig. 1, A7 structure). The haptens were coupled to BSA following the mixed anhydride method (6). As previously published, the hapten/BSA ratio could not be easily determined since radiolabelled hapten was not available and the conjugates were not totally soluble. However, an indirect characterization of the coupling was performed using mass spectrometry, and the immunological response obtained in rabbits using these immunogens confirmed that the coupling was efficient and the hapten/BSA ratio was sufficient (6).

Production of Hybridomas

Eight six-week-old female BALB/c mice (four for each conjugate) were immunized four times by intraperitoneal injection of hapten conjugates with an interval of 14 days between the first and the second injections and 7 days between the two remaining injections. Primary immunizations were composed of 250 µl of a 0.9% sterile NaCl solution containing 50 µg of immunogen emulsified in 250 µl of Freund's complete adjuvant. Subsequent immunizations were of the same volume, with incomplete adjuvant instead of complete adjuvant. Blood samples were taken three days after the fourth immunization to check for antibody production. A final intravenous boost consisting of 25 µg of immunogen in a 0.9% sterile NaCl solution was then administered to the two mice which gave the highest titres (one for each conjugate). Fusion was carried out following a modification of Köhler and Milstein's procedure (7). Cells from immunized mice spleen were removed three days after the final boost. These lymphocytes were fused with myeloma cells (10:1 ratio) in the presence of 1 ml of polyethyleneglycol 1500 maintained at 37°C. After careful washing with RPMI 1640 supplemented with antibiotics (1%) and L-glutamine (1%), the cell pellet was cautiously diluted in the fusion medium (Dulbecco's Mod Eagle Medium with 4.5 g/l glucose supplemented with 10 % NCTC 135 medium, 20% fetal calf serum, 1% sodium pyruvate, 1% antibiotics and 1% L-glutamine). After appropriate dilution, microwell plates were seeded with 50,000, 20,000 or 10,000 cells from the fusion mixture suspended in the fusion medium, without feeder cells. On the day following fusion, HAT medium (1% hypoxanthine, aminopterin and thymidine in fusion medium) was added to the culture medium. After five days, cultures were supplied with fresh HAT medium every two days until growing clones were observed under the microscope. The supernatant liquids of wells with positive cell growths were tested for the presence of antibodies specific for S 20499 using a liquid-phase radioimmunoassay technique



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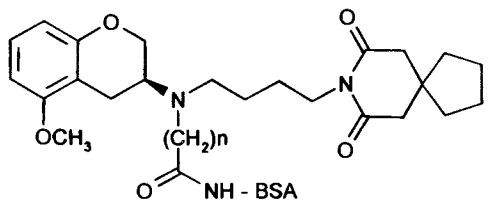


Immunogens : **2** n = 3, A4 structure
3 n = 6, A7 structure

Fig. 1. Structural formulae of S 20499 (1) and its conjugates (2) and (3).

Subcloning of Positive Hybridomas

All positive hybridoma for the production of anti-S 20499 antibodies were subcloned twice using the limiting dilution method (0.5, 1 and 5 cells/well). Stocks of expanded positive subclones were stored in liquid nitrogen. Ascites were induced in Pristane-primed 10-to 12-week-old female BALB/c mice by the intraperitoneal injection of 5 to 15 $\times$ 10^6 hybridoma cells per mouse to obtain large concentrations of anti-S 20499 MAbs. Ascitic fluids from mice or hybridoma culture supernatants (when ascite was not available) were characterized.

Liquid-Phase Radioimmunoassay

A liquid-phase radioimmunoassay (RIA) was developed to follow the antibody production, to test hybridoma supernatant for specific antibodies towards S 20499 and to characterize the monoclonal antibodies elicited.

Antibody Checking

To check the antibodies elicited, 50 or 100 µl of mouse antiserum were incubated along with 250 or 200 µl (depending on the antiserum volume added) of a 5 g/l BSA solution in citrate buffer (0.03 M, pH 6.4), and 200 µl of a 0.6 nM solution of [^{3}H]S 20499 in citrate-BSA buffer to give a final reaction volume of 500 µl. Results were compared with the control sample obtained from serum of non-immunized mice.

Hybridoma Screening Assay and Competitive Experiments

Supernatant fluids in hybridoma cultures were screened for the production of anti-S 20499 antibodies in the same way, replacing mouse antiserum with an equivalent volume of supernatant fluid, and adding 50 µl of human plasma (Centre de transfusion sanguine, Hôpital Lariboisière, France).

For competitive experiments, increasing amounts of unlabelled drugs in BSA-citrate buffer (100 µl, up to a concentration of 2,000 ng/ml) or BSA-citrate buffer (100 µl) were added to the incubation medium (containing 50 µl of human plasma, 100 µl of appropriate MAb dilution in citrate buffer and 200 µl of a 0.7 nM [^{3}H]S 20499 solution in citrate-BSA buffer), keeping the total reaction volume to 500 µl. Non-specific binding was determined by replacing MAb with citrate buffer.

In all experiments (Antibody checking, hybridoma screening assay and competitive experiments), reactions were allowed to proceed for 1.5 h at room temperature. Proteins were then precipitated with 500 µl of a saturated solution of ammonium sulfate. Bound [^{3}H]-S 20499 was separated from the free ligand by centrifugation. A 500 µl aliquot of the supernatant was added to 4 ml of Picofluor (Packard, France) and the radioactivity was counted in a Packard Tricarb counter (Packard, France).

Calculations

Displacement curves and binding parameters were obtained and calculated using the InPlot program, version 4.03 (GraphPad Software, 10855 Sorrento Valley Road, Suite 204B, San Diego, California 92121, USA). The IC_{50} was calculated as the ligand concentration required to displace 50% of the labelled ligand. In accordance with the criteria of Abraham (8), cross-reactivity was expressed as the percentage ratio for the IC_{50} of S 20499 with respect to the IC_{50} of the test ligand. Mean apparent affinity constants were estimated according to the method of Müller (9).

Determination of Partition Coefficients

Partition coefficients of S 20499, S 21555, and S 21656, between *n*-octanol and aqueous solutions and between heptane and aqueous solutions were measured, according to the dual-phase potentiometric titration technique described by F.H. Clarke and colleagues (10). Experiments were carried out at 25°C (ionic strength: 0.15 M KCl) on a Sirius PCA 101, following the experimental procedure proposed by B. Slater and coll. (11). Two series of experiments corresponding to various *n*-octanol/water ratios (0.5:20, 4:16, and 1:19; or 1:15, 5:10, and 1:19), were carried out for the three compounds at drug concentrations ranging from 0.2 to 0.7 mM. Partition coefficients between heptane and aqueous solutions (volume ratio: 0.5:20) were determined at 0.3 mM, 0.8 mM, and 0.1 mM for S 20499, S 21555, and S 21656, respectively (three experiments for each compounds).

Computational Methodology

Molecules were modeled by attributing standard bond lengths and angles, with the SYBYL program version 6.3 (Sybyl, Tripos Associates, Inc., 1699 South Hanley Road, St. Louis, Missouri, MO 63144, USA), running on an Indigo^{2TM} R4400 Extreme Silicon Graphics workstation. The geometries were roughly minimized *in vacuo*, using some steps of Simplex and then with the Conjugate Gradient of the Powell algorithm (12), provided with the Tripos force field (13). The resulting structures were then fully optimized using the AM1 semi-empirical method (14) within the Gaussian 92 program (15).

As already described (16,17), the effect of the conformational change on lipophilicity can be assessed by the lipophilicity range calculated over the conformational space of flexible compounds. Indeed, the most lipophilic and hydrophilic conformers were retained and the difference between their *virtual log P* values (*virtual log P* being the log P calculated for a given conformer (18)) was considered as the lipophilicity range accessible to a given solute in the neutral state. The lipophilicity range encompasses the ensemble of all virtual log P values of a solute, whereas the experimental log P is the weighted average of an unknown number of virtual log P values of the molecule (18). For each conformer, the *virtual log P* was calculated with the CLIP 1.0 software (CLIP 1.0; Institute of Medicinal Chemistry, University of Lausanne; 1996.) using a two-step strategy. The molecular lipophilicity potential, namely the continuum of lipophilicity existing around any molecule, was calculated using a Fermi type distance function (19) and the atomic lipophilic parameters of Broto et al (20). Then the *virtual log P* was calculated by integrating the MLP back to log P_{oct} values on the Solvent-Accessible Surface Area (SASA (18)) of each conformer using the following equation (21):

$$\begin{aligned} \log P_{oct} &= 2.86 \cdot 10^{-3} (\pm 0.24 \cdot 10^{-3}) \Sigma MLP^{+} \\ &+ 1.52 \cdot 10^{-3} (\pm 0.22 \cdot 10^{-3}) \Sigma MLP^{-} \\ &- 0.10 (\pm 0.23) \\ n &= 114;\ r^2 = 0.94;\ s = 0.37;\ F = 926 \end{aligned} \quad (1)$$

where ΣMLP^{+} and ΣMLP^{-} represent respectively the hydrophobic and polar parts of the molecule.

The conformational behavior of each compound was explored using a simplified conformational search strategy able to describe efficiently the main valleys of a conformational space (22). This conformational analysis was performed using an adaptation of the Quenched Molecular Dynamics (QMD) approach as already described (23).

RESULTS

Establishment of Hybridoma Clones

Sixty-six wells were tested positive by RIA for the production of anti-S 20499 antibodies with the A7 structure, while only 5 wells tested positive with the A4 structure. Only 22 clones (all from the A7 structure) were shown to be stable: these clones were further characterized.

Monoclonal Antibody Characterization

Isotype

The heavy chains of 19 out of the 22 MAbs obtained consisted of γ_1-chains, while two MAbs belonged to the IgG_3

subclass and 1 to the IgG_{2b}. κ-chains were used as light chains in all the MAbs (Table 1).

Association Constants (Ka)

The displacement curves for the MAbs examined gave calculated Kd values for S 20499 ranging from 3.6 to 86 nM according to the method of Müller (12), corresponding to apparent association constants ranging from 1.1×10^8 to 2.8×10^9 M^{-1} (Table 1).

Cross-Reactivities

Potential metabolites and analogs of S 20499 were studied for concentrations up to at least 2000 ng/ml, to calculate their cross-reactivities. The results obtained from the different compounds tested and their structures, namely cleavage compounds and those with modifications to the chiral center, the propyl side-chain and to both the chroman and azaspiro moieties, are given in Table 2. The least recognized compounds by antibodies were those in which the chroman or the azaspiro moiety were lost. Loss of the chroman moiety (compounds Y 395, Y 412 and Y 607) led to cross-reactivity below 0.2%, while cross-reactivity level for Y 528 (loss of the chroman moiety) ranged from 0.2 to 32%, suggesting that the paratope of elicited MAbs is more recognizant of the azaspiro moiety. Clear-cut stereoselectivity was found for all antibodies, with cross-reactivity level ranging from 1.9 to 26% for S 20500, the opposite configuration of S 20499. From these results, the wide panel of MAbs obtained allowed us to define four non-equivalent antibody families, none of them fully specific of S 20499.

Table 1. Properties of Monoclonal Antibodies

Antibody family	Antibody	Apparent association constant ($\times 10^9$ M^{-1})	Isotype*
A	A457	1.6	IgG_1, k
B	A443	1.2	IgG_1, k
	A431	2.8	IgG_1, k
	A435	0.3	IgG_1, k
	A57	1.2	IgG_1, k
	A517	0.8	IgG_1, k
	A526	1.4	IgG_1, k
	A75	1.5	IgG_1, k
	A85	1.7	IgG_1, k
C	A86	0.4	IgG_1, k
	A824	0.9	IgG_1, k
	B45	0.1	IgG_3, k
	B412	0.2	IgG_{2b}, k
	B55	0.8	IgG_1, k
	B515	0.7	IgG_1, k
	A444	0.7	IgG_1, k
	A516	0.5	IgG_1, k
	A625	0.6	IgG_1, k
D	A72	0.9	IgG_1, k
	A87	0.8	IgG_1, k
	A821	0.1	IgG_1, k
	A827	0.5	IgG_3, k

* Determined using the Isostrip™ mouse monoclonal antibody isotyping kit.

Experimental and Calculated Partition Coefficients

In an effort to explain the clearly different interactions between MAbs and S 21656 (6-hydroxyazaspiro derivative) compared to those obtained for S 21555 (2-hydroxyazaspiro derivative), partition coefficients were determined experimentally and calculated by two theoretical approaches. The results for S 20499, S 21555 and S 21656 are summarized in Table 3. Due to the high lipophilicity behavior of S 20499, two experimental procedures were followed for the determination of the pKa value (differences in the % of cosolvent used, ranging from 18 to 40%, and in the extrapolation technique to 100% water). Two pKa values, and hence two log P values were obtained, and reported in Table 3.

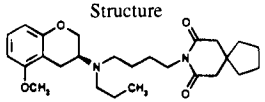
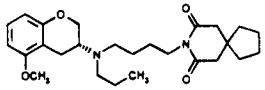
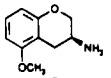
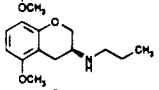
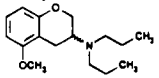
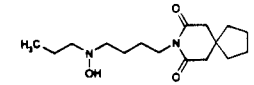
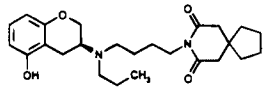
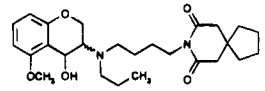
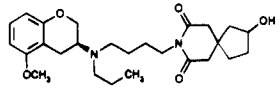
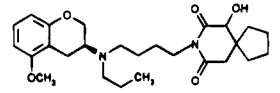
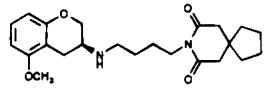
The partition coefficients measured both in octanol-water and heptane-water systems show intermediate values for S 21656 compared to S 20499 and S 21555. These results are supported by the calculated Clog P values and by the range of virtual lipophilicity calculated from the MLP (Table 3) confirming that S 21656 is more lipophilic than its isomer S 21555.

DISCUSSION

The main factors governing the production of specific antibodies include: the synthesis of an appropriate hapten, the correct choice of spacer arm (site, type and length) and the hapten/protein carrier coupling site. Two groups of immunogens were synthesized in an attempt to favor specificity and stereoselectivity, taking into account the potential metabolites of S 20499 and the location of the asymmetric carbon in the molecule (Fig. 1). As far as the metabolism of S 20499 was concerned, hydroxylation and de-alkylation reactions were expected: hydroxylation of the aminochroman and the azaspiro moieties and/or loss of the propyl chain and demethylation of the chroman methoxy group. The coupling site, based on a linkage through the propyl group, was chosen to maintain the integrity of the chroman and the azaspiro moieties of the drug. Several noteworthy studies have shown that antibody affinity is lowered when the structure of a hapten becomes less and less akin to the parent drug (24,25). Bearing this in mind, an immunogen was prepared with an overall structure similar to that of S 20499, with the effective addition of a carboxylic group between it and the BSA carrier protein (Fig. 1, A4 structure). A second immunogen was synthesized to distance the chiral center from the coupling site. This immunogen was prepared following the effective addition of a butanoic group between the S 20499 structure and BSA (Fig. 1, A7 structure). By adopting this strategy, cross-reactions with metabolites resulting from potential biotransformation on the propyl chain (de-alkylation, hydroxylation) were expected to decrease. Moreover, the introduction of a short, lipophilic, hence non-immunogenic spacer arm between S 20499 and BSA increased the probability of its non-recognition by the antibodies. This was an important point of note to obtain stereoselective antibodies: it is well known that at least three interaction sites between ligands and antibodies are required for tridimensional recognition.

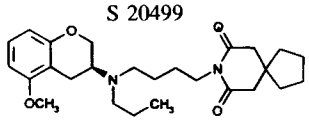
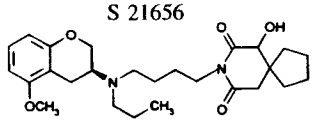
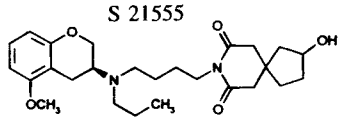
As expected, the cross-reactions indicated that the chroman and the azaspiro moieties formed part of the hapten's epitopic site (Table 2). Indeed, binding to antibodies decreased dramatically when one of the immunodominant parts of S 20499 was missing: cross-reactivities fell to below 32%, even below 0.2%

Table 2. Structural Formulae of S 20499 and Analogs[a]

Antibodies			A457	A443	A431	A435	A57	A517	A526	A75	A85	A86	A824	B45	B412	B55	B515	A444	A516	A625	A72	A87	A821	A827
Antibody family			A	B							C										D			
Ligands		Structure																						
native drug	S 20499		100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Modification:																								
on the chiral centre	S 20500		20	7.4	2.8	4.3	17	5.9	26	12	10	2.0	21	7.0	11	6.7	4.8	2.9	2.9	1.9	2.0	2.7	2.1	2.0
cleavage compounds	Y 395		<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.2	<0.2	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
	Y 607		<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.2	<0.2	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.2	<0.1
	Y 412		<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.2	<0.2	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.2	<0.1
	Y 528		0.9	0.9	3.3	8.1	19	18	9.8	3.0	12	7.1	0.2	32	32	13	1.5	5.0	13	7.8	9.1	6.0	16	6.1
on the chroman moiety	S 20580		168	171	89	71	121	114	154	149	136	94	130	113	98	108	88	47	79	80	58	68	77	75
	S 21556		70	56	57	58	71	92	60	100	88	99	86	94	78	69	76	44	60	68	51	67	54	71
on the azaspiro moiety	S 21555		63	19	4.6	6.1	15	11	4.3	17	12	4.6	13	2.3	2.9	6.7	8.3	1.7	3.9	4.7	3.0	3.6	1.2	3.2
	S 21656		67	25	69	118	108	79	72	116	84	62	112	74	100	71	89	27	52	65	48	41	50	56
on the propyl side chain	Y 464		13	5.9	143	117	281	240	219	421	419	148	232	121	102	229	144	69	121	118	96	85	85	119

[a] Cross-reactivity with anti-S 20499 monoclonal antibodies, expressed as the ratio (per Cent) of the IC_{50} for S 20499 to that of the test compound.

Table 3. Experimental Partition Coefficients and Molecular Modeling parameters

Ligands	pK_a	log P_{exp} oct./eau	log P_{exp} hep./eau	Δ log P_{exp} (oct-hep.)	C log P	Virtual log P range[a]
S 20499 	7.89 *7.18*	4.8 *4.4*	3.5 *2.8*	1.3 *1.6*	4.4	4.7–5.9
S 21656 	7.95[b]	4.1	2.8	1.3	4.0	3.6–4.8
S 21555 	7.95	3.6	0.4	3.2	2.4	3.3–4.2

[a] Intervals corresponding to the different identified conformers for each ligand.
[b] Extrapolated value.

when the loss involved the chroman moiety. However, the cross-reactivities of ligands with only minor chemical modifications with respect to S 20499 (loss of the propyl chain, demethylation or hydroxylation on the chroman moiety or the azaspiro moiety) yielded an assortment of results. The wide range of MAbs obtained allowed four non-equivalent families of antibody to be defined, unfortunately none of them were fully specific for S 20499. Two out of the four groups were composed of only one MAb. One of these groups (A family, A457b, Fig. 2) exhibited no specificity. With the exception of Y 464 (a potential metabolite of S 20499, cross-reactivity: 13.4%), high cross-reactivity levels were found with all of the ligands assayed. The second MAb, forming a group by itself (B family), A443, also gave a low cross-reactivity level for Y 464 (5.9%) and a moderate level in the presence of ligands hydroxylated on the azaspiro moiety. In comparison with the preceding MAb cross-reactivity, it is worth noting that the additional interactions with S 20499 allowed increased levels of stereoselectivity (7% cross-reaction towards the S 20499 enantiomer, instead of 20% for A457b MAb). High cross-reactivity levels were found with the

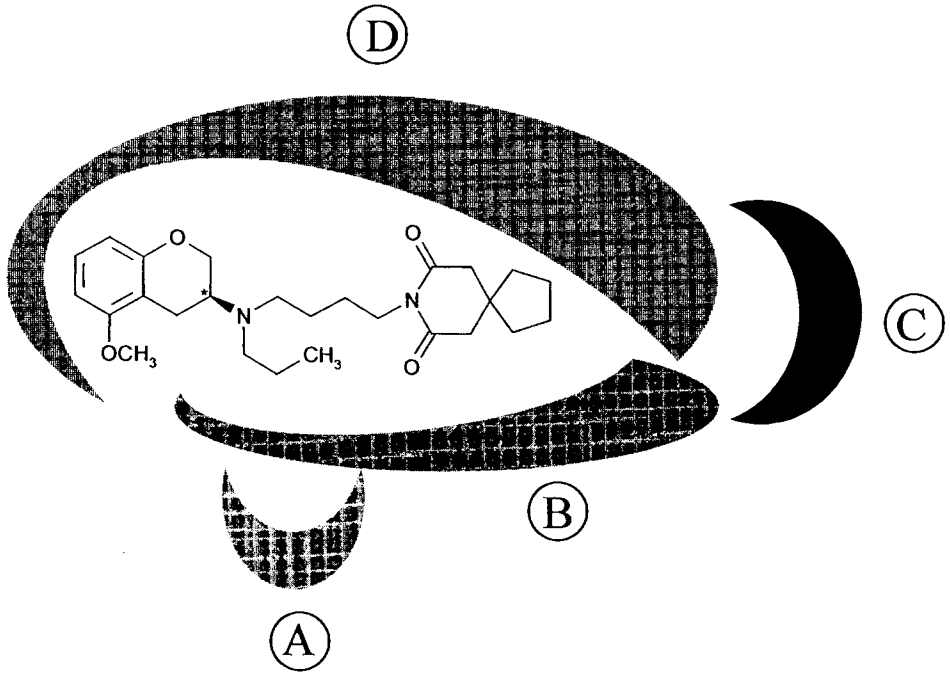


Fig. 2. Recognition sites of the four monoclonal antibody families produced. ⊗ denotes the different categories of family.

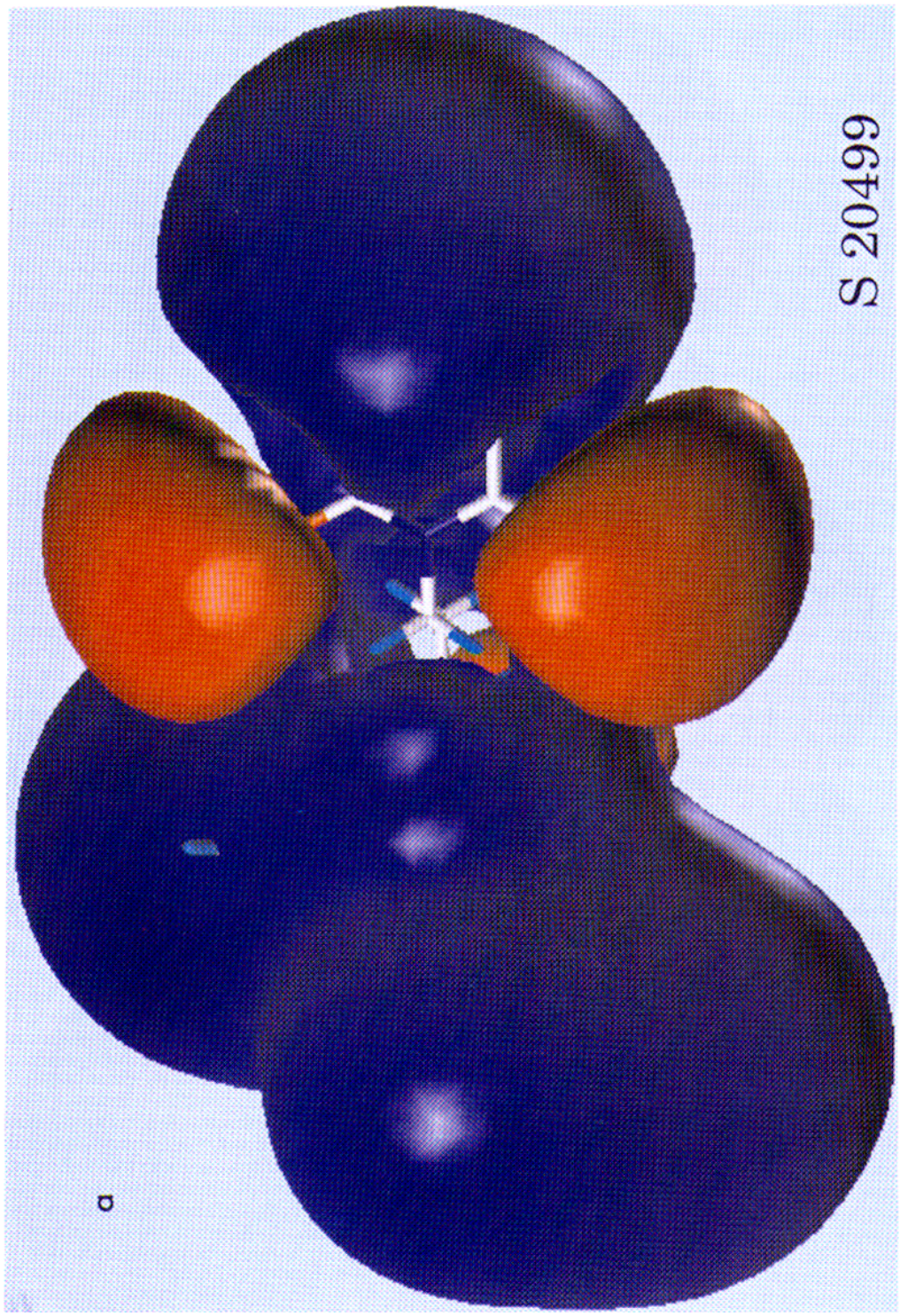


Fig. 3. 3D structure and lipophilicity potential of S 20499 and two analogs. Blue and red colors denote hydrophobic and hydrophilic sites, respectively.

13 MAbs belonging to the third group (C family) for all of the compounds slightly different, chemically speaking, in comparison with S 20499, except when the modification was sustained at a remote part of the azaspiro moiety (in the 2 or 3 position on the azaspiro moiety). In this case, cross-reactions ranged from 2.3% to 16.6%. For the 7 remaining MAbs, a wide specificity was once again found (D family). A slight decrease in the level of recognition was nonetheless observed with these MAbs for ligands with partially modified S 20499 structures.

Clearly, the hydroxyl group at the 2 (or 3) position on

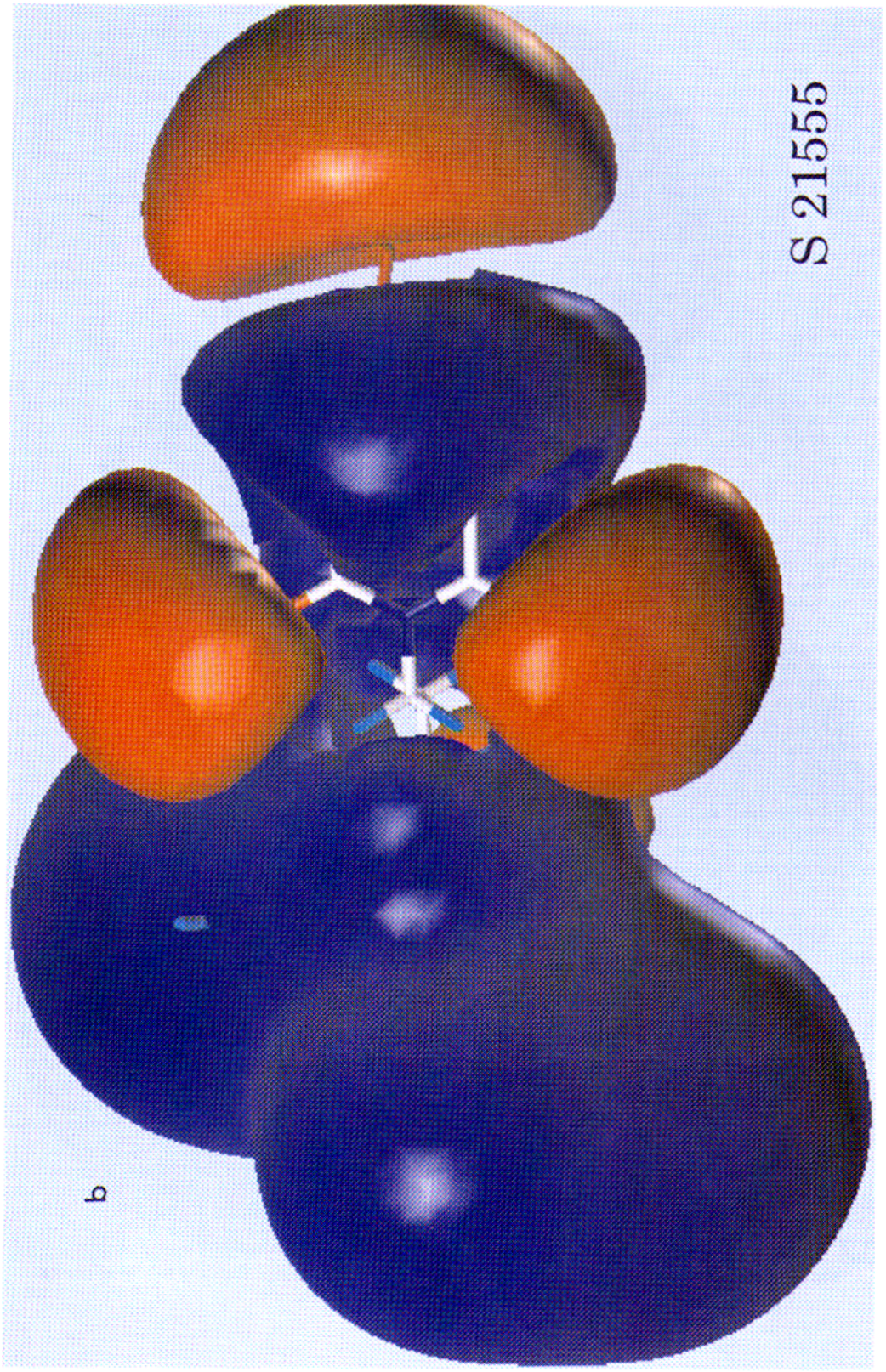


Fig. 3. Continued.

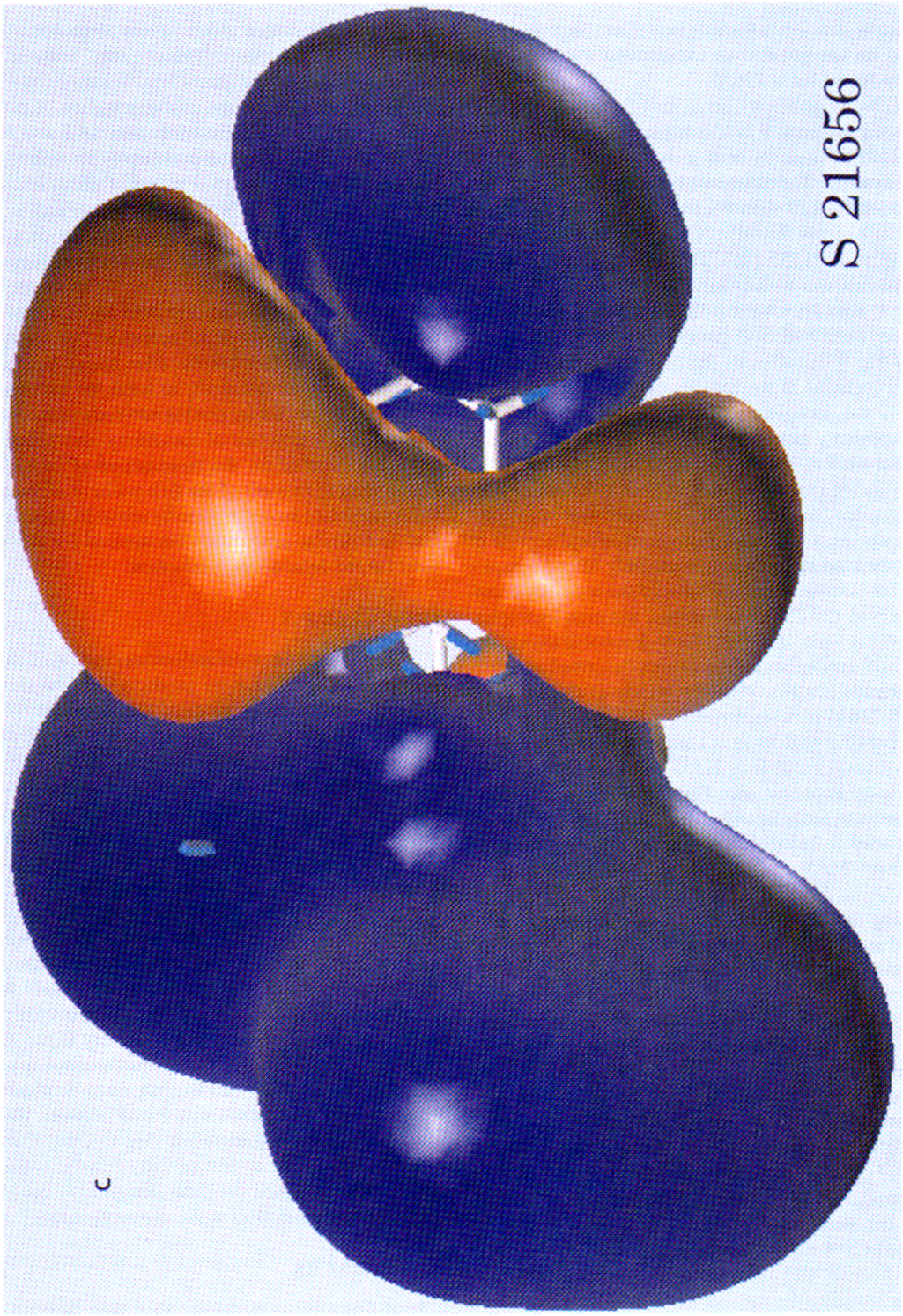


Fig. 3. Continued.

azaspiro moiety plays an important inhibitory role in antibody binding. On the other hand, cross-reactions are surprisingly high for S 21656, an isomer of S 21555 with a hydroxyl group in the 6 position. These findings can suggest a different conformational behavior or different lipophilicity properties of S 21656, S 21555 and S 20499.

The conformational space of these three compounds was explored by quenched molecular dynamics. For all the compounds, this approach identify folded and linear conformations. Whereas, the folded conformations are the more stable for S 21656 and S 21555, the energy difference between the folded and linear conformers remains low suggesting that both confor-

mations coexist in solution at equilibrium. Thus the conformational analysis do not provide an explanation for the higher cross-reactivity found for S 21656.

However, the lipophilicity behavior of these compounds reveals interesting features: S 21656 displays a higher lipophilicity than S 21555 (Table 3) both in *n*-octanol/water and *n*-heptane/water systems. The difference between the two partition coefficients ($\Delta\log P_{oct\text{-}hep}$) supports that the hydroxyl group in S 21656 cannot express its full polarity in contrast with the hydroxyl group of S 21555 (26). This effect is supported by Clog P calculations and *virtual* log P back-calculations from MLP. In Clog P algorithm a proximity correction term of 1.6 between carbonyl and hydroxyl group was added for S 21656 and in *virtual* log P calculations the polarity of the hydroxyl group is partly included in the polarity of the carbonyl group.

Moreover, the three-dimensional representation of the molecular lipophilicity potential confirms that S 20499 and S 21656 are quite similar, both analytes differing from S 21555 (Fig. 3). Sophisticated technics, such as thermodynamic or X-ray crystallographic studies, NMR spectroscopy, and site-directed mutagenesis, allow to study conformational aspects of antigen-antibody binding, and would certainly afford complementary insights into the structural basis of the antibody cross-reactivity observed (27,28). Nevertheless, the high hydrophobicity of S 20499 is most likely to favor hydrophobic interaction with anti-S 20499 antibodies, as it was well described and fully studied for steroids (29,30). The distribution of the MLP for S 20499 and S 21656 is characterized by the presence of three hydrophobic pockets englobing a unique hydrophilic region, whereas the hydroxyl group in S 21555 produces a discontinuous hydrophilic-hydrophobic site. This 'haptophore' is present for all conformers of these ligands. Thus, the MLP distribution may explain, why S 21555 is not recognized by antibodies and why a lower MAb cross-reactivity level was found for this compound.

Concerning the stereospecificity, all of the anti-S 20499 MAbs showed preferential recognition for S 20499, with cross-reactivity levels for S 20500 (the optical antipode of S 20499) ranging from 1.9 to 26%. Most of them showed less than 5% cross-reactivity against S 20500: this may enable a stereospecific assay to be set up. Of the four MAb families defined, the most stereoselective MAbs belonged to the D family. This may be attributed to the increased number of interaction sites between the antibody paratope and S 20499, as shown in Fig. 2, favoring higher Ab stereoselectivity levels (cross-reactivity for the S 20499 optical antipode ranging from 1.8% to 3.8%). On the other hand, A457b (A family), showed broad specificity. Interestingly, the stereoselectivity of this MAb is one of the lowest recorded (20% cross-reactivity towards S 20500). This leads one to believe that the spacer arm is included in its paratope, thus reducing the number of interaction sites with S 20499 itself. In addition, the low cross-reactivity towards the *N*-depropylated S 20499 (13.4%, Table 2) obtained for this antibody, showing that the propyl moiety is recognized by A457b, is in favor of this hypothesis. This reinforces the importance of having several interaction sites between the antibody paratope and ligand for optimal tridimensional recognition. For compounds with small molecular weights (S 20499 for example), a trade off must be found: an optimal distance must be achieved between the asymmetric center and the hapten/protein linkage site through the use of a short, non-immunogenic spacer arm. A longer spacer group favors stereoselectivity, while a short non-immunogenic spacer arm reduces interactions between the Ab and the spacer arm. In a previous study concerning the production and the characterization of polyclonal antibodies using both same immunogens (A4 and A7 structures, A), the choice of a hapten structurally resembling to S 20499 proved already to be a good means of obtaining stereospecific antibodies with high affinities, as the asymmetric center in the hapten was located farther away from the coupling site. For polyclonal antibodies, both approaches gave high levels of stereospecificity. While not decreasing the mean apparent affinity constant, the longer spacer improved antibody specificity. With the production of monoclonal antibodies, we wanted to confirm that an additional four carbon-atom bridge between the native drug and the protein carrier, to form the immunogen, should be a valuable tool for increasing antibody stereospecificity with no drawbacks in terms of specificity and affinity, and could favorably be used for the construction of other immunogens. Unfortunately five out of the five positive wells obtained from the A4 structure were shown to be unstable, although a concomitant and similar procedure than applied for A7 structure was used for the hybridoma production.

CONCLUSIONS

Specific monoclonal antibodies capable of eliciting stereoselective recognition were produced for the development of a chiral immunoassay for S 20499, a potent 5-HT_{1A} agonist chemically related to buspirone. The short lipophilic spacer arm chosen to ensure that the hapten bore a close resemblance to the native drug proved to be appropriate to preserve affinity. The coupling site was distanced from the chroman and azaspiro moieties of S 20499, which are susceptible to biotransformations. Nevertheless, cross-reactivities of potential metabolites were too high with these antibodies to allow a specific immunoassay to be developed for the unmodified drug (i.e. S 20499). Moreover differing results were found for the binding characteristics of S 20499 derivatives hydroxylated on the azaspiro moiety. These findings were attributed tentatively to the folding up of the azaspiro on the chroman moiety, masking the chemical modifications inside the azaspiro moiety to the antibody paratope. The exploration of the conformational space of the two isomers did not allow this hypothesis to be confirmed. On the other hand, a correlation was found between the lipophilicity properties of these compounds (log P, Clog P, and virtual log P values) and their cross-reactivity results, proving that these approaches proposed for drug design (17) could be valuable tools for the prediction of Ab cross-reactivity.

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