Table 2. Inhibition of soybean lipoxygenase activity by constituents of cannabis

Compound	Maximum conc. tested mg/ml	Maximum inhibition (%)	I.C.$_{50}$ (±SE) (μM)
1.	50	inactive	—
2.	50	40	—
3.	30	98	2.2 (0.6)
4.	30	96	2.9 (0.2)
5.	30	83	3.2 (0.5)
6.	30	79	9.2 (0.3)

Data was the mean of five experiments

these compounds on the enzymes of the prostaglandin pathway.

In this communication the actions of cannabis constituents on enzymes of arachidonate metabolism have been determined. The results confirm that the non-cannabinoid constituents are primarily cyclo-oxygenase inhibitors whilst the cannabinoids are dual inhibitors of both cyclo-oxygenase and lipoxygenase. Preliminary kinetic studies suggest that the cannabinoids share a common mode of action with other phenolic anti-oxidants.

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Monoclonal antibodies specific for 1–4 benzodiazepines

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Benzodiazepines (BZD)* are an important class of pharmacologically active molecules [1] widely used as anxiolytics, hypnotics, anticonvulsants or muscle relaxants. They exert their pharmacological effects through interaction with γ aminobutyric acid receptors [2, 3] localised on neurons, probably acting at the synaptic level [4]. "Peripheral" binding sites for BZDs have also been demonstrated in kidney, heart, lung and on thrombocytes [5, 6] but their function is not known.

Minor modifications to the BZD structure can lead to dramatic changes in pharmacological properties: thus the anticonvulsant drug clonazepam (for BZD structural formula see Fig. 1) is specific for receptors of the neuronal type whereas RO5-4864, which is structurally closely related, is specific for the peripheral binding sites [7] and does not show BZD-like pharmacological activity. Flunitrazepam, a centrally active BZD, binds to both types of receptor [8].

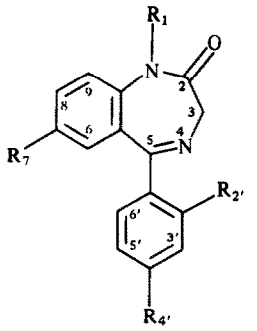
One approach to the analysis of the structure/activity relationships of these drugs is to use immunological methods to develop antibodies that can be considered as models for receptors. In this respect monoclonal antibodies specific for clonazepam have already been described [9, 10].

The present report concerns the production and purification of murine monoclonal antibodies directed towards flunitrazepam. The binding specificity and the immunochemical characteristics of three of these are described.

* Abbreviations used: BSA, bovine serum albumine; BGG, bovine γ-globulins; PBS, phosphate buffer saline; FNZ, flunitrazepam; BZD, benzodiazepine; ABTS, 2-2' azino-di-(3 ethylbenzthiazoline sulfonic acid); mAb, monoclonal antibody; tBu, tertiobutyl.

Materials and methods

Chemicals. Bovine serum albumin V, bovine γ globulin Cohn fraction II and adipic acid dihydrazide agarose were obtained from the Sigma Chemical Co., 1-(3-dimethylaminopropyl-)-3-ethyl-carbodiimide hydrochloride from



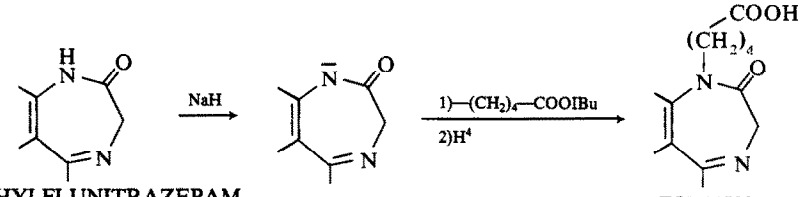
Flunitrazepam R_1 = CH_3, $R_{2'}$ = F, R_7 = NO_2
Clonazepam R_1 = H, $R_{2'}$ = Cl, R_7 = NO_2
Ro5 - 4864 R_1 = CH_3, $R_{4'}$ = Cl, R_7 = Cl

Fig. 1.

Janssen Chimica, [*N*-^{3}H Methyl] BZDs from Amersham (flunitrazepam, 79 Ci/mM) and New England Nuclear (RO5-4864, 79 Ci/mM) rabbit anti-mouse isotype specific antibodies were obtained from the Zymed laboratories. All other chemicals were provided by the Roussel UCLAF research centre (Romainville, France).

Flunitrazepam conjugates. The methyl group of flunitrazepam (R_1, Fig. 1) was replaced by an alkyl chain bearing an activatable carboxyl group according to the following reaction scheme:

The modified BZD was linked to BSA by the mixed anhydride method [11]. After reaction, the hapten protein conjugate was dialyzed at 4° against water/dioxan 1:1 then PBS and kept in the dark at −20°.



DESMETHYLFLUNITRAZEPAM RU 44502

Agarose conjugates were obtained as follows: to 10 g of adipic hydrophobe agarose gel washed with water/DMF 2:1 (2 × 50 ml) were added 0.1 g of modified flunitrazepam in 10 ml of DMF and 0.5 g of carbodiimide in 5 ml of water. The mixture was gently shaken for 2 hr at room temperature, then the gel was repeatedly washed with water/DMF 2:1. The amount of flunitrazepam covalently coupled was estimated by u.v. light absorption at 310 nm (ε_M^{310} FNZ N ~ 8 × 10^3) and was equal to 15 moles/mole of BSA and 10 μmoles/ml of agarose gel respectively.

Immunisation and somatic cell fusion. Eight-week-old Balb/c mice were immunized with 100 μg of the BSA ~ flunitrazepam conjugate emulsified in complete Freund adjuvant (50 μg in the hind foot pads and 50 μg subcutaneously in the flanks). Four weeks later a 50 μg boost was given in incomplete Freund adjuvant and another a week later (i.v. 20 μg).

After 2 days the spleens were excised for fusion. Fusion was performed with the Sp2 OAg cell line cultured in MEM medium supplemented with antibiotics glutamine and 10% v/v decomplemented horse serum. Hybridomas were selected in conventional medium (azaserine 10^{-5} M, hypoxanthine 510^{-5} M) and screened 10–15 days after fusion. Clones positive for ^{3}H FNZ binding (see below) were subcloned by limited dilution in 20% horse serum. Each positive subcloned cell line was grown in Petri dishes and them amplified into ascites for antibody production.

Monoclonal antibody purification. (a) *Ion exchange chromatography.* Ascitic fluids were precipitated with Na_2SO_4 (18% w/v final), and the pellet obtained after centrifugation resuspended and dialysed against 20 mM Tris pH 8.5. Proteins were loaded onto a DEAE-TSK 5WP HPLC column equilibrated with the same buffer and eluted (1 ml/min flow) with a 30 min linear gradient of 0 to 100% of 20 mM Tris pH 7.0 NaCl 0.3 M [12]. One-millitre fractions were collected and tested for ^{3}H FNZ binding (see below).

(b) *Affinity chromatography.* RU 44502-agarose (5 ml see above) was incubated at 4° for 1 hr with excess ascitic fluid in order to saturate all active groups of agarose. The gel was then washed with cold PBS until no protein could be detected in the buffer. Specifically bound 1 g were eluted at 20° with 0.1 M glycine HCl pH 2 and neutralized with 1 M Tris pH 9.

In both cases, mAbs were concentrated by ultrafiltration, dialyzed vs PBS and their purity checked by sodium dodecyl sulfate gel electrophoresis in reducing conditions.

Immunoassays with monoclonal antibodies. Binding and inhibition experiments with ^{3}H BZDs were performed as follows: the radioligand solution (100 μl in PBS/0.1% (w/v) BGG) was incubated 30 min at 4° with either hybridoma culture supernatants or purified mAb at the appropriate concentration in PBS/BGG (100 μl). The BZD-mAb complexes were precipitated with polyethylene glycol (PEG 8000, final concentration 17%), and separated from unbound ligand by centrifugation. The precipitates were dissolved in 0.5 ml of water for radioactivity counting. Non-specific binding in these experiments was measured (~5% of total) with 10^{-6} M FNZ in the incubation medium.

The mAbs isotype was determined as follows: 100 μl of RU 44502-BSA (50 μg/ml in carbonate–bicarbonate buffer 0.1 M pH 9.5) were incubated overnight at 4° in the wells of immunoplates. After saturation with BSA in PBS, purified mAb (5 μg/ml in PBS/BSA) were incubated 30 min at 20° then the wells were washed. Peroxidase-linked antibodies specific for mouse 1 g subclasses were added for 2 hr. Peroxidase activity in the wells was revealed with the H_2O_2–ABTS system.

Results and discussion

From one fusion experiment, more than 100 hybridoma cell lines were obtained, five of which were found to be positive for ^{3}H FNZ binding. Three of these were stable after cloning and subcloning.

The corresponding mAb (2C6, 3B3, 4B3) were purified from ascitic fluids either by HPLC (recovery yield after purification > 95%) or by affinity chromatography (yield ~ 80%). The purified antibody isotypes were determined by ELISA: they all had κ-light chains and γ_1 heavy chains.

Binding studies with the 3 mAbs were performed with ^{3}H FNZ and ^{3}H RO5-4864 in order to demonstrate possible binding specificity with respect to either the neuronal or the peripheral types of sites. For both ligands binding experiments showed saturable binding isotherms as expected for mAbs. The dissociation constants (K_D) determined by Scatchard analysis at 20° for the three mAbs are presented in Table 1. The K_D values lie in the 10^{-8}–10^{-9} M range for FNZ and in the 10^{-7}–10^{-8} M range for the RO5-4684 ligand. It is of note that the 2C6 mAb shows a much

Table 1. Binding of ^{3}H flunitrazepam and ^{3}H RO5-4864 to the anti-BZD monoclonal antibodies

	K_D (M)		
	2C6	3B3	4B3
Flunitrazepam	$4\ 10^{-9}$	$2\ 10^{-8}$	$8\ 10^{-9}$
RO5-4864	$8\ 10^{-7}$	$2.5\ 10^{-8}$	$4\ 10^{-8}$
$KD^{RO5-4864}/KD^{Flu}$	200	*1.25*	*5*

Scatchard plots were made by linear regression; in all cases correlation coefficients were >0.90. Supposing 2 binding sites/molecule of mAb and given the amount of purified mAb in the assay, the percentage of sites occupied at saturation was estimated and found to be ~85 ± 15% for the 3 mAbs.

higher affinity for FNZ than for RO5-4864 (KD RO5-4864/KDFNZ ~ 200), which is not the case for the 3B3 and 4B3 mAbs.

The specificity of the 3 mAbs towards a number of BZDs was also investigated by measuring their inhibition of ^{3}H FNZ binding to mAbs. The corresponding inhibition constants are presented in Table 2 which shows that the 3 mAbs have similar but not identical specificities: the highest K_i values are observed for BZDs with an alkyl group at the N_1 position (RU 44502, FNZ). Substitution at this position by a hydrogen atom (clonazepam, desmethyl FNZ) or by a polar group at the N_1 (flurazepam), R_3 (clorazepate) or R_4 position (chlordiazepoxide) leads to lower K_i values.

Non-BZD ligands with a high affinity for the neuronal BZD receptor were also studied. There was no measurable inhibition ($K_i > 10^{-4}$ M, results not shown in Table 2) for suriclone, the imidazodiazepine RO15-1788, the triazolopyridazine CI 218872, the pyrazoloquinolinone CGS 8216 and the β carboline methyl and ethyl esters, indicating that these mABs cannot be considered as exact mirrors of the receptor complex.

We have thus obtained three monoclonal antibodies highly specific for the 1–4 BZD structure. One of them, the 2C6 mAb shows a high affinity for the immunogen flunitrazepam but not for RO5-4864, despite the fact that these two compounds only differ by very minor structural features.

Such a narrow difference in specificity is also characteristic of the neuronal versus the peripheral types of BZD binding sites [7]. Moreover, since the K_D values for FNZ binding to both the neuronal type receptor and to the 2C6 mAb are similar (10^{-8}–10^{-9} M) [7, 8], the antibody may be considered as a possible model for FNZ binding to the former. A generalisation cannot be made, however, to the other BZDs tested since, for example, clonazepam, which shows high affinity and specificity for neuronal sites, has only low affinity for the 2C6 mAb.

Combining sites of macromolecules that recognize the same ligand may present structural homologies: we have raised polyclonal antibodies in rabbit against the antigen-

Table 2. Inhibition of ^{3}H FNZ binding to monoclonal antibodies to BZDs and to the neuronal type receptor

	K_i (M)			
	Monoclonal antibodies			Neuronal receptor
Ligand*	2C6	4B3	3B3	
RU 44502 $R_1 = (CH_2)_4$ COOH $R_2 = F$, $R_7 = NO_2$	$2.5\ 10^{-9}$	$3.1\ 10^{-9}$	$1.4\ 10^{-8}$	$1.4\ 10^{-7}$
Flunitrazepam $R_1 = CH_3$, $R_2 = F$ $R_7 = NO_2$	$1.1\ 10^{-8}$	$5.9\ 10^{-8}$	$3.7\ 10^{-8}$	$1.8\ 10^{-9}$
Diazepam $R_1 = CH_3$, $R_7 = Cl$	$3.0\ 10^{-8}$	$4.1\ 10^{-8}$	$3.1\ 10^{-8}$	$6.4\ 10^{-9}$
Clorazepate $R_3 = COOH$, $R_7 = Cl$	$1.7\ 10^{-6}$	$4.8\ 10^{-7}$	$2.1\ 10^{-6}$	$1.4\ 10^{-8}$
Clonazepam $R_2 = Cl$, $R_7 = NO_2$	$9.3\ 10^{-7}$	$8.5\ 10^{-7}$	$2.5\ 10^{-6}$	$2.6\ 10^{-9}$
Chlordiazepoxide $R_2 = NHCH_2$, $R_7 = Cl$ *N*-Oxide at position 4	$6.4\ 10^{-6}$	$2.1\ 10^{-6}$	$1.5\ 10^{-5}$	$3.0\ 10^{-7}$
Flurazepam $R_1 = (CH_2)_2 - N(Et)_2$ $R_2 = F$, $R_7 = Cl$	$7.0\ 10^{-7}$	$3.2\ 10^{-7}$	$2.8\ 10^{-7}$	$3.5\ 10^{-8}$
Desmethyl, Flunitrazepam $R_2 = F$, $R_7 = NO_2$	$9.3\ 10^{-7}$	$8.5\ 10^{-7}$	$7.4\ 10^{-7}$	$3.4\ 10^{-9}$

Antibody concentrations were 0.3 μg/ml, 0.28 μg/ml and 1.1 μg/ml for 2C6, 4B3 and 3B3 respectively. ^{3}H FNZ concentration in the assay was 2.85 nM. K_i values were determined with the following formula for competitive inhibitors: $K_i = IC_{50}/[1 + (L/K_D)]$ where L is the radiotracer concentration and IC_{50} is the concentration of inhibitor that blocks 50% of specific radiotracer binding to mAb. For comparison, the corresponding K_i values for the neuronal receptor, using a rat cortex preparation as described by Mohler and Okada [5], are also shown.

* R = H when not otherwise specified.

binding domain of the 2C6 molecule and studies are in progress to determine whether or not these antiidiotypic antibodies can recognize the BZD receptor complex.

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Effects of carbon dioxide on onsets of seizures in mice induced by antagonists of vitamin B_6

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The excitability of the central nervous system is affected considerably by procedures that change the carbon dioxide concentration in blood and tissues. For example, the increased blood concentration of CO_2 induced by the inhalation of CO_2 (5–20%) increases the threshold for minimal electroshock seizures [1], whereas the fall in arterial pCO_2 induced by hyperventilation increases electrical activity of the brain cortex [2]. Further, it is well known clinically that petit mal seizures can be induced by hyperventilation and abated by the addition of moderate amounts of CO_2 to the inspired air [3, 4].

In investigating the possible mechanism of the action of CO_2, it was deemed important to study the effect of the gas on experimental seizures, the mechanism of which has been, comparatively, made clear. Antivitamin B_6 is known to produce in experimental animals characteristic convulsions that are related to a decline in γ-aminobutyric acid (GABA, an inhibitory transmitter) because of an inhibition of glutamic acid decarboxylase in the central nervous system [5]. And it is assumed that a decrease in release of GABA from the nerve terminals and, thereby, a decline in binding of GABA to its receptor of the postsynaptic neuron produce an enhancement in central neuronal excitability [6]. In this paper, we have investigated the effect of inhalation of CO_2 on seizures induced by injection of DL-penicillamine (PeA) and thiosemicarbazide (TSC) which are known to be antagonists of vitamin B_6.

Methods

Exposure of animals to carbon dioxide. DDY mice weighing 20–25 g were used as experimental animals. The animals were exposed to various gas mixtures by placing them in individual plastic chambers (6 cm high × 12 cm deep × 6 cm long) with the following details of construction. A 3-cm circular opening was made on one side to introduce the animal into the chamber, and the opening was closed by a rubber stopper that was pierced with two glass-tubes (3 mm d) for entrance of the gas mixture into the chamber and exit of it from there. The tube that was the entrance to one chamber was connected to the tube that was the exit of the adjacent chamber, and the exit tube was connected to the entrance tube of another adjacent chamber.

The gas mixture to be delivered to the chain of chambers was prepared by mixing the gases in a large gasometer (24 liter bottle). The various gases, obtained from separate tanks of oxygen, nitrogen and carbon dioxide, were mixed in the gasometer in the proper proportions. In this manner, any mixture of gases desired for a particular experiment could be obtained; all mixtures contained 20% oxygen plus nitrogen as the diluent. By pouring water into the gasometer the gas mixture was expelled into all the chambers through the entrance tube of the first chamber.

After the air in the chambers had been replaced with a gas mixture to be tested, the animals were introduced through the openings into the respective chambers, and then the gas mixture flow through the animal chambers was maintained at a constant rate. The rate of flow of the gas was 2 liters per 10 min. The behavior of the animals was observed continuously through the plastic walls of the chambers.

Induction of convulsion by convulsants. PeA and TSC were used as convulsants. Solutions of these drugs were prepared daily in 0.9% NaCl solution, the pH being adjusted to 7 immediately before use. The final concentration of the drugs was adjusted so that the required dose was administered in a volume equivalent to 1% of the body weight of the animal. All injections were intraperitoneal, and the injected animals were kept in their usual cage until they were introduced into their chambers. After the injections, food and water were withheld from the animals during experiments.

Results

The exposure of mice to a 5% CO_2 gas mixture, 100 min after the administration of PeA (2 mmol/kg), had little effect on the total number of animals convulsing but had a