International Journal of Pharmaceutics, 36 (1987) 113–120
Elsevier

IJP 01215

Photobinding of flunitrazepam and its major photo-decomposition product *N*-desmethylflunitrazepam

Ruud W. Busker, Gerard M.J. Beijersbergen van Henegouwen, Brigitta M.C. Kwee and Jos H.M. Winkens

Center for Bio-Pharmaceutical Sciences, Division of Medicinal Chemistry, Gorlaeus Laboratories, Leiden (The Netherlands)

(Received 29 October 1986)
(Accepted 17 November 1986)

Key words: Flunitrazepam; *N*-desmethylflunitrazepam; Photoaffinity label; Photobinding; Human serum albumin; Benzodiazepine

Summary

Photodecomposition of flunitrazepam (FNZP) and its photolabeling to human serum albumin (HSA) under oxygen-rich and -poor conditions were studied. The kinetics of both photodecomposition and photolabeling in oxygen-flushed solutions correspond completely with those found under normal aerobic conditions, as usually applied in photoaffinity labeling (PAL) of benzodiazepine receptor sites. This correspondence also holds for the photoproducts found. Nitroreduction products photochemically formed in the absence of oxygen, appeared not likely to contribute in PAL, in contrast to what has been suggested earlier. FNZP photodecomposes up to about 55% into *N*-desmethylflunitrazepam (D-FNZP) under aerobic conditions. In this case photolabeling to HSA proceeds far more efficiently, and results in twice as much covalent binding, as in an oxygen-poor environment. D-FNZP also photobinds to HSA to an extent comparable to that of FNZP. The results may in part explain the low percentage (20–25%) of photolabeled sites in PAL with [methyl-^{3}H]FNZP by: (i) loss of label by photodecomposition of FNZP into-D-FNZP; (ii) photobinding of (unlabeled) D-FNZP leading to a decrease of receptor sites available for labeled FNZP; and (iii) destruction of receptor sites by singlet oxygen produced by photoexcited nitrobenzodiazepine.

Introduction

Flunitrazepam (Reg. no. 1622-62-4), 5-(2′-fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-(2H)-1,4-benzodiazepin-2-one (FNZP), is a benzodiazepine derivative which belongs to the class of potent tranquilizing drugs (Dement et al., 1973; Kales and Scharf, 1973; Monti et al., 1972). [Methyl-^{3}H]FNZP has been shown to act as a photoaffinity label of the benzodiazepine neuroreceptor (Battersby et al., 1979; Johnson and Yamamura, 1979). Because of its specificity and high efficiency as a label for the benzodiazepine–GABA receptor complex it has received considerable attention in this regard (Moehler et al., 1980). The molecular basis of the irreversible photobinding of FNZP to its receptor has been studied (Sherman-Gold, 1983) but is still unclear. Several investigators, working with membrane-bound receptor sites, have reported that only 20–25% of the total sites present can be irreversibly labeled by [methyl-^{3}H]FNZP and that after labeling, the remaining sites show a much lower affinity for FNZP (Thomas and Tallman, 1981). The underlying ba-

Correspondence: R.W. Busker, Center for Bio-Pharmaceutical Sciences, Division of Medicinal Chemistry, Gorlaeus Laboratories, P.O. Box 9502, 2300 RA Leiden, The Netherlands.

sis of this discrepancy is not completely understood. In this view the photochemical behaviour of FNZP in the presence of proteins needs further investigation.

The photodecomposition of FNZP in oxygen-poor solution was looked into: Cornelissen and Beijersbergen van Henegouwen (1979, 1981a) succeeded in isolating and identifying the nitroreduction products. In this journal Givens et al. (1986) recently suggested that nitroreduction, which takes place in oxygen-poor medium, may be the most important photochemical pathway leading to irreversible binding of FNZP to receptor protein.

Surprisingly the photolysis of FNZP in oxygen-containing or oxygen-rich media has not received much attention. This is striking since PAL with FNZP is commonly performed with oxygen being present. Moreover phototoxicity of FNZP is only seen under oxygen-rich conditions (Cornelissen et al., 1981b), indicating that photochemical activity certainly takes place in the presence of oxygen. Therefore we investigated the photodecomposition in an oxygen-rich medium. In the experiments we used human serum albumin (HSA) as a model protein. It appeared that irradiation of FNZP in the presence of HSA in an oxygen-rich medium leads to nearly twice as much irreversible binding as in an oxygen-poor medium. The major photoproduct of FNZP, under atmospheric conditions, was identified as *N*-desmethylflunitrazepam (D-FNZP). Binding experiments showed that both FNZP and D-FNZP are able to bind covalently. The possible implications of this for PAL are discussed.

Materials and Methods

Chemicals

Flunitrazepam (FNZP), *N*-desmethylflunitrazepam (D-FNZP), hydroxylamino- (HA-FNZP) and aminoflunitrazepam (AM-FNZP) were kindly provided by Hoffmann-La Roche (Basel) and were used without further purification. [Methyl-^{3}H]FNZP (> 80 Ci/mmol) was purchased from New England Nuclear (Boston). Human serum albumin was purchased (Orha 20/21, Behringwerke, Marburg) and used as such. Salts used in buffers: potassium hydrogenphosphate (Baker Chemicals, Deventer, The Netherlands), disodium hydrogenphosphate 2-hydrate (Merck, Darmstadt), sodium dihydrogen phosphate 1-hydrate (Baker Chemicals, Deventer) and sodium chloride (OPG, Utrecht, The Netherlands). Sodium hydroxide and trichloroacetic acid were purchased and used as such. Solvents used were chemically pure or purified by distillation if necessary.

General methods

Analytical HPLC separations were performed on a prepacked, 30 cm long, 4 mm (i.d.) stainless steel column, containing a μBondapak C-18 reversed-phase 10 μm microparticulate packing (Waters), the mobile phase used was methanol/isopropanol/phosphate buffer (1 M, pH 3.25)/water (400 : 40 : 1 : 449) (Strojny et al., 1978); a LKB 2150 HPLC-pump with a Rheodyne injector and an UV-detector (LKB 2238 uvicord) or a diode array detector (Hewlett-Packard 1040A) and in some cases an integrator (Hewlett Packard 3390A) were used.

For preparative HPLC we used a column Lichrosorb 10 RP 18, 25 × 2.27 cm (i.d.) (Chrompack, Middelburg, The Netherlands).

The UV-spectra were recorded with a Perkin Elmer EPS-3T spectrometer or with a diode array detector. The HPLC mobile phase was used as a solvent. The pH values were determined with a Metrohm Herisan E 350B equipped with a glass electrode.

The mass spectra were recorded on an AEI MS-902 (70 eV).

Irradiations were performed with a Rayonet Photochemical Reactor (RPR-208; Southern New England Ultraviolet Co., New Haven) equipped with 8 lamps (RUL-3000A). Light intensity 1.5 mW/cm^2. In order to irradiate them with an equal light dose the samples were placed in a merry-go-round.

Photoproducts in an oxygen-rich medium

2.0 ml FNZP (1 mM) in methanol and 4.0 ml phosphate buffer pH 7.4 (0.03 M) were mixed. Oxygen was led through the solution for 10 min. The solution was irradiated in a quartz cell (15 × 1 × 1 cm) with 300 nm light for 1 h. After irradia-

tion the composition of the mixture was examined by analytical HPLC.

To prepare the major photoproduct 450 ml of 0.33 mM FNZP solution (see above) was flushed with oxygen for 10 min and irradiated for 1 h. The major photoproduct was isolated by subsequently extracting with dichloromethane, removing the solvent under reduced pressure, dissolving the residue in methanol/DMSO (0.3 ml + 0.7 ml), and separation by preparative HPLC.

The purity of the isolated product, determined by analytical HPLC, was more than 97%. The structure of the photoproduct was elucidated by comparison of its UV and mass spectrum and HPLC-retention time with those of an authentic sample of D-FNZP; photoproduct: UV 218, 253, 310 nm; mass spectrum m/e (rel.int. %) 224 (50), 234 (14), 252 (47), 271 (70), 280 (28), 299 (100); HPLC retention time 9.21 min. Standard sample: UV 220, 255, 311 nm; mass spectrum m/e (rel.int. %) 224 (49), 234 (12), 252 (50), 271 (69), 280 (28), 299 (100); HPLC retention time 9.17 min.

Photobinding experiments

Several quartz cells were filled with 4 ml of a mixture of FNZP (1 mM)/phosphate buffer pH 7.4 (0.03 M)/HSA (0.1% w/v) (1 : 2 : 1). They were kept for half-an-hour at 4 °C. After flushing some of the solutions with either nitrogen or oxygen for 10 min, they were irradiated with 300 nm light for varying times in a Rayonet Photochemical Reactor with 8 lamps (in most cases methanol was used to dissolve FNZP in the aqueous medium; maximum amount in the solvent 30% v/v). After irradiation the mixtures were incubated for half-an-hour in the dark at 20 °C after which 1 ml TCA 20% was added. The pellets obtained after centrifugation, were washed 3 times by resuspension, each time followed by TCA precipitation, to remove free and reversibly bound [methyl-^{3}H]FNZP, and were finally dissolved in 2 ml sodium hydroxide 0.1 N. An aliquot of 1 ml was mixed with 5 ml Dynagel and counted in a liquid scintillation counter (Packard Tri-carb 4640); a 0.2 ml aliquot for protein assay (Lowry et al., 1951). In some cases UV spectra were recorded as well.

Another set of irradiated and incubated samples, containing only unlabeled benzodiazepine were submitted to fast protein liquid chromatography (FPLC) with a LKB 2150 pump equiped with a Glas Pac column (LKB) and a diode array detector. The eluent consisted of 0.1 M phosphate buffer pH 6.8 and 0.1 M NaCl in water.

Results and Discussion

The photochemistry of flunitrazepam (FNZP) is strongly dependent on the amount of oxygen present. Cornelissen and Beijersbergen van Henegouwen (1979) reported that irradiation of FNZP in methanol with exclusion of oxygen results in photoreduction of the 7-nitro group, with formation of the 7-nitroso (NO-FNZP), the 7-hydroxylamino (HA-FNZP), and the 7-amino (AM-FNZP) derivative. According to Givens et al. (1986) these nitroreduction photoproducts are likely to play an important role in the irreversible photoaffinity labeling (PAL) of FNZP. In order to be able to confirm this, the photolysis of FNZP — in an oxygen-poor medium — needs to be investigated with respect to the quantitative importance of the nitroreduction as a function of the irradiation time. Table 1 shows that FNZP is very efficiently photodecomposed into HA-FNZP and AM-FNZP: e.g. after 2 h UV-B irradiation 84 molar % of nitroreduction products is present. The concentration of NO-FNZP measured was always less than 1 molar %.

However, because PAL is usually performed under atmospheric conditions, the photochemistry of FNZP under these conditions also needs fur-

TABLE 1

Time-dependent decomposition of FNZP (1 mM in MeOH/H_2O = 1 : 4 v/v) and formation of HA- and AM-FNZP after irradiation of the nitrogen-flushed solution

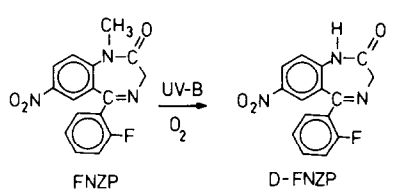
Irradiation/time (h)	FNZP	HA-FNZP	AM-FNZP
0	100	0	0
1	49	31	1
2	15	80	4
3	6	68	17
4	4	25	46

Each value represents molar % of starting FNZP.

ther investigation. Cornelissen and Beijersbergen van Henegouwen (1981a) have already found that FNZP and other 7-nitro-1,4-benzodiazepines are far more photostable in the presence of oxygen than under nitrogen; deactivation of the excited states of nitrobenzodiazepines by oxygen partly results in the formation of singlet molecular oxygen. In the case of FNZP it appeared that beside singlet oxygen (an)other reactive species are (is) formed, the formation of which is also oxygen dependent.

We found that irradiation of FNZP in methanol/water in an oxygen-rich medium with 300 nm light gave one major photoproduct, which appeared to be photolabile itself. This derivative was also formed when methanol was omitted. After examining the ultraviolet and mass spectrum and the HPLC retention time this compound was concluded to be identical to *N*-desmethylflunitrazepam (D-FNZP, Fig. 1). To examine whether this D-FNZP may play a role in the photolabeling process, we determined the percentage of FNZP that decomposed into D-FNZP.

Because D-FNZP is photochemically labile itself, this percentage cannot be determined directly. Therefore we used a kinetic model (de Mol et al., 1979) which takes into account the pseudo-first-order decomposition of FNZP and D-FNZP (shown in Fig. 2), and the time-dependent appearance of D-FNZP in an irradiated 1 mM FNZP solution. The decomposition of FNZP and of D-FNZP as a function of time was determined by HPLC analysis of samples taken during irradiation of a solution of FNZP and of D-FNZP, respectively. Analyses of the samples from the photodecomposition of FNZP also gave the concentration of D-FNZP present at each moment of the FNZP irradiation. Out of slopes of the decomposition curves (semi-logarithmic), and the data from the concentration/time curve of D-FNZP during irradiation of FNZP, the % of FNZP that decomposed into D-FNZP was calculated: $55 \pm 5\%$. So in oxygen-rich solutions D-FNZP is quantitatively the most important photochemical decomposition product.



FNZP D-FNZP

Fig. 1. Photochemical decomposition of flunitrazepam into its main photoproduct *N*-desmethylflunitrazepam in an oxygen-containing medium.

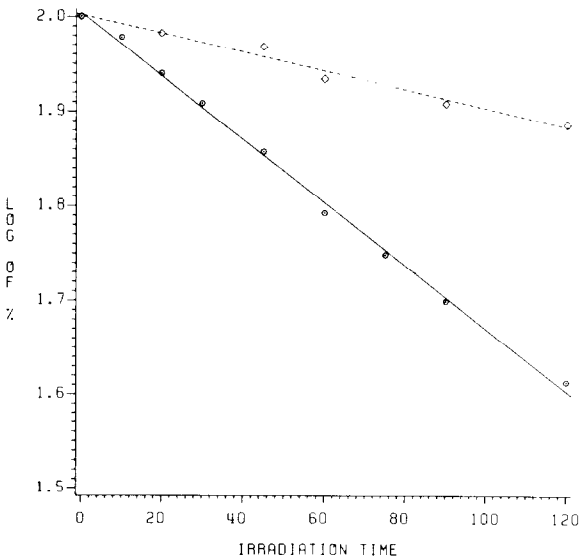


Fig. 2. Photodecomposition in the presence of oxygen of methanol/water solutions of FNZP (0.25 mM) (◇— — —◇) and D-FNZP (0.25 mM) (○———○) in log of molar % of starting material. Light intensity at 310 nm is 1.5 mW/cm^2. Irradiation time in min.

Under atmospheric conditions the photolysis of FNZP was just as fast as when oxygen was bubbled through, and also $55 \pm 5\%$ FNZP decomposed into D-FNZP. Moreover under these conditions no nitroreduction products could be observed, so that the photochemical pathway from FNZP via D-FNZP certainly has to be taken into consideration with regard to photobinding of FNZP.

We used human serum albumin (HSA) as a model protein to perform covalent binding studies. HSA is frequently used for screening photo-allergens (Barratt and Brown, 1985). When [methyl-^{3}H]FNZP was irradiated in the presence of HSA substantial irreversible binding was observed (Fig.

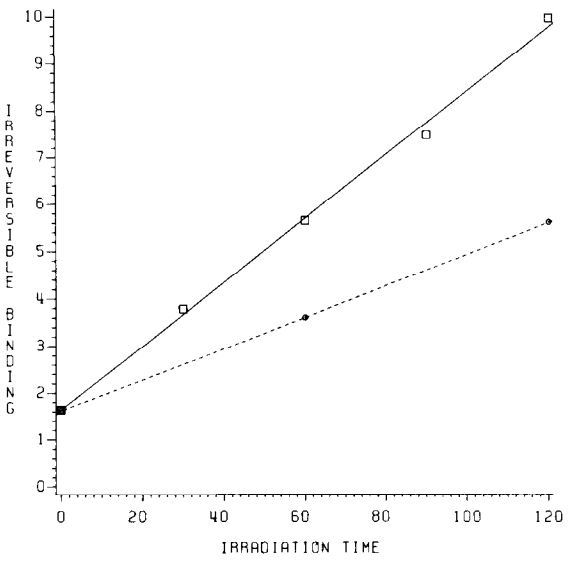


Fig. 3. Irreversible photobinding of FNZP (0.25 mM) to HSA (0.1% w/v). Light intensity at 310 nm: 1.5 mW/cm^2). Oxygen flushed □———□; nitrogen-flushed: O– — —O. The vertical axis represents the irreversible photobinding in nmol FNZP/mg HSA, and the horizontal axis the irradiation time in min.

3). The irreversible character of the binding was confirmed by dialysis under sink conditions (data not shown).

In an oxygen-rich medium the binding was twice as much as in nitrogen-flushed solutions. It should be realized that in the latter case FNZP is less stable (Table 1, Fig. 2), e.g. after 120 min of irradiation in the presence of nitrogen 96% of FNZP is decomposed, whereas this is only 20% under oxygen-rich conditions. (It should be noted that in the case where the solution was flushed with nitrogen the FNZP concentration is 4 times that in the oxygen-flushed situation; thus in the former case there will be a larger inner filter effect of FNZP resulting in relatively slow decomposition, so the difference between the figures would have been still larger if the starting concentration of FNZP had been equal in both situations.) So taking the % of photodecomposed FNZP into account, the binding under oxygen is far more efficient.

The decomposition of FNZP in the presence of oxygen and HSA is about as fast as without HSA; however, the amount of D-FNZP present is smaller (5% after 60 min irradiation) than without HSA (15% after 60 min irradiation). Probably the binding is formed at the cost of D-FNZP formation.

When nitrogen-flushed FNZP solutions are irradiated for 60 min and HSA is added immediately afterwards, irreversible binding is still observed: 3.2 nmol/mg (compared to 5.4 nmol/mg if HSA was already present). When irradiated solutions were first kept at 37 °C for 1 h before HSA was added, still 3.0 nmol/mg was bound. In the latter case, however, we determined with HPLC analysis that at the moment that HSA was added — 1 h after the irradiation — only $< 5\%$ of the original amount of HA-FNZP was left and only $< 1\%$ of NO-FNZP. Still it is possible that the small amounts of HA- and NO-FNZP left can be responsible for the observed binding: binding to HSA would then already be saturated by these small amounts. However, as can be seen in Table 1, after 1 h of radiation already 49% of HA-FNZP is formed, which is given enough time to react with HSA, but nevertheless the maximal binding has not yet been obtained as after 2 h the binding still increases (Fig. 3). So from all this it was concluded that the labile nitroreduction products, HA- and NO-FNZP, did not contribute to a considerable extent to the irreversible binding.

In oxygen-flushed solutions and in those which were irradiated under normal atmospheric conditions the photobinding to HSA only takes place when FNZP is irradiated in the presence of HSA, and not when HSA is added after irradiation. Since both the photodecomposition of FNZP, without HSA being present, and the photobinding to HSA under atmospheric conditions strongly resemble the oxygen-rich situation, the irradiation in the presence of oxygen seems to be the best reflection of the photochemical behaviour of FNZP PAL. This conclusion is supported by the observation of Thomas and Tallman (1981) that the PAL of FNZP is not caused by long-lived intermediates.

It is very interesting that [methyl-^{3}H]FNZP is used as photoaffinity label, because it is the labeled methylgroup which is photochemically cleaved in an oxygen-rich medium (Fig. 1). Loss of the labeled methyl group during PAL means that less radioactivity becomes bound to the receptor sites.

In our experiments with HSA, HPLC analysis combined with radiochemical detection showed that part of the radioactivity was present in the injection peak, probably as [^{3}H]methanol; the injection peak showed no benzodiazepine-like spectrum.

Besides that loss of label leads to less radioactivity bound to receptor sites, the percentage of binding of the labeled compound may also be decreased by photobinding of unlabeled D-FNZP, formed by photodecomposition of FNZP, competing for the same binding sites. In order to support this suggestion it is important to know the affinity of D-FNZP to the benzodiazepine receptor compared to that of FNZP. A K_i of 0.6 nM for D-FNZP, and of 0.5 nM for FNZP, was determined at our laboratory (with a radioligand binding study using calf-brain, Dr. A.P. Yzerman, personal communication). So the affinity of the photoproduct D-FNZP is equal to that of FNZP.

In this connection we investigated the possible irreversible photobinding of D-FNZP with HSA under atmospheric conditions. Because labeled D-FNZP was not available, the binding experiments with D-FNZP were performed spectrometrically. After irradiation of FNZP or D-FNZP with HSA non-covalently bound benzodiazepine was removed by repeated precipitation and resuspension of HSA. Thereafter the UV spectrum was recorded. It turned out that the spectra of HSA, irradiated in the presence of FNZP, and in the presence of D-FNZP, were clearly different from the albumin spectrum, but the spectra corresponded well to each other: in the 335 nm range a new chromophore was present. This absorption at 335 nm (which indicates formation of adducts to HSA) increases linearly with the irradiation time. When the slope of this absorption/time plot is calculated, one obtains 0.08 for FNZP and 0.12 for D-FNZP. (If equal extinction coefficients for both FNZP- and D-FNZP HSA-adducts are presumed, D-FNZP binds even more than FNZP does.)

Because repeated precipitation may result in denaturation of HSA a more refined method was also used. Irradiated FNZP-HSA and D-FNZP-HSA mixtures were analyzed with FPLC (gel filtration) combined with diode array detection.

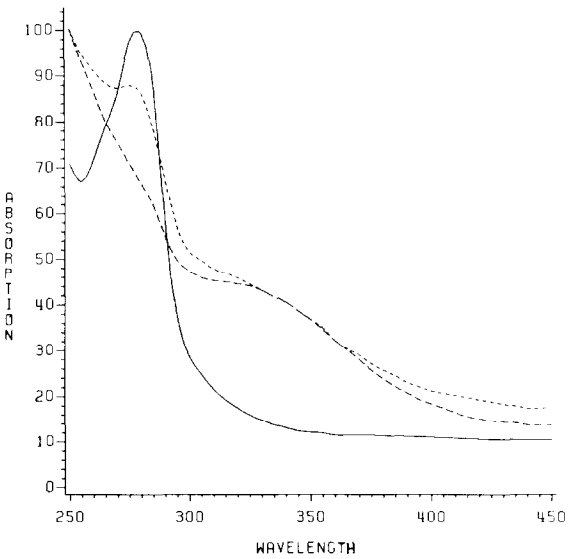


Fig. 4. UV-vis spectra of FNZP-HSA adduct (- - - - -), D-FNZP-HSA adduct (— — —) and irradiated HSA (———), obtained after FPLC, using diode array detection. Addition of FNZP or D-FNZP after irradiation did not change the HSA spectrum. On the vertical axis the absorption in mAU is shown, on the horizontal axis the wavelength in nm.

As can be seen in Fig. 4 the spectrum of HSA irradiated in the presence of either FNZP or D-FNZP shows an absorption peak at 335 nm. This can be ascribed to irreversibly bound benzodiazepine because this peak was not found when HSA, irradiated or not, was incubated with either the pure or the irradiated benzodiazepines.

Because the results of both methods correspond, it can be concluded that, not only FNZP, but also its most important photoproduct D-FNZP is capable of photobinding. This is not that surprising since the structurally related clonazepam, which also lacks the methyl group, can be used as a photoaffinity label (Sieghart and Moehler, 1982).

In PAL using [methyl-^{3}H]FNZP and irradiation with 366 nm light only 20–25% of the total amount of binding sites can be labeled. Several explanations for this have been suggested; e.g. for each binding site labeled, 3 sites may be inactivated (Thomas and Tallman, 1981). However, this explanation is in contradiction with more recent findings of Herblin and Mechem (1984)

who were able to label > 40% of the binding sites with FNZP after irradiation with 254 nm light. Moehler et al. (1984) were able to photolabel > 90% of the benzodiazepine receptor sites with another benzodiazepine. So it seems that not only the characteristics of the receptor, but also the photochemical behaviour of FNZP determines the amount of labeling which can be obtained. This can be understood by realizing the following: (i) [methyl-^{3}H]FNZP partly photodecomposes into D-FNZP, and so loses its label; (ii) the (unlabeled) D-FNZP can also irreversibly photobind to HSA and thus possibly occupy [methyl-^{3}H]FNZP binding sites; and (iii) the number of binding sites may also decrease by destruction by singlet oxygen which is a concurrent process following photo-activation of FNZP and D-FNZP (Cornelissen and Beijersbergen van Henegouwen, 1981a). Singlet oxygen is an excited state of oxygen and is reactive towards bio(macro)molecules (Singh and Petkau, 1978).

It can be concluded that from a photochemical point of view the usefulness of [methyl-^{3}H]FNZP in photobinding studies is limited. To what extent the above-mentioned factors contribute to the low photolabeling percentage of FNZP found, can be learned by using e.g. aromatic ring-labeled FNZP and D-FNZP.

Acknowledgement

We are grateful to Dr. A.P. Yzerman for determining the affinities of FNZP and D-FNZP for the benzodiazepine receptor.

References

Barrat, M.D. and Brown, K.R., Photochemical binding of photoallergens to human serum albumin: a simple in vitro method for screening potential photoallergens. *Toxicol. Lett.*, 24 (1985) 1–6.

Battersby, M.K., Richards, J.G., and Moehler, H., Benzodiazepine receptor: photoaffinity labeling and localization. *Eur. J. Pharmacol.*, 57 (1979) 277–278.

Cornelissen, P.J.G. and Beijersbergen van Henegouwen, G.M.J., Photochemical decomposition of 1,4 benzodiazepines. Nitrazepam. *Photochem. Photobiol.*, 30 (1979) 337–341.

Cornelissen, P.J.G. and Beijersbergen van Henegouwen, G.M.J., Photochemical activity of 7-nitro-1,4-benzodiazepines. Formation of singlet molecular oxygen, isolation and identification of decomposition products. *Pharm. Weekbl. Sci. Ed.*, 3 (1981a) 96–105.

Cornelissen, P.J.G., Beijersbergen van Henegouwen, G.M.J., and Mohn, G.R., Photobiological activity of 1,4-benzodiazepines. Study on the phototoxic effects of nitrazepam, clonazepam, and flunitrazepam using a bacterial indicator strain. *Photochem. Photobiol.*, 34 (1981b) 345–351.

Dement, W.C., Zarcone, V.P., Hoddes, E., Smythe, H. and Carskadon, M., Sleep laboratory and clinical studies with flurazepam. In Garattini, S., Mussini, E. and Randall, L.O. (Eds.), *The Benzodiazepines,* Raven, New York, 1973, pp. 599–611.

Givens, R.S., Gingrich, J. and Mecklenburg, S., Photochemistry of flunitrazepam: a product and model study. *Int. J. Pharm.*, 29 (1986) 67–72.

Herblin, W.F. and Mechem, C.C., Short-wave ultraviolet irradiation increases photoaffinity labeling of benzodiazepine sites. *Life Sci.*, 35 (1984) 317–324.

Johnson, R.W. and Yamamura, H.I., Photoaffinity labeling of the benzodiazepine receptor in bovine cerebral cortex. *Life Sci.*, 25 (1979) 1613.

Kales, A. and Scharf, M.B., Sleep laboratory and clinical studies of the effects of benzodiazepines on sleep: flurazepam, diazepam, chlordiazepoxide, and RO 5-4200. In Garattini, S. Mussini, E. and Randall, L.O. (Eds.), *The Benzodiazepines,* Raven, New York, 1973, pp. 577–598.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., Protein measurement with the Folin phenol reagent. *J. Biol Chem.*, 193 (1951) 265–275.

de Mol, N.J., Beijersbergen van Henegouwen, G.M.J. and Gerritsma, K.W., Photochemical decomposition of catecholamines. The extent of aminochrome formation from adrenaline, isoprenaline and noradrenaline induced by ultraviolet light. *Photochem. Photobiol.*, 29 (1979) 479–482.

Moehler, H., Battersby, M.K. and Richards, J.G., Benzodiazepine receptor protein identified and visualized in brain tissue by a photoaffinity label. *Proc. Natl. Acad. Sci. U.S.A.*, 77 (1980) 1666–1670.

Moehler, H., Sieghart, W., Richards, J.G. and Hunkeler, W., Photoaffinity labeling of benzodiazepine receptors with a partial inverse agonist. *Eur. J. Pharmacol.*, 102 (1984) 191–192.

Monti, J., Trenchi, H.M. and Morales, F., Effects of RO 5-4200, a benzodiazepine derivative, on the EEG and the sleep cycle in man (abstract). *Electroencephalogr. Clin. Neurophysiol.*, 32 (1972) 583.

Sherman-Gold, R., Photo affinity labeling of benzodiazepine-receptors: possible mechanism of reaction. *Neurochem. Int.*, 5 (1983) 171–174.

Sieghart, W. and Moehler, H., [^{3}H]Clonazepam, like [^{3}H]flunitrazepam, is a photo affinity label for the central type of benzodiazepine receptors. *Eur. J. Pharmacol.*, 81 (1982) 171–173.

Singh, A. and Petkau, A., Singlet oxygen and related species in chemistry and biology. *Photochem. Photobiol.*, 28 (1978) 429–933.

Strojny, N., Puglisi, C.V. and de Silva, J.A.F., Determination of chlordiazepoxide and its metabolites in plasma by high pressure liquid chromatography. *Anal. Lett.*, B11 (1978) 135–160.

Thomas, J.W. and Tallman, J.F., Characterization of photoaffinity labeling of benzodiazepine binding sites. *J. Biol. Chem.*, 256 (1981) 9938–9842.