Bioorganic & Medicinal Chemistry Letters, Vol.3, No.12, pp. 2587-2592, 1993
Printed in Great Britain

0960-894X/93 $6.00 + .00

AZIDO-FUNCTIONALIZED THIOUREA ANALOGUES ACTING AS EFFICIENT PHOTOAFFINITY PROBES FOR THE UREA CHANNEL.

Hervé Lamotte†, Françoise Dégeilh†, Bernard Rousseau†*, Philippe Neau‡, Pierre Ripoche‡

Département de Biologie Cellulaire et Moléculaire, Service des Molécules Marquées† and Service de Biologie Cellulaire‡, C.E.A. SACLAY, 91191 Gif sur Yvette, FRANCE

(*Received in Belgium* 6 *July* 1993; *accepted* 28 *September* 1993)

Abstract : A selected series of photoactivable thiourea analogues has been prepared as potential probes for the urea channel. We demonstrated that at least two compounds 4 and 5 can be used to label specifically and covalently the urea channel.

It is well known that urea crosses the plasma membrane of different kinds of cells such as mammalian red cells, mammalian kidney cells and amphibian urinary bladder epithelial cells, by a facilitated diffusion mechanism involving a membrane protein[1] which acts as a channel.

Certain inhibitors of the urea flux have been described in these tissues, among which are various low specific inhibitors, such as p-chloromercuribenzenesulfonic acid[2] and phloretin[3]. Urea analogues have been shown to be much more specific in inhibiting the urea facilitated transport since they behave as competitive inhibitors[4].

No information is as yet available on the urea channel structure and on the molecular mechanism of the transport. Isolation and thus characterisation of this protein is required. One approach consists in isolating the carrier by the use of specific and covalent markers. In the absence of antibodies against the urea carrier, urea analogues would appear to be the most appropriate candidates for the specific recognition of the protein.The possibility of labelling the urea channel with urea analogues was therefore considered.

An analysis of the relationship between structure and affinity of 53 urea analogues suggested that the one which inhibit the urea flux across red blood cells with the highest affinity has the following structure[4] (Figure 1) where R is an hydrophobic and an electron-withdrawing group. Interestingly, the terminal NH_2 group is not required for binding. Among tested analogues, 1-(3,4-dichlorophenyl)-2-thiourea has the best affinity with a Ki=0.01 mM (Figure 2).

Figure 1

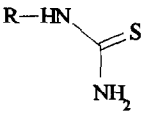
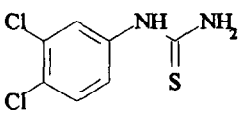


Figure 2



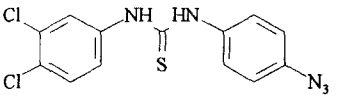
Photoaffinity probes interact reversibly with the receptor in the absence of light but on irradiation, they inactivate the biological function through covalent bond formation with the amino acid residues of the receptor binding site. Highly reactive species are required for that purpose. Diazo compounds, arylazido and aryldiazonium derivatives are usually used as precursors for the corresponding reactive species, respectively carbene, aryl nitrene and aryl cation derivatives[5]

Accordingly, we synthesized seven new photoactivable reagents (**1**, **4**, **5**, **7**, **8**, **12**, **13**) as specific urea inhibitors. All of these compounds share two common structural features:

- a thiourea group bearing an hydrophobic (aromatic) N-substituent.
- an azido or a diazonium group as the photoaffinity part.

The first diarylsubstituted thiourea **1**[6] (Figure 3) was synthesized by reaction of 4-azidophenylisothiocyanate and 3,4-dichloroaniline [5 eq /EtOH/1h : 65 %]. This analogue has been discarded because of unusable solubility for biological assay.

Figure 3

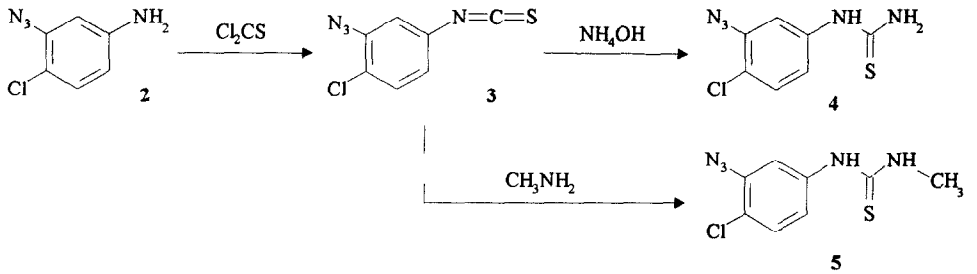


1

The synthesis of thiourea incorporating a photoactivable group (azido or diazonium) raised several problems such as the necessity to introduce the photoactivable group at the beginning of the synthesis and to achieve the synthesis in the dark. As a matter of fact, it is well known that thiourea and alkyl thiourea react rapidly with nitrous acid in aqueous solution to form an equilibrium concentration of coloured S-nitroso-compounds[7,8,9]. Consequently, the strategy we proposed for the synthesis of **4** and **5** was the introduction of the azido group followed by the thiourea moiety.

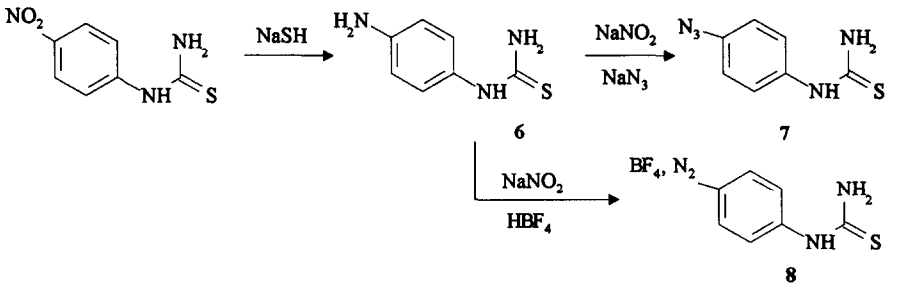
The products **4** and **5** have been synthesized from 4-chloro-3-nitroaniline in 6 steps (scheme 1)[6]. The synthesis of the requisite 3-azido-4-chloroaniline **2** has been previously reported by Boschetti and al[10]. The isothiocyanate **3** was obtained by action of thiophosgene [20 eq./PhMe-$NaHCO_3$aq/4h : 70 %]. The condensation with aqueous ammonia [$CHCl_3$/0°C/15 mn · 75%] or aqueous methyl amine [$CHCl_3$-NaOH 1M/0°C/15 mn : 62 %] produced respectively **4** or **5**.

Scheme 1 : Synthesis of 4 and 5.



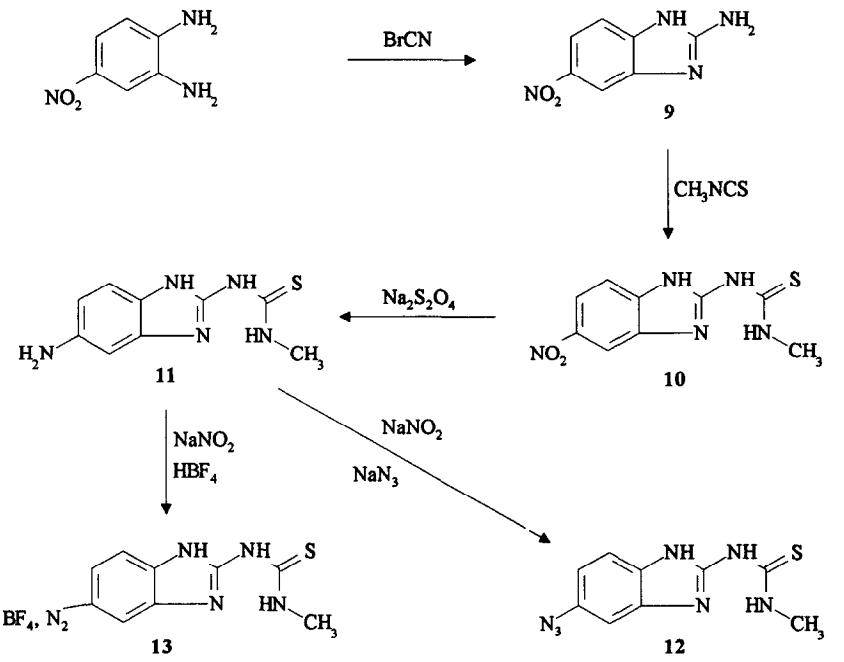
1-(4-azidophenyl)-2-thiourea **7** was prepared in order to study the reduction of a nitro group and the introduction of an azide in the presence of a thiourea function. The reduction of nitro group raised no problem using sodium hydrosulfide in ethanolic solution [50°C/2h : 75 %]. The introduction of the azide was carried out under standard conditions to obtain the product **7**. The diazonium salt **8**[11] was obtained from amine **6** by reaction of sodium nitrite in fluoboric acid [HBF_4 3 eq./5°C/3 mn][12]. No coloured S-nitroso compound was observed in the course of these reactions.

Scheme 2 : Synthesis of 7 and 8.



The third kind of analogues was photoactivable thiourea analogues with benzimidazole structure and they were prepared from 4-nitrophenylene-1,2-diamine in 5 steps. First cyanogen bromide was reacted with 4-nitrophenylene-1,2-diamine [H_2O/24h : 95%] to give 2-amino-5-nitrobenzimidazole **9** in good yields[13].

Scheme 3 : Synthesis of photoactivable benzimidazolthioureas 12 and 13.

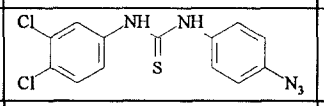
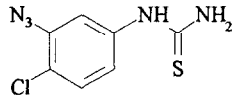
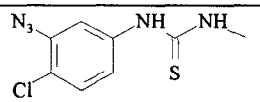
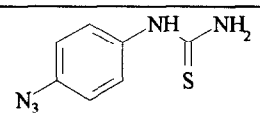
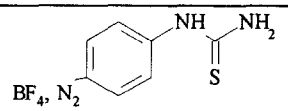
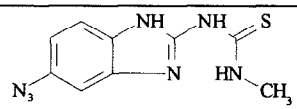
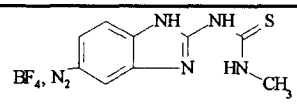


Methylisothiocyanate may react with the primary or the secondary amine of **9** or with both [C_6H_5N/reflux/24h]. However the reaction with the primary amine was the only relevant one for our purpose[14]. The thiourea analogue **10** was finally obtained in poor yields by successive recrystallizations. The nitro group of **10** was reduced with sodium dithionite [4 eq./EtOH-H2O/reflux/2h] and the azido group was obtained under standard conditions. To obtain the diazonium salt **13**[11], only 0.5 equivalent of sodium nitrite should be used . The unreacted amine was removed by HPLC.

This series of thiourea analogues were screened for their ability to inhibit the urea facilitated transport system[15].

Biological tests were performed using the urinary bladder of female frogs according to the technique described by Parisi and al[16]. Bladders were removed from pithed animals and mounted horizontally on a nylon mesh between two lucite chambers, the mucosal side facing downwards. The exposed bladder area was 3.15 cm^2. Both mucosal (12 ml) and serosal (2 ml) compartments were filled with the same Ringer solution.

Table 1 : Biological results for photoactivable urea analogues.

Substrate	Structure	Concentration	Inhibition	Irreversibility after illumination
1		very low solubility		
4		6.03×10^{-5} M	50%	100%
5		3.16×10^{-5} M	50%	100%
7		5×10^{-4} M	25%	100%
8		not tested*		
12		5×10^{-4} M	29%	100%
13		not tested*		

* Due to its high unstability this product was not biologically tested.

Unidirectional urea flux were measured after addition of [^{14}C]-urea to the mucosal compartment measuring the [^{14}C]-urea appearance in the serosal medium. Before inhibition experiments, the preparation was stimulated by 4.4 x10^{-8} M oxytocin added at the serosal side. Inhibitors were introduced at different times and at different concentrations in the mucosal compartment and a percentage of inhibition could be calculated. Results for the synthesized urea analogues are collected in table 1.

The inhibition constants (IC_{50}) of these photoactivable analogues were found between 10^{-5} and 10^{-3} M. Analogue **5** proved especially interesting with an IC_{50} comparable to the Ki value obtained by Mayrand[9] with the not photoaffinity molecule, 1-(3,4-dichlorophenyl)-2-thiourea. When **4** or **5** was applied at a concentration of 0.1 mM to the mucosal side of frog urinary bladder (previously stimulated with oxytocin), then irradiated for 30 mn with a polychromatic beam, an inhibition of 70 % of the urea flux was observed. The mucosal medium was finally replaced by one lacking compound **4** or **5**, the urea flux was not increased showing that the inhibition was irreversible.

As a conclusion, we have synthesized a series of photoactivable thiourea analogues as potential probes for the urea channel. The tritium labelled compound **5** with the lowest IC_{50} should be useful in attempt to biochemically characterize the urea channel.

ACKNOWLEDGEMENTS

We thank Mrs L. Sergent for NMR experiments, Mrs I. Rochas for liquid scintillation counting and infrared spectra, Mr A. Valleix for mass spectra and Dr T. Le Gall for critical reading of this paper. We express our gratitude to Dr Charles Mioskowski for valuable discussions.

REFERENCES AND NOTES

1. Brahm, J. *J. Gen. Physiol.* **1983**, *82*, 1-23.
2. Macey, R.I.; Farmer, R.E.L. *Biochem. Biophys. Acta* **1970**, *211*, 104-106.
3. Owen, J.D.; Solomon, A.K. *Biochem. Biophys. Acta* **1972**, *290*, 414-418.
4. Mayrand, R.R.; Levitt, D.G. *J. Gen. Physiol.* **1983**, *81*, 221-237.
5. Bayley, H. *Laboratory Techniques in Biochemistry and Molecular Biology*, T.S. Work and R.H. Burdon editors **1983**, *12*.
6. All new compounds provided mass spectra in accord with the molecular weight. IR spectra displayed the 2120 cm^{-1} azido band and ^{13}C NMR displayed the thiourea peak (δ 182)
   ^{1}H NMR :
   - **1** δ 7 (d, C_2-H, C_6-H, J=8Hz), δ 7.42 (dd, C_6-H, J=8Hz, J'=3Hz), δ 7.5 (d, C_3-H, C_5-H, J=8Hz), δ 7.6 (d, C_5-H, J=8.5Hz), δ 7.9 (d, C_2-H, J'=3Hz), δ 10 (s, 2x NH).
   - **4** δ 7.2 (d, C_6-H, J=9Hz), δ 7.4 (d, C_5-H, J=9Hz), δ 7.75 (s, C_2-H), δ 9.9 (s, NH), the NH_2 signal was masked by the aromatic protons and disappeared on the addition of D_2O.
   - **5** δ 3.1 (d, N-CH_3, J=5Hz), δ 6 (s, NH), δ 6.95 (d, C_6-H, J'=8Hz), δ 7.05 (s, C_2-H), δ 7.4 (d, C_5-H, J'=8Hz), δ 7.8 (s, NH); ^{13}C NMR (300 MHz) δ 182 thiourea, δ 116, 122, 124, 132, 135, 139 aromatic carbons.
   - **7** δ 7 (d, C_2-H, C_6-H, J=8Hz), δ 7.4 (d, C_3-H, C_5-H, J=8Hz), δ 6-7.8 (NH_2 thiourea), δ 9.7 (s, NH).
   - **12** δ 3.1 (d, NCH_3, J=6Hz, with D_2O s), δ 6.85 (dd, C_6-H, J=9Hz, J'=3Hz), δ 7.25 (d, C_4-H, J'=3Hz), δ 7.5 (d, C_7-H, J=9Hz), δ 10.9 (s, CH_3N-H), δ 11 2 (s, 2 NH).

7. Collings, P.; Al Mallah, K.; Stedman, G. *J. Chem.Soc. Perkin Trans. 2* **1975**, 1734.
8. Meyer, T.A.; Illiam, D.L.H. *J. Chem.Soc. Perkin Trans. 2* **1981**, 361.
9. Al Mallah, K.; Collings, P.; Stedman, G. *J. Chem.Soc. Dalton Trans.* **1974**, 2469.
10. Boschetti, A.; Tellenbach, M.; Gerber, A. *Biochem. Biophys. Acta* **1985**, *810*, 12-19.
11. UV spectra displayed λ_{max}=368 nm for diazonium in ethanol.
12. Adams, R.; Bachmann, W.E.; Blatt, A.H.; Fieser, L.F.; Johnson, J.R. *Organic Reactions*, John Wiley and Sons, Inc.: New York 1949; vol. 5 (tome 2) Chap. 4, pp. 198-206.
13. Paget, C.J. *J. Med. Chem.* **1969**, *12(5)*, 1010.
14 Ganbaum, H.; Martin, D. *J. Prakt. Chem.*. **1982**, B 324, *5*, 809-815.
15. Martial, S.; Neau, P.; Dégeilh, F.; Lamotte, H.; Rousseau, B.; Ripoche, P. *Eur. J. Physiol.* **1993**, *423*, 51.
16. Parisi, M.; Bourguet, J.; Ripoche, P.; Chevalier, J. *Biochem. Biophys. Acta* **1979**, *556*, 509-523.