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SYNTHESIS OF A CHEMILUMINESCENT PROBE USEFUL FOR THE PURIFICATION OF STEROID 5α-REDUCTASE

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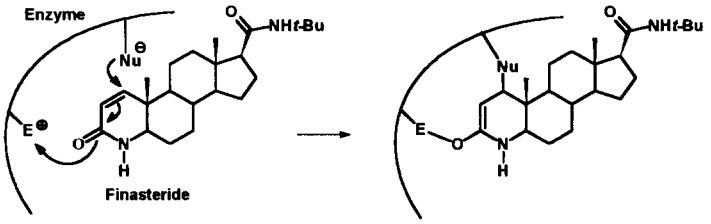
Abstract: Steroid 5α-reductase is a system of two isozymes (5αR-1 and 5αR-2), which is related to several human diseases. The synthesis of a chemiluminescent probe (AZA-LU) useful for purification of 5αR is described. AZA-LU has a chemiluminescence detectable up to 10^{-15} M and is a good inhibitor (IC_{50} = 0.5 μM) for rat 5αR-1 expressed in transformed *Saccharomicyes cerevisiae* yeast. Incubation of AZA-LU with yeast lysates, followed by SDS-PAGE, led to the detection of two luminescent protein regions with estimated molecular weights of 26 kDa and 21 kDa. Copyright © 1996 Elsevier Science Ltd

The steroid 5α-reductase is a family of two isozymes, named type 1 (5αR-1) and type 2 (5αR-2), which catalyses the NADPH-dependent reduction of testosterone (T) to dihydrotestosterone (DHT); also other 3-oxo-4-ene steroids (e.g., progesterone, corticosterone, etc.) may be reduced by these isozymes. The 5α-reductase and its product DHT play an important role in the pathogenesis of several human diseases, e.g. benign prostatic hyperplasia (BHP), prostatic cancer, acne, alopecia, pattern baldness in men and hirsutism in women. The discovery of potent and selective inhibitors for the two isozymes appears of great importance for the pharmacological treatment of these human diseases.[1-3] The knowledge of the enzyme structure would be of significant help in order to design new inhibitors. The primary sequences of both 5αR-1 and 5αR-2 proteins have been determined after the isolation and characterization of their c-DNA's; [4] however, their complete structural determination has been impossible so far, owing to the difficulties in the isolation and purification of these two isoenzymes from normal and pathological tissues,[5-8] or even from mammalian or non mammalian cells transfected with the corresponding cDNA's.[9,10] This difficulty arises from the small amount of these enzymes normally present in cellular systems and from the loss of the activity during the intensive purification of the protein. The first of these limiting factors has been solved by using non mammalian cells (the yeast *Saccharomyces cerevisiae*) transformed with expression plasmids bearing cDNA's coding respectively for each of the two isozymes 5αR-1 and 5αR-2; this allows the production of high levels of enzymatic proteins. In this system, the enzymes produced represent up to 1% of total yeast proteins.[11,12] Attempts to solve the second problem (i.e. an intensive purification of the protein without loss of activity) have been made by several groups[13-17] using techniques, aimed at stabilizing the protein by reconstitution of an environment similar to the

nuclear membrane in which the enzyme is usually deeply embedded. However, the possibility to produce and purify an amount of protein sufficient for crystallization and X-ray determination appears at the moment of difficult realization with these techniques. The formation of stable complexes between the protein and photoreactive labeled substrates,[18] inhibitors[18,19] or cofactors [20] represent a new approach, which could help to solve the problem of enzyme purification. By photolabeling the rat 5αR-1 isozyme with a ^{32}P-labeled photoreactive cofactor, Bhattacharyya et *al* [21,22] have isolated and purified the protein in amount sufficient to identify the cofactor binding domain. However, to date, no information has been obtained by this approach on the substrate binding domain, or on the structure of the enzyme.

In this paper we describe the formation of stable enzyme-inhibitor complexes of rat 5αR-1, obtained by using an irreversible inhibitor which had been previously conjugated with a chemiluminescent molecule. The labeling of proteins with chemiluminescent molecules is a well established technique which represents a real alternative to the use of radioactive labels. The applications of chemiluminescence technique are spread over many different fields, their common features being the extreme sensitivity of the chemiluminescence-based determinations and the complete safety. [23,24] The use of a specific and irreversible 5αR inhibitor bound to a chemiluminescent marker, instead of the direct linking of a photoreactive probe to the enzyme, in order to obtain a covalently bound enzyme-inhibitor complex, should increase the specificity of the complex formed, because all non specific linkages with other proteins should be absent. This could therefore improve the efficiency of the purification and decrease the number of steps involved.

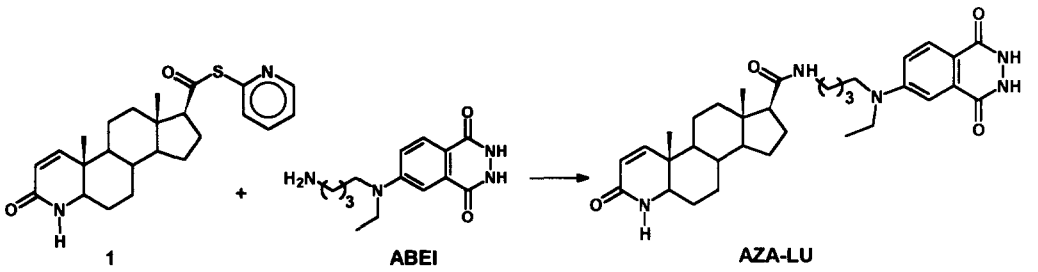
Among the several 5αR inhibitors tested, some of them showed a time-dependent inhibition which is an indication of irreversibility. Finasteride, or MK-906 (17β-(N-*ter*-butylcarbamoyl)-4-aza-5α-androstan-1-en-3-one), is a time-dependent, irreversible inhibitor of both isoforms of human steroid 5αR, but more active against the 5αR-2 isoform and therefore used for the treatment of BHP.[25] Its *in vitro* and *in vivo* potency has been explained on the basis of an irreversible enzyme inhibition.[26-28] This is probably due to the formation of a covalent linkage between an electrophilic residue of the protein with the carbonyl group in the 3 position of finasteride subsequent to the attack to the electrodeficient position 1 by a nucleophilic residue of the protein.[29] (Fig 1).



The stability of this covalent linkage with the human 5αR has been proved under a variety of denaturing conditions and appears specific for the 4-azasteroids which have, like finasteride, a double bound in 1-2 position.[27, 29] On the basis of these observations, we have planned the synthesis of a chemiluminescent probe for a covalent linkage to the 5αRs; the approach selected was a modification of position 17 of finasteride, which allowed the introduction of a chemiluminescent group instead of the *ter*-butylamine group. It is known that the potency of steroidal 5αR inhibitors depends on the skeleton structure and the type of substitution at position 17.[30] Several azasteroids, substituted at the C-17 position with groups of different structure and size,[31] are accepted by the enzyme without loss of inhibitory potency and maintain their irreversible mode of action.[32]

These findings suggested us the hypothesis that the introduction of the chemiluminescent group at position 17 of finasteride could be compatible with the maintainance of the inhibitory activity.

The synthesis of this compound, named AZA-LU, was achieved by reaction of 17β-thiopyridyl ester **1**[33] with aminobutylethylisoluminol (ABEI), a potent chemiluminescent compound.[34] (Fig 2).



The compound AZA-LU was purified by column chromatography followed by HPLC and full characterized by NMR and FAB mass-spectrometry.

The luminescence of AZA-LU was measured with a luminometer by automatic addition of NaOH, H_2O_2 and micro-peroxidase. Under these conditions, the hydrolysis and oxidation of the phtaloylhydrazide group, present in the chemiluminescent molecule, occurs; this is accompained by emission of light which can be detected by a luminometer and transformed into an electronic signal (which is expressed in counts, an arbitrary unit). The luminescence of AZA-LU alone or in the presence of a protein [bovine serum albumin (BSA), 5ng] was linear in the range of 10-100 fmol (10^{-15} M) (Fig. 3). A decrease of the chemiluminescence of AZA-LU alone (which was maintained in the presence of BSA) was observed when compared to the chemiluminescence of ABEI; however, the chemiluminescence of AZA-LU appears to be sufficient for its application as a probe for the 5αR.[35]

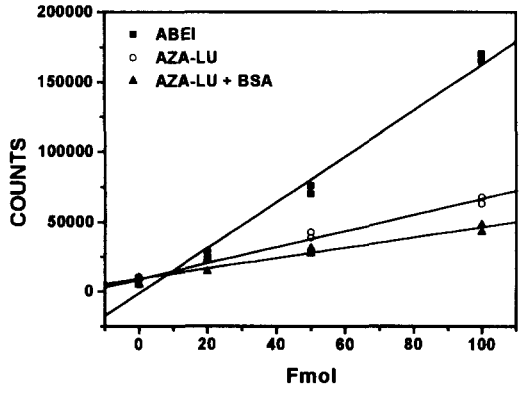


Fig. 3 Chemiluminescence of ABEI, AZA-LU and AZA-LU + 5 ng of BSA.

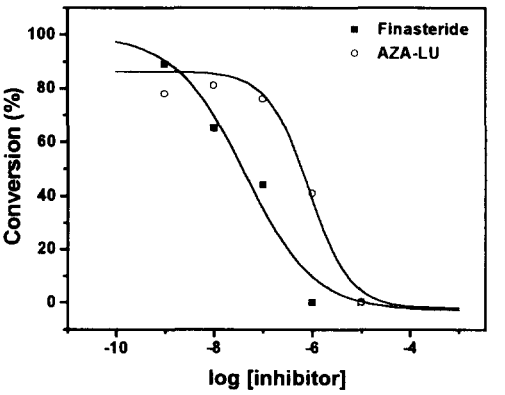


Fig. 4 Inhibition curves of AZA-LU and Finasteride towards rat 5αR-1 expressed in transformed yeast *Saccharomyces cerevisiae*.

The inhibitory potency of AZA-LU was measured, in comparison with finasteride, using the rat 5αR-1 expressed by the transformed yeast *Saccharomyces cerevesiae*.[12] For the inhibition experiments, the transformed yeast cells were grown, harvested and then treated with a specific glucuronidase to produce spheroplasts. These were then lysated by osmotic shock with hypotonic solution. An amount of lysates corresponding to 25 μg of protein was incubated with NADPH (100 μg) and labelled testosterone (2 μM of T containing 1μCi of [^{3}H]-T) in the presence or absence of a different amount of inhibitors. AZA-LU showed an inhibition potency (IC_{50} = 0.5 ± 0.2 μM) lower than that of finasteride (IC_{50} = 40 ± 18 nM), with a ratio of 12 between the two inhibition potencies (Fig. 4). However, the decrease of inhibition potency induced by the introduction of chemiluminescent group does not prevent the use of AZA-LU as probe for the 5αR.

The irreversible formation of a stable complex between the rat 5αR-1 and AZA-LU was achieved in the following experimental conditions. A sample of 1.5 ml of yeast cell lysates (containing about 10 mg of proteins) was centrifuged at 50,000 xg for 20 min. The surnatant was discarded and the pellet (containing 0.8 mg of proteins) was resuspended in 200 μl of TRIS buffer (pH 7.5) and divided in two identical portions; the two suspensions were separately incubated for 30 min at 37°C with NADPH (0.2 mg) in the presence of AZA-LU (0.1mM) (sample 1) or in absence of AZA-LU (sample 2). The same amount of AZA-LU was incubated, under the same conditions with the lysates derived from untrasformed yeast cells (sample 3), as control. The incubation was stopped by cooling at -20°C and the samples 1-3 were repeatly centrifuged at 50,000xg for 20 min. The surnatants (containing in samples 1 and 3 a great part of non bound AZA-LU) were discarded, the pellets were resuspended and denaturated in SDS-PAGE sample buffer,[36] and electrophoresed on 12% SDS-PAGE (400 A, 200 V, 45 min). The resolved proteins were then electrotransferred to nitrocellulose and stained with Ponceau staining to determine the correct molecular weight by comparison to protein standards. The 3 different lanes were loaded with equal amounts of proteins. The bands of the proteins observed in the different lanes (1-3) were removed by cutting a 3 mm large nitrocellulose portion; each nitrocellulose fragment was heated with NaOH 2N at 50°C for 30 min directly in the luminescence assay tubes. The chemiluminescence of each protein band was measured by a luminometer (Berthold Biolumat LB9500, Wilbad, Germany) with 10 sec of delay partial integration after the automatic addition of 100 μl of micro-peroxidase (0.01 mM) and 100 μl of H_2O_2 (0.3% w/v).

Table. Chemiluminescent protein regions after SDS-PAGE of the samples 1-3

Protein regions (kDa)	Sample 1 (Counts)	Sample 2 (Counts)	Sample 3 (Counts)
21	273900	22900	11000
26	129300	18000	9400
45	11600	8700	8500
66	11300	9800	9000
97	8700	8200	8000

The results (shown in the Table) indicate that in sample 1, in which the yeast lysate containing 5αR-1 was incubated with AZA-LU, the majority of the chemiluminescence was found associated to two portions of the nitrocellulose corresponding to the migration regions of the standards 21 kDa (63% of the total luminescence of sample 1) and 26 kDa (30% of the total luminescence); only the 7% residual luminescence was spread in the migration regions of the other protein bands centered at 45 kDa, 66 kDa and 97 kDa. A completely different distribution pattern of the chemiluminescence signals was observed in the samples in which the addition of the chemiluminescent probe was obmitted (sample 2) or in those performed on yeast cells lacking the 5αR-1

enzyme (untrasformed cells, sample 3). Moreover, the chemiluminescence values observed in the regions of 45, 66 and 97 kDa of sample 1 were not significatively different from the luminescence detected in the same regions of nitrocellulose of the experiment controls 2 and 3. In all these regions the luminescence levels were very close to the background luminescence (estimated as 8000-10000 counts).[37]

The present data show that the chemiluminescent probe has allowed the identification of two protein bands centered at 21 and 26 kDa. These results agree with the previous studies of Enderle-Schmidt *et al.*[18] which have also found two labeled proteins at 20 kDa and 26 kDa by photoaffinity labeling of nuclei and enriched nuclear membrane fractions of rat ventral prostate with [4-^{14}C]testosterone or [1,2-^{3}H]21-diazo-4-methyl-4-aza-5α-pregnane-3,20-dione. In a different experiment, Bhattacharyya *et al.*[22] have isolated by preparative gel electrophoresis in 8% SDS-PAGE a 26 kDa protein by photoaffinity labeling of rat liver microsomes with [2'-^{32}P]-2-azido-NADP$^+$; the apparent molecular mass of 26 kDa is consistent with a photolabeled rat 5αR-1 protein. The chemiluminescence-labeled band detected in our experiment at 26 kDa may therefore correspond to the electrophoretic mobility of the 5αR-1 isozyme. This protein has an estimated molecular mass of 29 kDa on the basis of cDNA sequence analysis;[38] the difference between predicted and found molecular mass of rat 5αR-1 could be due to an aberrant electrophoretic mobility on SDS polyacrylamide gel in relation to the high hydrophobic amino acid content of this protein.[39] The presence of the chemiluminescence associated to the diffuse protein band spread around 21 kDa could be due, in our case, to a proteolytic degradation of the protein bound to AZA-LU.[40] We can exclude that this luminescence may derive from the presence of free AZA-LU, because in the lane 3 of the control experiment no significant luminescence was found in the corresponding portion of the gel.

In conclusion, the detection of the chemiluminescent protein band centered at 26 kDa only in the experiment where AZA-LU and 5αR-1 enzyme were both present is consistent with the formation of a stable complex between the chemiluminescent probe AZA-LU and the rat 5αR-1 protein. The compound AZA-LU is very easy to synthesize, also in large amount, is completely safe, and detectable at very low concentrations. It is an irreversible inhibitor of rat 5αR-1, forming an enzyme-inhibitor complex detectable at 10^{-15} M concentration, which is stable in various conditions in which the proteins are usually denaturated. The formation of this complex seems to be very specific because it is associated to the irreversible process of enzyme inhibition, and occurs only in the presence of rat 5αR-1. These features make it an effective probe for the substrate binding domain of 5αR and an effective marker during the purification of the enzyme. A study on the application of this probe for the purification of 5α-reductase isozymes is in progress.

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34. Aminobutylethylisoluminol (ABEI) was purchased from Sigma, St.Louis MO (USA).
35. Twenty fmol of AZA-LU in the presence of 5 ng of BSA gave a chemiluminescence (18500 counts) which is significantly higher than the background luminescence (8000 counts).
36. The buffer gel contains 0.2 ml (0.05 % w/v) of bromophenol blue, 0.4 ml of 2-β-mercaptoethanol, 1.6 ml of SDS 10%, 0.8 ml of glycerol and 1 ml of TRIS-HCl at pH 6.8 in 28 ml of water.
37. The slight increase of chemiluminescence observed in the protein bands centerd at 21 and 26 kDa of lane 2, could derive by a little imprecision in the cutting the strong chemiluminescent bands of the adjacent lane 1.
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