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Fluorescent and Photoaffinity Labeling Probes for Retinoic Acid Receptors

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Summary: A fluorescent probe for retinoid receptors (RARs) was designed and prepared. The probe consists of a retinoid moiety and a dansyl moiety, i.e., 2-[3-(5-dimethylaminonaphthalene-1-sulfonyl)-aminopropyl-1-oxy]-4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carboxamido]benzoic acid: DAM-3. DAM-3 specifically bound RARs. Additionally, a photoreactive RAR fluorescent probe was designed and prepared, i.e., 2-[3-(5-azidonaphthalene-1-sulfonyl)aminopropyl-1-oxy]-4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carboxamido]benzoic acid (ADAM-3). ADAM-3 irreversibly and specifically bound RARs using ultraviolet irradiation. © 1991 Academic Press, Inc.

Retinoic acid is significantly involved in the control of cell proliferation, cell differentiation and embryonic development.[1] The genes for the three classes of retinoic acid receptors (RARs) have been characterized in humans and mice, with these genes being denoted as RAR-α, RAR-β, and RAR-γ.[2-5] RARs are ligand-inducible transcriptional enhancer factors which belong to the steroid/thyroid hormone receptor superfamily, and are believed to mediate the action of retinoids at the gene expression level.[2-7] RAR-proteins will undoubtedly give clues which clarify the retinoidal action molecular mechanisms, thus their purification/characterization is considered to be essential. However, there is limited information concerning the RAR-proteins themselves. RAR protein purification/characterization has not yet been performed in detail due to their instability and low abundance, therefore probes are desired which can stably

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label RARs in order to allow high sensitivity detection after complex formation.

A large number of retinoic acid analogs (retinoids) have been prepared.[5] We have also developed a series of novel synthetic retinoids, i.e., retinobenzoic acids,[8-9] which have proven to be useful tools in the investigations of retinoidal action molecular mechanisms.[10-12] Tritium-labeled retinobenzoic acids[10-12] and affinity chromatography gels liganded with retinobenzoic acid skeletons[13] have been developed, and have partially fulfilled the requirement for obtaining superior RAR-purification/characterization probes, yet they have not been effectively applied to either large scale or multistep-analyses. This led to the presented study focused on characterizing RARs as stable fluorescent complexes formed after covalent labeling with a suitable probe, with initial developmental results being discussed which utilize this new technique.

MATERIALS AND METHODS

2-[3-(5-Dimethylaminonaphthalene-1-sulfonyl)aminopropyl-1-oxy]-4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-carboxamido]benzoic Acid (DAM-3): 2-(Aminopropyloxy)-4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carboxamido]-benzoic acid (aminopropyloxy-Am580) was prepared as previously described.[13] Coupling of aminopropyloxy-Am580 with dansyl chloride in CH_3CN gave DAM-3 (y:30%). mp.121-122℃. FAB/MS($M+H^+$): 658. ^{1}H-NMR($CDCl_3$): 1.31 (s,6H), 1.37 (s,6H), 1.73 (s,4H), 2.02 (m,2H), 2.85 (s,6H), 3.11 (q,2H, J=6.0Hz), 4.24 (t,2H, J=6.0Hz), 6.10 (s,1H), 6.95 (dd,1H, J=2.0, 8.0Hz), 7.11 (d,1H, J=8.0Hz), 7.43 (d,1H, J=8.0Hz), 7.45 (dd,1H, J=8.0Hz), 7.51 (dd,1H, J=8.0 Hz), 7.56 (dd,1H, J=2.0, 8.0Hz), 7.87(d,1H, J=2.0Hz), 7.90 (d, 1H, J=2.0Hz), 7.97 (s,1H), 8.08 (d,1H, J=8.0Hz), 8.26 (d,1H, J= 8.0Hz), 8.34 (d,1H, J=8.0Hz), 8.51 (d,1H, J=8.0Hz).

2-[3-(5-Azidonaphthalene-1-sulfonyl)aminopropyl-1-oxy]-4-[5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carboxamido]-benzoic Acid (ADAM-3): 5-Azidodansylsulfonic acid sodium salt was converted to acid chloride by $SOCl_2$. Then the acid chloride was condensed with aminopropyloxy-Am580 to give ADAM-3 (y: 23%, mp.63-65℃). FAB/MS($M+H^+$): 656. ^{1}H-NMR($CDCl_3$): 1.31 (s,6H), 1.33 (s,6H), 1.73 (s,4H), 1.95 (m,2H), 3.16 (m,2H), 4.07 (t,2H, J=5.5 Hz), 6.90 (dd,1H, J=2.0, 8.5Hz), 7.24 (d,1H, J=7.5Hz), 7.42 (d, 1H, J=8.0Hz), 7.49 (dd,1H, J=8.5Hz), 7.51 (dd,1H, J=8.5Hz), 7.57 (dd,1H, J=2.0, 8.0 Hz), 7.85 (d,1H, J=2.0Hz), 7.88 (d,1H, J=2.0 Hz), 8.09 (d,1H, J=8.5Hz), 8.20 (s,1H), 8.30 (d,1H, J=7.5Hz), 8.31 (dd,1H, J=2.0, 8.0Hz), 8.59 (d,1H, J=8.5 Hz).

Preparation of a Dansyl Group Bearing Analog of Retinoic Acid, DRA-6: Methyl-4-oxo-retinoate prepared by oxidation of methyl-retinoate with MnO_2 was oximated by NH_2OCH_2COOH, and then converted to activated ester by N-hydroxysuccinimide. The activated ester was then condensed with dansylethylenediamine to give DRA-6. mp.114-115 ℃. ^{1}H-NMR($CDCl_3$): 1.11 (s,6H), 1.64 (t,2H, J=7.0 Hz), 1.87 (s,3H), 2.03 (s,3H), 2.37 (d,3H, J=1.1Hz), 2.69 (t,2H, J=7.0Hz), 2.89 (s,6H), 3.05 (dt,2H, J=6.0, 6.0Hz), 3.40 (dt,2H, J=6.0, 6.0Hz), 5.25 (t,1H, J=6.0Hz), 5.82 (s,1H), 6.22 (d,1H, J= 11.5Hz), 6.24 (d,1H, J=16.5Hz), 6.33 (d,1H, J=16.5Hz), 6.34 (d, 1H, J=15Hz), 6.68 (t,1H, J=6.0Hz), 7.03 (dd,1H, J=11.5, 15.0Hz), 7.19 (d,1H, J=7.5Hz), 7.51 (dd,1H, J=7.5, 9.0Hz), 7.57 (dd,1H, J

=7.5, 9.0Hz), 8.21(dd,1H, J=1.0, 7.5Hz), 8.24 (d,1H, J=9.0Hz), 8.55 (d,1H, J=9.0Hz).

Preparation of RAR-α Fraction From HeLa Cells: RAR-α fraction was prepared as previously described,[11,12] and was used after dilution and/or concentration to adjust the sample's salt concentration to 0.15 M. The fraction thus prepared contained RAR-α with less than 10% contamination of RAR-β.

Binding Competition Assay: The incubation mixture contained [^{3}H]Am80 (1 nM), the RAR-α fraction (1 mg protein/ml: 0.15 M KCl/NaCl - 20 mM Tris (pH 8.0)), and various concentration of test compounds. A binding competition assay using [^{3}H]Am80 was performed as previously described.[10-13]

Dextran-coated Charcoal Method: A previously reported dextran-coated charcoal method was used to determine the bound amounts of the fluorescent probes and of ADAM-3.[14] Briefly, either DAM-3 or ADAM-3 (1μ M) were incubated (4℃, 5 hrs) with the RAR-α fraction (1 mg protein/ml: 0.15 M KCl/NaCl - 20 mM Tris (pH 8.0) - 10%v/v DMSO) in the presence or absence of 20μ M of Am80, with the incubation mixture (0.5 ml) having added to it 0.2 ml of a dextran-coated charcoal mixture [1% activated charcoal and 0.1% dextran]. The mixture was allowed to stand on ice for exactly 1 min and centrifuged (10^4 rpm, 5 min), with the supernatant's fluorescent intensity then being measured.

RAR Photoaffinity Labeling with ADAM-3: After incubation of ADAM-3 and the RAR-α fraction, the mixture was irradiated at ice-cold conditions for 10 min using a high pressure mercury lamp (450 W). For liberation of the non-covalently bound ADAM-3 or its photo-decomposed product(s), the mixture was then treated for 1 min with 0.1% SDS at 100℃. The determination of the bound amounts of ADAM-3 was performed by dextran-coated charcoal method exactly as described above.

RESULTS AND DISCUSSION

RAR Fluorescent Probes

Two types of RAR fluorescent probes were initially developed, both consisting of a biologically active retinoid moiety and a fluorescent dansyl moiety. The retinoid moiety was selected to be 4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carboxamido]benzoic acid (Am580, a potent retinobenzoic acid)[8] and retinoic acid, both of which can be converted to a respective dansyl derivative, i.e., DAM-3 and DRA-6 (Fig. 1). It was found using the analysis of the structure-activity relationships of retinobenzoic acids that the hydrophobic part (corresponding to the tetrahydrotetramethylnaphthalenyl moiety of Am580) and the carboxyl group are essential for RAR binding. In addition, the structural correlation (superimposition) between retinoic acid and Am580 has clarified that Am580's tetrahydrotetramethylnaphthalenyl moiety corresponds to retinoic acid's trimethylcyclohexenyl moiety.[15] It was therefore believed that DRA-6 and DAM-3 could be respectively utilized as derivatives to introduce a dansyl moiety to a retinoid's hydrophobic and carboxyl parts. Both the DAM-3 and DRA-6 showed strong fluorescence (350 nm: excitation, 520 nm: emission).

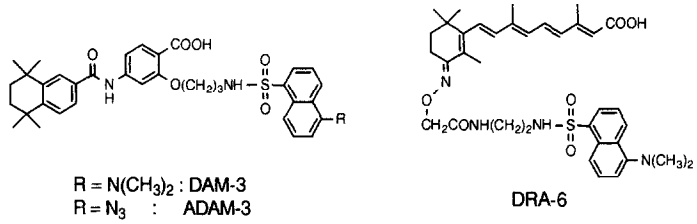

Fig. 1. Structures of DAM-3, DRA-6, and ADAM-3.

The DAM-3/DRA-6 binding activities with RAR-α fraction were examined using tritium labeled 4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbamoyl]benzoic acid ([^{3}H]Am80). Binding competition experiments using [^{3}H]Am80 are advantageous because Am80 does not efficiently bind cellular retinoic acid binding protein (CRABP).[6] Other types of retinobenzoic acids, (E)-4-[3-oxo-3-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (Ch80) and (E)-4-[3-(3,5-di-tert-butylphenyl)-3-oxo-1-propenyl]benzoic acid (Ch55),[9] were also used. The [^{3}H]Am80 binding competition assay results are shown in Table I. Specific [^{3}H]Am80 binding was determined by subtracting the amount of bound [^{3}H]Am80 measured in the presence of excess Am80 from that in the absence of Am80, with this value being defined as 100%. Notice no effective binding compe-

Table I. Results of Binding Competition Assay

Competitor	(M)	[^{3}H]Am80 Specifically Bound	Competitor	(M)	[^{3}H]Am80 Specifically Bound
none		100%	Ch80	(10^{-7})	63%
Am80	(10^{-8})	52%	Ch80	(10^{-6})	34%
Am80	(10^{-7})	14%	Ch80	(10^{-5})	1%
Am80	(10^{-6})	0%	zCh80	(10^{-7})	68%
DAM-3		79%	zCh80	(10^{-6})	0%
DAM-3	(10^{-5})	60%	zCh80	(10^{-5})	4%
DAM-3	(10^{-3})	-1%	Ch55	(10^{-9})	49%
DRA-6	(10^{-6})	102%	Ch55	(10^{-8})	20%
DRA-6	(10^{-5})	96%	Ch55	(10^{-7})	-4%
DRA-6	(10^{-4})	89%	zCh55	(10^{-9})	36%
ADAM-3	(10^{-6})	62%	zCh55	(10^{-8})	18%
ADAM-3	(10^{-5})	42%	zCh55	(10^{-7})	-2%
ADAM-3	(10^{-3})	-5%			

tition between DRA-6 and [^{3}H]Am80 is shown. Therefore, it is concluded that DRA-6 do not effectively bind to RAR-α, and accordingly cannot be used as RAR fluorescent probes. On the other hand, specific binding of [^{3}H]Am80 was completely inhibited by addition of 1 mM of DAM-3, and because this binding was also similarly inhibited by Ch80/Ch55, the observed [^{3}H]Am80 binding represents binding to RAR-α and not to Am80-specific binding protein(s), if any, or to CRABP. Using the DAM-3 concentration necessary to cause 50% inhibition of the [^{3}H]Am80 binding, the binding constant to RAR-α was estimated to be on the order of 10^6 M^{-1}.

Since retinoic acid and Am580 are spatial superimposable at their carboxyl and hydrophobic parts,[15] the observed results showing inefficient DRA-6 and efficient DAM-3 binding to RAR-α indicate that the introduction of a large group into the hydrophobic part hinders ligand binding to RAR-α. It is therefore suggested that the ligand's hydrophobic part interacts with RAR-α at the protein's interior site, whereas the carboxyl group interaction site is located near the RAR protein's surface.

The capability of using DAM-3 as fluorescent probes to detect RAR-α was examined next, with its bound amount being measured by the fluorescence of an incubation mixture which had previously had the unbound DAM-3 removed using the dextran-coated charcoal method.[14] As shown in Fig. 2, the DAM-3 binding was

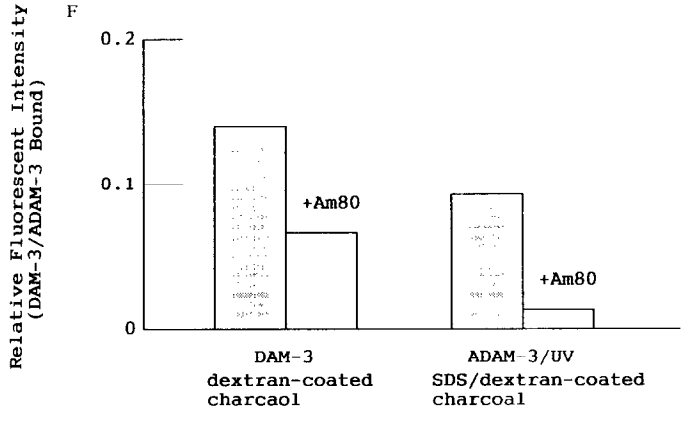

Fig. 2. Binding of DAM-6 and ADAM-3.
Vertical scale: Amounts of bound DAM-3 or ADAM-3 measured by means of relative fluorecence intensity. Free DAM-3 was removed by dextran-coated charcoal method. Free ADAM-3 was removed by dextran-charcoal method after ultraviolet irradiation and the SDS treatment. Excited at 350 nm, emission at 520 nm. Shaded bar: Incubated with DAM-3/ADAM-3 alone. Open bar: Incubated with DAM-3/ADAM-3 in the presence of excess amounts of Am80.

in competition with Am80, although this specific binding disappeared when the cell extract sample was treated with antisera raised against RARs (data not shown), thus indicating that DAM-3 and Am80 bind to RAR-α in a mutually competitive manner. The quantity/concentration detection limit of the RAR-α /DAM-3 complex was approximately 100 fmole/100 pM, thereby indicating the advantages of using DAM-3 as a fluorescent probe for RAR-α (and possibly for other RAR-subtypes).

Photoaffinity Labeling of RARs

The DAM-3 was found to be useful as a lead compound for fluorescent photoaffinity RAR labeling probes. We designed 2-[3-(5-azidonaphthalene-1-sulfonyl)aminopropyl-1-oxy]-4-[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carboxamido]benzoic acid (ADAM-3, Fig. 1). Under ultraviolet (UV) irradiation, ADAM-3 was stable in pure form, yet easily decomposed in the presence of high nucleophile concentrations, e.g., UV irradiation of aqueous ADAM-3 solution in the presence of phenol efficiently yielded an ADAM-3/phenol adduct (M^+ 721).

With respect to RAR-α binding, ADAM-3 competed with [^{3}H]-Am80 with almost the same efficieny as that of DAM-3 where the estimated association constant was in the order of 10^6 M^{-1} (Table I). UV irradiation of the RAR-α fraction did not affect the [^{3}H]Am80 binding to RARs under the experimental conditions used, nor did UV irradiation of [^{3}H]Am80 and/or Am80 change the RAR-α binding activity of these compounds. Surprisingly, UV irradiation of Ch80 and Ch55 did not affect the compounds' binding activity even though both Ch80 and Ch55 were isomerized under UV irradiation to (Z)-isomers. In fact, purified zCh80/zCh55 bound to RAR-α with almost the same efficiency as that of their (E)-isomers (Table I).

[^{3}H]Am80 and ADAM-3 bound to RAR-α was liberated by treating RAR-α /[^{3}H]Am80 and RAR-α /ADAM-3 complexes with sodium dodecyl sulfate (SDS), thus indicating non-covalent bonding. Bound [^{3}H]Am80 was similarly liberated by the same SDS treatment of UV irradiated RAR-α /[^{3}H]Am80 complex. However, approximately 70% of the bound ADAM-3 was not liberated by the same SDS-treatment after UV irradiation of RAR-α /ADAM-3 complex. In addition, ADAM-3's irradiation-dependent undissociable binding was efficiently reduced by pre-incubation of RAR-α fraction with ADAM-3 in the presence of Am80 (Fig. 2). These results strongly suggest that ADAM-3 covalently and specifically binds to RAR-α at the ligand binding site. Therefore, ADAM-3 is concluded to be a useful fluorescent RAR-α photoaffinity labeling agent. Iso-

lation, purification and characterization of RARs covalently bound with ADAM-3 are under investigation.

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