Available online at www.sciencedirect.com
ScienceDirect
Insect Biochemistry and Molecular Biology 37 (2007) 865–875
Insect Biochemistry and Molecular Biology
www.elsevier.com/locate/ibmb

A photoaffinity, non-steroidal, ecdysone agonist, bisacylhydrazine compound, RH-131039: Characterization of binding and functional activity

Tarlochan S. Dhadialla[a,*], Dat Le[b], Subha R. Palli[c], Alexander Raikhel[d], Glenn R. Carlson[e]

[a]*Dow AgroSciences, LLC, Indianapolis, IN 46268, USA*
[b]*PolySciences, Warrington, PA 18976, USA*
[c]*Department of Entomology, University of Kentucky, Lexington, KY 40546, USA*
[d]*Department of Entomology, University of California Riverside, CA 92521, USA*
[e]*305 Britt Road, North Wales, PA 19454, USA*

Received 5 March 2007; received in revised form 15 May 2007; accepted 16 May 2007

Abstract

In this paper we describe the synthesis, ligand-binding and functional activity characteristics of the photoaffinity, non-steroidal, ecdysone agonist, bisacylhydrazine compound, 3-benzoyl-benzoic acid N-tert-butyl-*N*′-(2-ethyl-3-methoxy-benzoyl)-hydrazide (RH-131039). Tritiated RH-131039 is the first non-steroidal photoaffinity compound that was shown to bind specifically to ecdysone receptors (EcRs) from insects belonging to the orders Diptera and Lepidoptera. The spruce budworm (*Choristoneura fumiferana*) ecdysone receptor (CfEcR) bound with high affinity ($K_d = 2.23 \pm 0.27$ nM) to this compound. When irradiated with UV light ($\lambda = 350$ nm) under equilibrium ligand-binding conditions, RH-131039 attached specifically and covalently to the CfEcR ligand-binding domain (LBD). RH-131039 also bound to cloned ecdysone receptor proteins from three dipteran insects, *Drosophila melanogaster*, *Aedes aegypti* and *Chironomous tentans*. This paper also describes and invokes caution in interpretation of ligand-binding results obtained using crude cellular extracts containing target receptors, as illustrated with the use of *Drosophila* Kc cells that have functional EcR and L57 cells (derivatives of Kc cells in which EcR-B isoforms have been knocked out by "parahomologous" recombination). Tritiated RH-131039 is a useful tool to dissect ligand-binding and functional differences for EcRs from different arthropod species.

Keywords: Photoaffinity labeling; Limited proteolysis; Ecdysteroid receptor; Non-steroidal ecdysone agonist; Photoaffinity ecdysone agonist; Dipteran EcR; Lepidopteran EcR

1. Introduction

With the discovery of the first non-steroidal ecdysone agonist, unsubstituted bisacylhydrazine (RH-5849) and cloning of the ecdysone receptor (EcR) from *Drosophila melanogaster* (Koelle et al., 1991) and many other arthropod species (Henrich, 2005; Palli et al., 2005), significant research interest was generated in the use of the non-steroidal ecdysone agonists in understanding their biological effects *in vivo* and *in vitro* (Oberlander et al., 1995; Dhadialla et al., 1998, 2005; Dhadialla and Ross, 2007) Since the discovery of the first insect active bisacylhydrazine, RH-5849 (Wing, 1988), four bisacylhydrazine compounds (tebufenozide, methoxyfenozide, halofenozide, and chromafenazide) have been commercialized (Hsu, 1991; Heller et al., 1992; RohMid, 1996; Yanagi et al., 2000; Carlson et al., 2001).

During insect development, growth and morphogenesis are regulated by the steroid hormone 20-hydroxyecdysone (20E) and the sesquiterpene juvenile hormones. Amongst the insects studied, the activity of 20E is manifested via interaction with nuclear receptor heterodimers consisting of the EcR (Koelle et al., 1991) and ultraspiracle proteins (USP, Oro et al., 1992; Henrich et al., 1990), which recognize specific ecdysone receptor response elements

*Corresponding author. Tel.: +1 317 337 3755.
E-mail address: tdhadialla@dow.com (T.S. Dhadialla).

0965-1748/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ibmb.2007.05.009

(EcRE) present in the promoter regions of 20E regulated genes (Yao et al., 1992; Yao et al., 1993). It is this stable interaction of the quaternary complex (20E, EcR/USP and EcRE) which brings about the transcription of 20E-responsive genes. Although all the data from ligand-binding experiments indicated that 20E binds to amino acid residues in the EcR ligand-binding domain (LBD) (Koelle et al., 1991; Yao et al., 1992, 1993; Dhadialla et al., 1998, 2006; Hu, 1998), the visual proof for this came from crystal structures of liganded and unliganded EcR LBDs from cotton bollworm, *Heliothis virescens*, and the sweet potato whitefly, *Bemesia tabacci* (Billas et al., 2003; Carmichael et al., 2005). While in both studies, the potent phytoecdysteroid, ponasterone A was used as the steroidal ligand, Billas et al. (2003) also co-crystallized the *H. virescens* EcR (HvEcR) LBD with a non-steroidal bisacylhydrazine compound. The results of this study confirmed the earlier hypothesis (Kumar et al., 2002, 2004) that 20E and active bisacylhydrazines occupy different but overlapping spaces in the ligand-binding pocket of EcRs.

Before the EcR crystal structures were published, there was a great deal of interest in understanding the 3D interaction of 20E or ponasterone A with EcR LBDs. This interest was further escalated after the molecular basis of the action of the non-steroidal bisacylhydrazine insecticidal compounds was understood (reviewed by Dhadialla et al., 1998, 2005; Dhadialla and Ross, 2007). Due to the novel mode of action of these insecticidal compounds, and in particular, the selective toxicity of tebufenozide, methoxyfenozide, and chromafenozide to predominantly lepidopteran insects, it was felt that by understanding the interaction of these compounds with specific amino acid residues in EcR LBDs, it would be possible to design and discover new ecdysone agonists using molecular modeling approaches for activity against pests from other insect orders such as Homoptera, Hemiptera, Orthoptera etc. Different labs adopted different approaches that included homology modeling of EcR LBDs based on crystal structures of vertebrate steroid receptors and ligand docking (Kumar et al., 2002, 2004) and using photoaffinity radiolabeled bisacylhydrazines (briefly discussed in Dhadialla et al., 2005) as well as ecdysteroids (Bourne et al., 2002) We pursued both these approaches. Here, we describe the synthesis of a photoaffinity radiolabeled bisacylhydrazine and characterization of its ligand-binding properties to several EcRs.

2. Materials and methods

Chemical synthesis and radio-labeling of the RH-131039 is described in the on-line supplementary material.

2.1. EcR and USP proteins

EcR protein from the spruce budworm, *Choristoneura fumiferana* (CfEcR) lacking the *N*-terminal A/B transactivation domain and full-length ultraspiracle protein (CfUSP) were expressed as GST fusions in the expression vector, pGEX-3X (Pharmacia, Bai d'Urfe, Quebec, Canada, Palli S.R., unpublished) for use in ligand-binding studies and photoaffinity labeling experiments. Truncated CfEcR proteins were produced as described previously (Perera et al., 1999a, b). *D. melanogaster* EcR and USP (DmEcR and DmUSP) proteins were produced in *E. coli* as His-Tag fusions from constructs provided by Drs Peter and Lucy Cherbas, Indiana University, IN. EcR and USP from the yellow fever mosquito, *Aedes aegypti* (AaEcR and AaUSP), were produced by in vitro transcription and translation of cloned AaEcR and AaUSP as described (Kapitskaya et al., 1996). *Chironomus tentans* EcR and USP (CtEcR, CtUSP) bacterial GST-fusion proteins were produced from cloned DNA encoding the two proteins, kindly provided by Professors Klaus and Margaret Spindler, University of Ulm, Germany.

2.2. Radiolabelling of EcR proteins and limited proteolysis experiments

Ligand-induced conformational changes in EcR were investigated using an ecdysteroid, muristerone A, and non-steroidal ecdysone agonists, RH-5992 and RH-131039, and ^{35}S-labelled DmEcR. DmEcR-GST fusion proteins were metabolically labeled by including ^{35}S-methionine (specific activity >1000 Ci/mmol; Perkin-Elmer, Shelton, CT) in in vitro transcription-and-translation reaction mixture (Promega, Madison, WI) using DmEcR-GST LBD cDNA cloned in a pGEM vector following manufacturer's instructions. From the resulting translation mixture, unincorporated ^{35}S-methionine was removed by centrifuging the reaction mixture in a micro-centricon tube (MWCO 10,000). In each experiment, bacterial fusion proteins of ^{35}S-labelled DmEcR and unlabeled DmUSP (1:10 ratio v/v) were incubated in the absence or presence of muristerone A, RH-5992 or RH-131039. Following equilibrium ligand–receptor binding, the reaction mixture was subjected to limited proteolysis with increasing amounts of proteinase K at room temperature. Proteolysis was stopped after 20 min by adding 2× SDS-PAGE sample buffer to the reaction mixture followed by heating the sample to 95 °C for 5 min. The samples were analyzed using SDS-PAGE and fluorography.

2.3. Radiometric ligand-binding assays

Ligand-binding assays using tritiated ponasterone A were performed as described earlier (Kapitskaya et al., 1996; Pereira et al., 1999a, b). Briefly, 1 μl each of bacterial extracts containing EcR or USP (tagged or not) were mixed in a final reaction volume of 100 μl buffer (10 mM Tris–HCl, pH 7.2, 1 mM DTT) and 2.39×10^{-9} M [^{3}H] ponasterone A (specific activity of 170 Ci/mmol; NEN, Boston, MA, now Perkin-Elmer, Shelton, CT), and incubated for 2 h at 4 °C in the absence (total binding) or presence of either 20E (10^{-4} M; non-specific binding) or