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Amiodarone and thyroid hormone receptors

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" ... further experiments which will be needed to explain satisfactorily
the Janus' face of amiodarone being capable of inducing
either thyrotoxicosis or hypothyroidism".
Wiersinga WM, Eur J Endocrinol. 1997; 137:15-7

Onderzoek doen is als reizen;
nieuwsgierig onderweg zijn in een gekozen richting,
naar onbekende plaats van aankomst.

Voor Jesper en Jasmijn

CHAPTER 1

Introduction

1.1. Amiodarone

1.1.A. Pharmacology of amiodarone

Amiodarone (AM) was synthesized in 1961 by Tondeur and Binon in the Labaz Laboratories in Belgium. Characteristic in the chemical structure of the drug is its high iodine content, two iodine atoms per molecule, and its resemblance to thyroxine. It was introduced in clinical medicine in 1962 for the treatment of angina pectoris. In the beginning of the seventies it was seldom prescribed because of the many side effects. However, it was recognized later that AM is a very potent antiarrhythmic drug, and it is now widely used for life-threatening arrhythmias resistant to other agents.

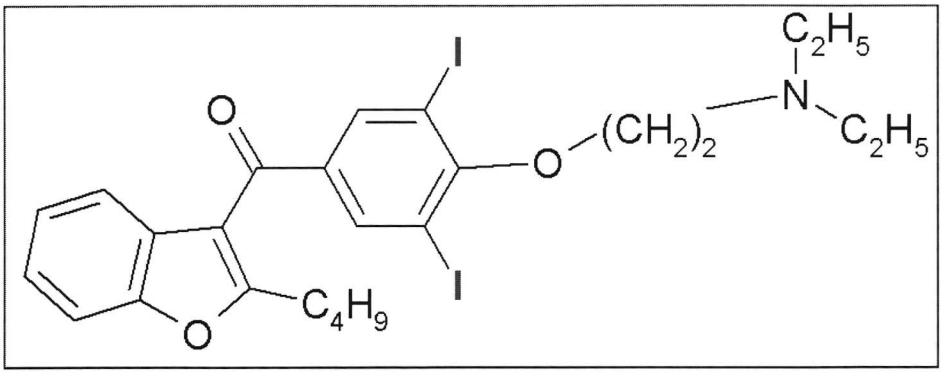


Fig 1: The molecular constitution of amiodarone.

Physicochemical properties. Amiodarone, chemical name: 2-butyl-3-[3, 5 diiodo-4(β-diethylamino-ethoxy) benzoyl] benzofuran, is a benzofuran derivative with two iodine atoms (Fig. 1). It is an amphiphilic drug with hydrophilic (tertiary amine) and lypophilic (benzofuran and di-iodinated benzene ring) moieties.

Amiodarone is poorly soluble in aqueous or polar media, but highly soluble in chloroform or other nonpolar organic solvents and well soluble in light polar organic solvents like ethanol. The pK_a of AM is 6.56, which is normal for a tertiary amine, indicating that, at physiological pH, amiodarone is essentially ionized. The molecular weight of amiodarone and its main metabolite desethylamiodarone (DEA) are 645.3 and 617.3 respectively.

Amiodarone as a di-iodinated benzofuran derivative has some structural similarities to thyroid hormones. The drug is prescribed as amiodarone

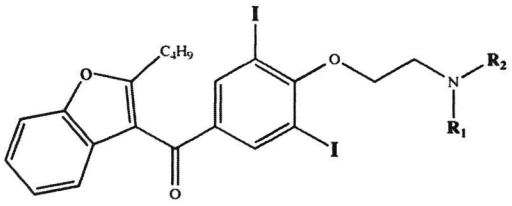
hydrochloride (MW 681.8), in the Netherlands known under the trade name Cordarone. Amiodarone contains 39.3% iodine by weight, amiodarone chloride 37.2%. A tablet of 200 mg amiodarone hydrochloride contains 75 mg iodine, a dose that via bio transformation in vivo releases an estimated 6 mg of iodide (1).

Tissue distribution. AM and DEA both accumulate in various tissues during long-term AM-treatment. Concentrations of AM and DEA in human tissues obtained at autopsy are reported in various studies (2;3): a typical example is given in Table 1. The highest concentrations of AM and DEA are found in adipose tissue, liver and lung; moderate levels occur in heart and kidney, and lowest levels in muscles, thyroid and brain. In all described tissues except adipose tissue, the DEA concentration is 4 -7 times higher compared to AM (Table 1) (2). The myocardial concentration of AM is 10-50 times higher that in plasma (3). As for the sub cellular distribution, AM medication induces cytoplasmic multilamellar inclusion bodies in many tissues including human myocardium (4). Evidence obtained from in vivo and in vitro studies indicate an intralysosomal localization for AM and DEA (5). This is not surprising because AM belongs to the group of cationic amphiphilic compounds that accumulate in lysosomes.

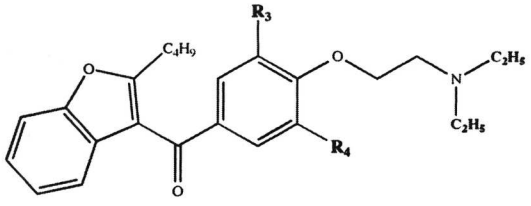
Table 1. Tissue distribution of amiodarone (AM) and desethylamiodarone (DEA) in human autopsies after long term AM-treatment

Tissue	AM µg/g	AM µmol/kg	DEA µg/g	*DEA µmol/kg*
Adipose tissue	316	490	76	*123*
Liver	391	606	2354	*3815*
Lung	198	307	952	*1543*
Kidneys	57	88	262	*425*
Heart	40	62	169	*274*
Muscle	22	34	51	*83*
Thyroid	14	22	64	*104*
Brain	*8*	*12*	*54*	*88*

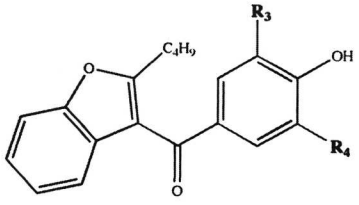
Figures are mean concentrations of seven to nine observations (Holt et al. 1983).



	R_1	R_2
amiodarone (A, L 3428)	C_2H_5	C_2H_5
desethylamiodarone (DEA, L 33520)	C_2H_5	H
desdiethylamiodarone (DDEA, L 33530)	H	H



	R_3	R_4
amiodarone	I	I
monoiodoamiodarone (MIA, L 6355)	I	H
desdiiodoamiodarone (DDIA, L 3937)	H	H



	R_3	R_4
L 3373	I	I
L 6424	I	H
L 3372	H	H

Fig. 2. The molecular constitution of amiodarone analogues, deethylated analogues (top), deiodinated analogues (middle), and benzofuran derivatives (bottom)

Metabolism. The two most important metabolic pathways of amiodarone are N-dealkylation and deiodination. These two pathways alone or in combination lead to the metabolites presented in Fig. 2.
N-dealkylation of amiodarone results in the major metabolite desethyl-amiodarone (DEA). Subsequent N-dealkylation leads to the primary amine desdiethylamiodarone, which has been detected in dogs but barely in humans. Deiodination of amiodarone results in monoiodoamiodarone, desdiiodo-amiodarone and desethyldesdiiodoamiodarone. These metabolites have been detected in human plasma, lung and liver samples (6, 7). In patients on long-term amiodarone treatment deiodination of amiodarone leads to pharmacological quantities of iodine, which can be considered as chronic iodine excess.
O-dealkylation leads to compound L3373, which is subject to further deiodination (Fig. 2). Hydroxylation may occur in the benzofuran ring. These metabolites so far have not been identified in human tissues.

Indication for use. Amiodarone is a highly effective drug against a wide range of cardiac arrhythmias (8). It is primarily used in patients presenting with sustained ventricular tachyarrhythmia, ventricular tachycardia, or ventricular fibrillation refractory to other treatment modalities. It is also effective in suppressing ventricular arrhythmia and in reducing mortality from sudden death and cardiac disease in patients with non-sustained ventricular tachycardia and cardiomyopathy. Amiodarone is also effective in maintenance of sinus rhythm in patients with atrial fibrillation (9)

1.1.B. Side Effects

The side effects of AM are numerous, involving many organs. These side effects occur in approximately 80% of patients. Most of the non-thyroidal side effects seem to be related to the accumulation of AM and DEA in tissues (5). The incidence of many side effects increases over time, and the site effects are related to the cumulative dose of the drug reflecting accumulation of the drug in the tissues. Lysosomal inclusion bodies in cells of many tissues are reported in amiodarone-treated patients. These inclusion bodies give rise to severe impairment of the lysosomal system, resulting in toxicity for the cell (10).

The main side effects of amiodarone are as follows:
HEART – Sinus bradycardia, heart block, proarrhythmia. Only in 2-3% of patients the arrhythmia gets worse, an unusually low incidence for an antiarrhythmic drug which is efficacious in ventricular arrhythmia.
LUNG - Pulmonary toxicity is the most serious noncardiac side effect (2-17% of patients) (11;12). Also in other studies pulmonary toxicity and pulmonary fibrosis are reported (13;14).
STOMACH and INTESTINE – Anorexia with or without weight loss, nausea with or without vomiting, abdominal discomfort, constipation, foul taste, aguesia. These symptoms are very common (80%).
LIVER – Abnormal liver function tests are seen in 25%.
KIDNEY – An increase in serum creatinine is observed in 9% if the patients.
EYE - In all patients corneal micro deposits of amiodarone are found, but also lens changes (22%) and dry eyes (9%)). In some patients abnormal blue colour vision is observed (15).
NERVE- Neurological side effects are reported in 20-40% of patients, including tremor, ataxia, peripheral neuropathy, and headache (11).
SKIN – Several types of dermatological reactions have been reported, including allergic rash, photosensitivity, and a typical blue-grey skin discoloration.
THYROID – The side effects on the thyroid are not only due to the accumulation of AM and DEA, but also to the excess of iodine that reach the thyroid after deiodination of AM. Thyrotoxicosis or hypothyroidism occurs in 14-18% of patients.

1.1.C. Effect on the thyroid

Thyroxine (3, 5, 3', 5'-tetra-iodothyronine, T_4, Fig. 3) is synthesized in the follicular cells of the thyroid gland within the protein thyroglobulin (16). Bound to plasma proteins T_4 is transported to the target tissues, where it can be deiodinated into the bioactive form T_3 by the enzyme 5'-deiodinase (D1) and into the inactive form rT_3 by the enzyme 5-deiodinase (D3).
In all patients on AM-treatment a decrease in plasma T_3 and an increase in plasma rT_3 is observed. These changes can already be seen two weeks after the beginning of treatment. After chronic administration of the drug low T_3 and high rT_3 and T_4 levels are maintained. A steady state in plasma hormone

concentration is reached after 12-16 weeks (17). Several animal studies (18, 19), which are in good accordance with each other, show similar results as observed in humans: a decrease in plasma T_3 and an increase in plasma T_4 and rT_3.

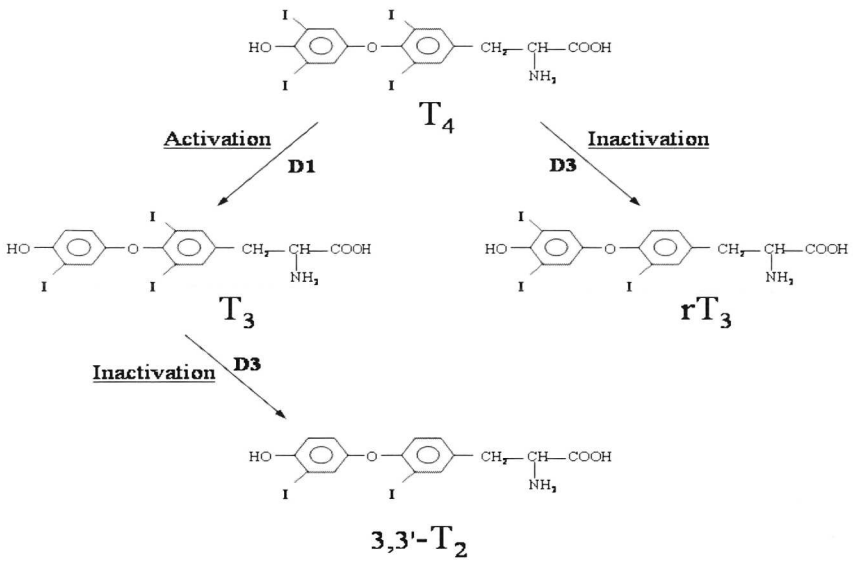


Fig. 3: The molecular constitution of T_4, T_3, rT_3 and T_2. T_4 is activated into T_3 by the enzyme 5'-deiodinase (D1). T_4 and T_3 are inactivated into rT_3 and 3, 3'-T_2 respectively by the enzyme 5-deiodinase (D3).

The changes in plasma T_3, T_4 and rT_3 are due to: 1. An effect of AM on the thyroid via the excess of iodine and 2. An effect of AM and DEA on extrathyroidal thyroid hormone metabolism and transport.

The pharmacological quantities of iodine released during biotransformation of AM result in an increased absolute thyroid iodide uptake. The chronic iodine excess will transiently inhibit thyroid hormone synthesis and release of thyroid hormones resulting in lower plasma T_3 and T_4 and consequently a small rise in TSH. Usually the thyroid escapes from this inhibitory (Wolff-Chaikoff) effect, and plasma T_4 and TSH return to normal values. However patients on chronic AM-treatment do have high T_4 and FT_4 plasma levels. The increase in plasma T_4 is most probably caused by the inhibitory effect of AM on the transport of T_4 across the plasma membrane especially in the liver (20). This inhibitory effect of AM on transport results in a lower T_4 concentration in the cell. In organs in

which no local conversion of T_4 into T_3 occurs (such as muscle), the decrease in tissue T_3 content is in direct proportion to the decrease in the plasma of T_4 into T_3. However intracellular T_3 is dramatically decreased by AM and DEA in all tissues where there is a local conversion of T_4 into T_3. The decrease in tissue T_3 goes together with an increased rT_3 in all tissues except muscle. In all tissues DEA has a more pronounced effect than AM (18, 19). These findings suggest inhibition of D1, the enzyme that catalyses the 5'-deiodination of T_4 into T_3 and of rT_3 into 3, 3'-T_2 . Although the mRNA of the gene encoding for D1 is expressed normally in the liver of amiodarone-treated rats (21), the activity of the enzyme is inhibited by AM as evident from various studies (22).

Although all patients on AM-treatment will experience a decrease in plasma T_3, most patients remain clinically euthyroid with a normal plasma TSH. Only a subset of AM-treated patients (14-18%) develops amiodarone-induced hypothyroidism (AIH) or amiodarone-induced thyrotoxicosis (AIT).

The iodine excess generated by AM may cause amiodarone-induced hypothyroidism (AIH). The underlying mechanism for this is the failure of the thyroid gland to escape from the Wolff-Chaikoff effect. Whereas a normal thyroid is capable of escaping from the inhibitory effects of iodine excess on thyroid hormone synthesis and release, this is less so in patients with chronic autoimmune thyroiditis; especially women with high concentrations of circulating TPO antibodies are at risk (23).

Amiodarone can also induce thyrotoxicosis (AIT) (23) by two mechanisms. Firstly excess of iodine may cause excessive thyroid hormone synthesis and release in patients with an underlying thyroid abnormality such as Graves' disease or nontoxic goitre; it is known as AIT type I. However AIT also occurs in patients without any evidence of pre-existent thyroid disease (24). In this type called AIT type II there is excessive thyroid hormone release caused by destructive thyroiditis. Structural changes in the thyroid gland were studied in rats treated with AM or with a comparable amount of sodium iodide. The results of this study are that AM-treatment causes extensive damage to the thyroid gland far beyond that caused by equivalent doses of iodine (25, 26).

1.2. Thyroid hormone receptors

1.2.A. TR protein structure

The thyroid hormone receptor (TR) was first cloned from chicken embryo (27) and human placenta (28) in 1986. It became clear that there are two thyroid hormone receptor genes: c-erbA-α that encodes TRα_1 and TRα_2 isoforms and c-erbA-β that encodes TRβ_1, TRβ_2 and TRβ_3 isoforms. The TRα_2 splice variant, which is only found in mammalians, cannot bind T_3 but can act as a constitutive repressor of TR action.

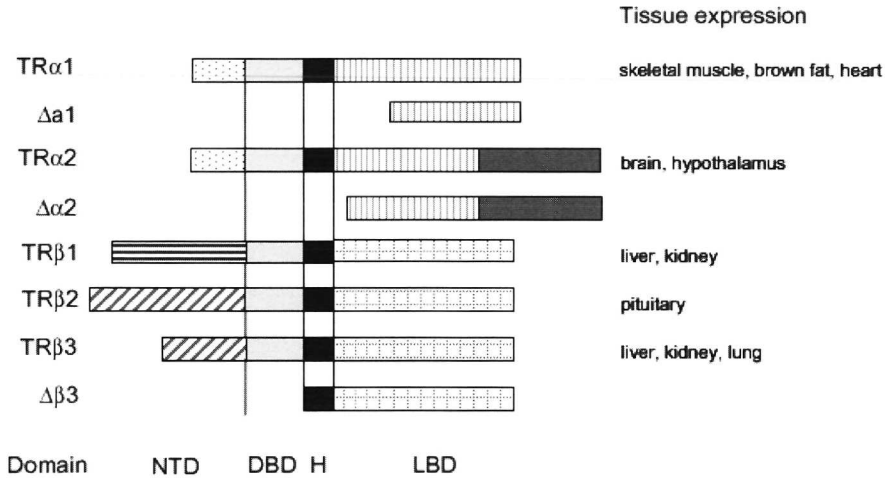


Fig. 4. Schematic representation of the domain structure of the thyroid hormone receptor isoforms and their tissue specific expression. In this schema the domain structure is divided into three function domains: N-terminal domain (NTD), DNA binding domain (DBD) and ligand binding domain (LBD). DBD and LBD are connected by the hinge region (H).

These receptors, like all members of the nuclear receptor super family, have a similar domain-like structure and each of these domains have a specific role in the interaction of the receptor with ligands, other nuclear proteins and DNA. The structure of the TRs is divided in three domains: 1. The N- terminal domain (NTD), differs in size and sequence between TRα and TRβ isoforms; it differs in TRβ_1 and TRβ_2, but is similar between TRα_1 and TRα_2; 2. The central DNA binding domain (DBD) is highly conserved in all nuclear receptors; 3. The C-terminal ligand-binding domain (LBD) binds ligand and is involved in co-

repressor and co-activator interaction. Between the DBD and the LBD is a movable (flexible) part of the protein named the hinge region (Fig. 4).
The NTD contains a constitutive activation function (AF-1) and is supposed to be involved in interaction with transcription factors and co-activators. This mechanism is poorly understood due to the fact that its activity is highly dependent on the species or cell types that are studied. The DBD mediates, via two zinc fingers, DNA recognition. Each zinc finger contains 4 cysteine residues coordinated by a zinc ion and 4 functional sites. The first zinc finger is important in the specific association with the thyroid hormone responsive element (TRE) sequences on the DNA. The LBD is involved in nuclear localization, homo- and/or heterodimerization, and most importantly T_3 binding. The structures of the TRα and TRβ LBDs have been elucidated by X-ray crystallography (29, 30). The LBD consists of a single structural domain packed in three layers, composed of 12 α-helices, H1-12, and of short β-strands, S1-4 forming a mixed β-sheet. The ligand is buried in the centre of the domain and forms the hydrophobic core of the structure. The LBD in its inactive form is able to bind so-called co-repressor molecules that help the receptor in keeping the gene silent.
When T_3 is bound inside the receptor pocket, helix 12 is displaced from "hanging" at the outside of the receptor to a position in which it closes the binding pocket like a lid on a box (Fig. 5). This also causes helix 12 to be packed over the lower part of the co-repressor binding surface resulting in a disruption of co-repressor binding. Co-repressors, like N-CoR and SMART, can bind to a hydrophobic cleft on the outside surface of the LBD of the TR that is composed of residues from helices (H) 3, 5, 6 and 12. They bind to the TR using two or three interaction domains (IDs) that contain the core consensus amino acid sequence I/LXXI/VI (31, 32).
Co-activators also bind to the same hydrophobic cleft of the LBD. Molecular studies have established that an LXXLL amino acid motif within the co-activators mediates this interaction with the T_3 activated receptor (34). This is confirmed by the crystal structure of the TR / glucocorticoid receptor interacting protein (GRIP) NR box peptide complex (35). The consensus I/LXXI/VI motif of the co-repressors resembles the consensus LXXLL motif of the co-activators suggesting that co-repressors and co-activators interact with overlapping sequences on the TR. Despite these similarities there must be a

mechanism that determines that only un-liganded TRß binds to N-CoR and only liganded TRß binds to GRIP1. A good candidate is the exact position of helix 12 that is supposed to have an important role in this TR-co-repressor / TR-co-activator exchange (36).

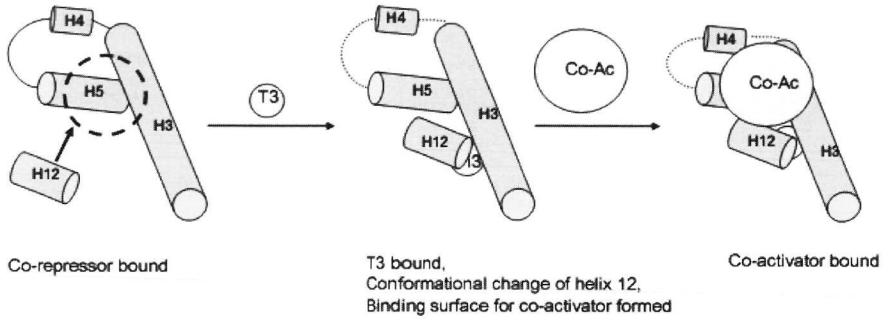


Fig. 5. Model for the exchange of co-repressor to co-activator induced by binding of T_3 to the TRα or TRβ. When T_3 is bound helix 12 (H12) moves from the outside of the receptor to close the receptor pocket partly over the co-repressor binding surface. Co-repressor will be released and the surface needed for co-activator binding is formed. Co-activators are recruited and bind to the hydrophobic cleft of the LBD. (Modified after Webb P. (33))

1.2.B. TR gene structure

Thyroid hormone receptors (TRs) are members of the super family of nuclear receptors. They are encoded by two different gene loci, TRα (locus c-erbA-alpha on chromosome 17q11.2 (human), 10 (rat) and 11 (mouse)) and TRβ (locus c-erbA-beta on chromosome 3p24.3 (human), 15 (rat) and 14 (mouse)) (37).

The TRα locus contains 10 exons and on this gene several TRα isoforms are encoded. The most important TRα isoforms are TRα_1 and TRα_2. TRα_2 is generated from an alternative splice site in exon 9 of the primary RNA-transcript (Fig. 6). The TRα_2 splice variant differs from the TRα_1 transcript in the part that encodes the hormone-binding domain and can therefore not bind T_3. TRα_2 can however act as a dominant negative regulator of transcription by forming dimers with TRα_1, TRβ_1 and possibly other nuclear receptors. Two other isoforms, TR α1 and TR α2, also arise from the TRα gene because of an internal promoter located in intron 7 of the TRα gene (Fig. 6). These truncated receptors are not able to bind to DNA and T_3 , but they can act as repressors of the other TR isoforms (38).

The TRβ locus encodes the isoforms $TR\beta_1$, $TR\beta_2$, $TR\beta_3$ and also a truncated form TR β_3 (unable to bind T_3) which are all generated via alternative splicing and/or differential promoter usage (39). They differ only in the amino terminal part of the receptor. The TRβ locus contains 11 exons, of which exons 3-8 are common to all TRβs and also show a high homology with the TRα isoforms. Alternative splicing of exon 1 and 2 results in the N-terminal part of $TR\beta_1$. Later it was discovered that exon a encodes the N terminal part of $TR\beta_2$ and that exons A and B encode $TR\beta_3$ and its variant (Fig. 6).

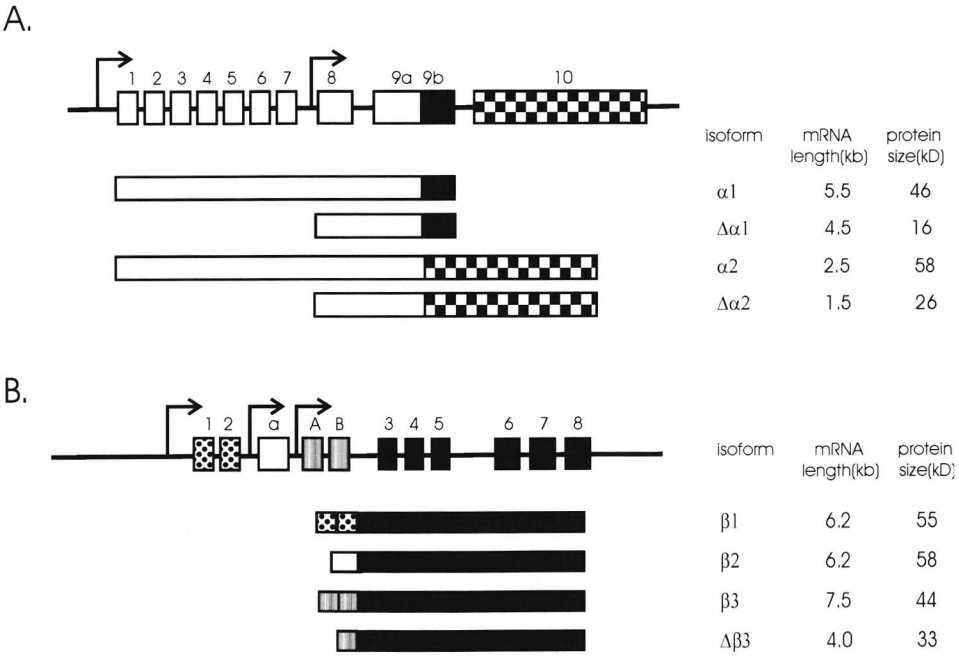


Fig. 6.
A. Schematic representation of the transcripts of the TRα gene. The white blocks are common to the α_1 and α_2 isoforms. The black box is the last part of the TRα primary transcript encoding for the ligand-binding domain of the $TR\alpha_1$. The blocked box is the transcript of exon 10 that encodes for the C-terminal part of $TR\alpha_2$.
B. Schematic representation of the transcripts of the TRß gene. The black boxes are transcripts from exon 3-8, and are common in all TRß isoforms. The differences between the TRß isoforms are located in the N-terminal part. They are generated via alternative splicing and/or differential promoter usage.
For each isoform the length of the mRNA transcript as kilo base pairs (kb) and the protein size as kilo Dalton (kD) are displayed. The protein size for TRα is estimated from mouse and the protein size for TRß from rat.

1.2.C. TR mechanism of action

The action of thyroid hormone in the cell is mediated by the binding of T_3 to its specific receptor in the nucleus. The zinc fingers in the DBD of the TRs bind DNA on specific thyroid hormone responsive elements (TREs) in the promoter region of target genes, most commonly as heterodimers with the retinoid X receptor (RXR). In order to understand the mechanism of TR action better, it is important to know that the chromosomal DNA in the nucleus of the cell is wound round a nucleosome, which is composed of positively charged proteins known as histones. Acetylation of these proteins allows the formation of a more loosely packed nucleosome structure because the methyl groups cover the (+) charge. The acetylation and deacetylation of specific lysine residues in histones is regulated by a balance between the activity of several different histone-acetyl-transferases (HATs) and histone de-acetylases (HDACs) (40). This equilibrium of acetylation and deacetylation is dependent on the binding of an activator (i.e. a ligand bound receptor). Thyroid hormone receptors are unique among their family in that they can influence gene expression whether bound by ligand or not. This is the result of the fact that the TR can bind to a TRE without hormone. The unliganded TR recruits so called co-repressors such as N-CoR and SMRT, which, via interaction with conserved hydrophobic sequences, causes the gene to be silent. Both N-CoR and SMRT contribute to a large complex that contains the histone de-acetylases (HDACs) and represses gene transcription by condensing chromatin in the proximity of the TR regulated promoter. When T_3 is bound the TR homodimer releases the co-repressor and disintegrates into monomers. The ligand-bound TR monomer now heterodimerizes with the retinoic X receptor (RXR) and binds again to the same TRE. Due to a conformational change within the LBD (shift of helix 12) it is now able to recruit p160 co-activators such as GRIP1 and/or SRC-1 (41). The p160 class of co-activators forms a large complex that contains the histone-acetyl-transferases (HATs), CBP/p300 and pCAF and the arginine methyl-transferases CARM1/PTMT1. This complex enhances gene transcription by opening the chromatin situated close to the TR-regulated gene so that the target DNA can be reached by RNA polymerase II. For reasons that are not completely elucidated, TRs interact only transiently with the p160 (42). After p160 dissociation, the TR binds a co-activator called TRAP220, again via interaction with LXXLL motifs. After TR binding to TRAP220, which is

also part of a large complex (TRAP/DRIP/SMCC/mediator), transcription is enhanced by contacting the basal transcription machinery (36) (Fig. 7).

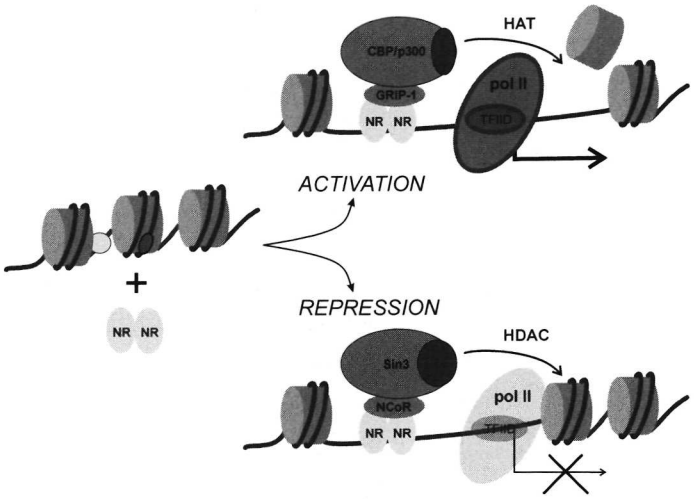


Fig. 7. General model of activation and repression after nuclear receptor binding on the DNA of ligand–dependent genes. When ligand is bound to the receptor, co-activators bind to the receptor, allowing chromatin structure to be opened and basal transcription machinery to start. In repression the unliganded receptor recruits co-repressors, histones will be deacetylated and chromatin can rewind.

1.3. Interactions of amiodarone and thyroid hormone receptors

1.3.A. Hypothyroid-like effects of amiodarone

Amiodarone is prescribed in clinical medicine for life-threatening cardiac arrhythmias and angina pectoris. Pharmacological actions of AM in the heart are bradycardia and depression of myocardial oxygen consumption, but the most important direct electrophysiological effect of AM is lengthening of the cardiac action potential duration (QTc interval; c means correction for heart rate). Changes in QTc interval caused by AM are prevented by the simultaneous administration of T_4 or T_3 (43). It has therefore been hypothesized that one of the mechanism of action of AM is by the induction of a local 'hypothyroid-like' condition in the heart (44). These electrophysiological changes seen in AM-treated patients resemble those observed in hypothyroid patients (45). The hypothetical scheme of this mechanism of action in presented in Fig. 8. In this model the duration of the cardiac action potential is viewed as a post receptor effect of nuclear thyroid hormone receptors (TRs) in the heart (46).

Besides in the heart, these hypothyroid-like effects of AM are also seen in liver, adipose tissue and in the pituitary. In these tissues comparable effects are induced by hypothyroidism and AM administration, and these effects can be normalized by simultaneous administration of T_3 (Table 2), (46, 47). The low tissue content of T_3, which resulted in a decrease of nuclear T_3 receptor occupancy, was hypothesized to be the working mechanism of AM. This idea was supported by the findings that in rats AM and DEA decrease intracellular T_3 levels in all tissues as a result of lower T_3 production in the thyroid and inhibition of deiodination from T_4 to T_3 (19).

From in vivo studies in rats it became clear that AM affects T_3-dependent gene expression. The liver enzyme PEPck, which is an enzyme of the metabolic pathway, is under T_3 control. The enzyme activity of PEPck and mRNA expression are decreased in liver of AM-treated rats compared to controls, whereas the nuclear thyroid hormone receptor occupancy in the livers of these animals did not change (48). Another important observation in patients on long-term AM-treatment is a dose-dependent increase in plasma cholesterol.

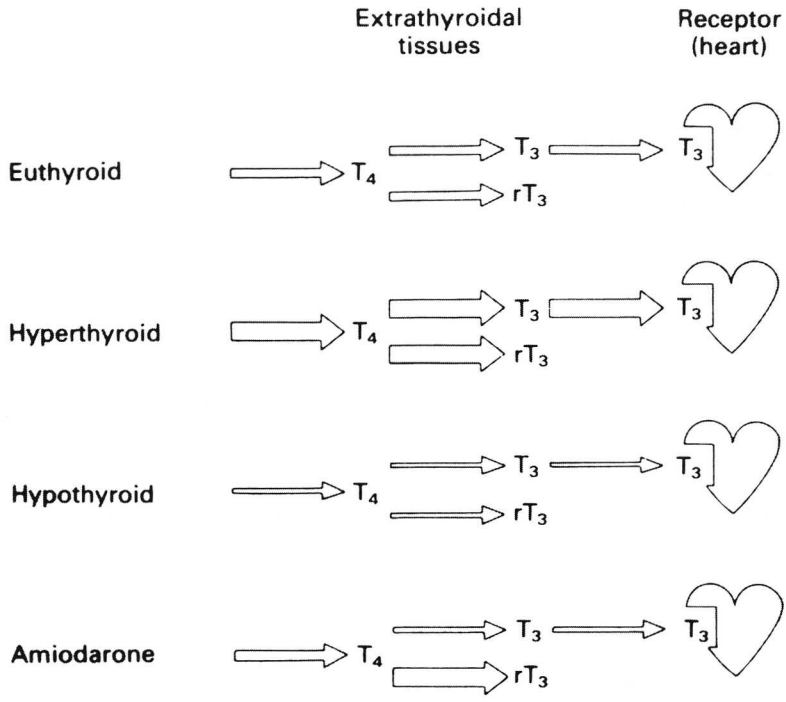


Fig. 8. Hypothetical scheme of the mechanism of action of amiodarone by the induction of a local "hypothyroid-like" condition of the heart. The duration of cardiac action potentials is viewed as a post receptor effect of nuclear T_3 binding to TRs in the heart. Receptor occupancy is decreased in hypothyroid and in amiodarone-treated patients, resulting in identical lengthening of the action potential. (Reproduced with permission from Wiersinga and Trip 1986)

In patients on oral AM administration for a mean duration of treatment of 17 months the observed rise in plasma cholesterol is correlated to the cumulative dose of AM, and is independent of thyroid function (49). This hypercholesterolemia is also observed in rats on AM-treatment (49), which was later explained as a result of a decrease in the T_3-dependent expression of the LDLr gene on mRNA and protein level in liver (21, 50).

Studies with the iodinated cholecystografic agent iopanoic acid that inhibit deiodinase activity and reduces the conversion of from T_4 to T_3 as strong as AM did not show the hypothyroid-like effects and did not prolong the QTc interval (51). In rodent myocardium no appreciable conversion from T_4 to T_3 is seen, and data from this and other studies suggest that the heart is uniquely dependent on plasma T_3 (52). These findings indicate that the lower T_3

concentrations in the cell cannot be the sole mechanism for the AM-induced action in the heart, but that there must be another working mechanism as well. It is known that an indirect electrophysiological effect of AM is due to its antiadrenergic action in which AM acts as a noncompetitive α- and β-adrenoceptor antagonist (8, 53). In analogy of the adrenoceptor it could well be that there is also an effect of AM on the binding of T_3 to the TR in the nucleus. The high resemblance of AM with T_3 led us to hypothesize that AM itself can bind to the TR or that AM hinders the binding of T_3 to the TR.

Table 2. Hypothyroid-like effects of amiodarone in various tissues

Tissue effect	Hypothyroidism	Amiodarone	Amiodarone + T_3
Heart			
QTc interval	↑	↑	N
Heart rate	↓	↓	N
β-Adrenoceptor density	↓	↓	N
SERCA [1])	↓	↓	N
Myosine Heavy Chain α mRNA	↓	↑	N
Myosine Heavy Chain β mRNA	↑	↑	N
Liver			
LDL receptor density	↓	↓	N
Spot 14 mRNA	↓	↑	N
PEPck [2]) protein and mRNA	↓	↓	N
Adipose tissue			
Lipoprotein lipase activity	(↑)	↑	N
Pituitary			
TSH synthesis and release	↑	↑	N
Prolactin mRNA	↓	↓	N

↑, increase; ↓, decrease; N, return to normal; (↑), increase not significant.

[1]) sarcoplasmic reticulum Ca^{2+} ATPase

[2]) phosphoenolpyruvate carboxykinase

1.3.B. Scope of the thesis

The effects of amiodarone (AM) and its major metabolite desethylamiodarone (DEA) are studied on the binding of thyroid hormone (T_3) to the thyroid hormone receptor α1 (TRα_1) and thyroid hormone receptor β1 (TRβ_1) protein. Isoform specific cDNAs were expressed in bacterial cells and the TRα_1 and TRβ_1 proteins were isolated. Interaction of AM and DEA on the binding of T_3 to TRα_1 and TRβ_1 made it possible to get more insight in the mechanism of action shown by the different TR isoforms.

In chapter 2 it is described how the solubility problem of the lypophilic compounds AM and DEA was solved. After it had become possible to keep AM and DEA in solution in the aqueous incubation-binding buffer, the effect of AM and DEA of the binding of T_3 to the thyroid hormone receptor β1 (TRβ_1) protein could be studied. From this study it became clear that not AM itself, but its major metabolite DEA, is the inhibitor of the binding of T_3 to TRβ_1 and that the inhibition of T_3 binding to TRβ_1 by DEA is noncompetitive in nature.
In chapter 3 the effect of AM and DEA is studied on the binding of T_3 to the other important T_3 binding isoform: thyroid hormone receptor α1 (TRα_1). DEA is a much more potent inhibitor of T_3 binding to TRα_1 than AM. But in contrast to TRβ_1, DEA is a competitive antagonist with respect to TRα_1.
In chapter 4 the relationship is studied between TRα_1 and TRβ_1 proteins and a number of AM metabolites and analogues to gain insight in which atoms and/or structures in the drug molecule are essential (important) for the inhibitory properties. The compounds were tested in an in vitro T_3 binding assay with TRα_1 and TRβ_1 proteins. Compounds formed by natural metabolism like deethylation and deiodination were tested as well as short-chain benzofuran derivatives with various deiodination grade.
In chapter 5 the structure function relationship between DEA and TRβ_1 is studied to get further information on the localization of the DEA binding site on the receptor. To get more insight in this T_3 binding studies were performed in the presence of DEA and with various mutations in the TRβ_1 protein. The naturally occurring and artificial mutations that are tested are known to decrease T_3 affinity, hormone-dependent transactivation, homo- or heterodimerization.

Chapter 6 and 7: From the mutation study a possible binding site for DEA on the hydrophobic outside of the LBD in helix 12 of the TRβ_1 was hypothesized. In the period that the mutation studies were performed, interaction of the same hydrophobic region of the TRβ_1 with the LXXLL motif of co-activators was described (34, 35). The previous two findings brought up the following question: Does DEA interfere with the binding of co-activator GRIP1 to the TRβ_1? To study this, a non-radioactive pull-down assay was developed, which is described in chapter 6. The results of the interference by DEA on the binding of co-activator GRIP1 to TRβ_1 are presented in chapter 7.
In chapter 8 the inhibitory properties of a new anti-arrhythmic drug dronedarone (Dron), without iodine, on the binding of T_3 to the TRα_1 and TRβ_1 was studied. The *in vitro* inhibitory effects of Dron and its major metabolite debutyldronedarone (DBDron) on the binding of T_3 to the TRα_1 and TRβ_1 are reported. From the results of the binding studies it was hypothesized that Dron is a TRα_1-selective antagonist. To test this hypothesis an *in vivo* study was performed in which rats were treated with Dron or AM. The effect of the drugs on plasma thyroid hormone and cholesterol parameters as well as the post receptor effects in heart and liver are described. In both studies the effects of AM to those of Dron are compared.

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CHAPTER 2

Desethylamiodarone is a noncompetitive inhibitor of the binding of thyroid hormone to the TRβ_1 protein

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2.1. Abstract

It has been hypothesized that amiodarone (AM), a potent antiarrhythmic and antianginal drug, induces a local hypothyroid-like condition in extrathyroidal tissues. This might be related to competitive antagonism of AM for the thyroid hormone receptor (TR) reported in some studies but denied in others. These conflicting results are presumably due to the poor solubility of AM in a hydrophilic environment. We, therefore, studied the effect of the drug and its major metabolite, desethylamiodarone (DEA), on the *in vitro* binding of thyroid hormone (T_3) to its receptor protein using the rat TRβ_1 expressed in *Escherichia coli*. AM and DEA stayed in solution up to 10^{-4} M when 0.05% Triton X-100 was added to the incubation buffer, as evidenced by a recovery of 80-90% for both chemicals, as measured by HPLC.

DEA, but not AM, had a clear inhibitory effect on the binding of T_3 to its receptor (IC_{50}, 1-3 x 10^{-5} M). Scatchard analysis in the presence of DEA demonstrated a dose-dependent decrease in the K_a as well as the maximum binding capacity. Lineweaver-Burke analysis indicated noncompetitive inhibition. Plots of the intercepts of Lineweaver-Burke plots *vs.* DEA concentration were linear ($y = 0.334 + 0.098x$), giving a K_i of 30 μM for the binding of DEA to the occupied receptor. Plots of the slopes *vs.* inhibitor concentration were parabolic ($y = 3.01 + 0.06x + 0.16x^2$), indicating a progressively stronger effect of DEA on the unoccupied receptor as concentrations rise. This preference for the unoccupied receptor is reflected in experiments that show a progressive loss of T_3 binding when the receptor was incubated for increasing periods with DEA before adding T_3.

We conclude that DEA is a noncompetitive inhibitor of the binding of T_3 to the TRβ_1 protein, interacting preferably with the unoccupied TR. (Endocrinology 134: 1665-1670, 1994)

2.2. Introduction

Amiodarone (AM), an iodinated benzofuran derivative, is a potent antiarrythmic and antianginal drug. The pharmacological actions of AM include bradycardia, depression of myocardial oxygen consumption, and lengthening of the cardiac action potential, which can be prevented by concomitant administration of a physiological dose of T_4 (1). Furthermore, it reduces cardiac ß-adrenergic receptor density (2), alters the distribution of cardiac myosin isoenzymes (3), and increases plasma cholesterol (4, 5) in a way similar to that observed in hypothyroid animals. It has, therefore, been hypothesized that one of the mechanisms of action of AM could be the induction of a local hypothyroid-like condition in extrathyroidal tissues (6, 7). The well known inhibition of T_4 5'-deiodination by AM (8, 9) is unlikely to be the sole mediator of this effect, because other agents that are equally potent inhibitors of the peripheral conversion of T_4 to T_3 do not have antiarrythmic activity in man (10).

AM could also induce a local hypothyroid-like state by interfering with the activation of thyroid hormone-responsive gene expression via the TR. Indeed, several groups have shown that AM inhibits the binding of T_3 to its nuclear receptor (11-13) and decreases thyroid hormone-dependent gene expression (13-16). This has led to the proposal that AM acts as a competitive antagonist for the TR, but various *in vivo* and *in vitro* studies have shown conflicting results (11-16). Putative reasons for the observed discrepancies could be the solubility of AM or the different purities of receptor preparations. We, therefore, evaluated the effect of AM and its major metabolite desethylamiodarone (DEA) on the *in vitro* binding of T_3 to the purified TRβ_1 protein, which was expressed in *Escherichia coli*. We conclude that DEA, but not AM, is a noncompetitive inhibitor of T_3 binding to the TRβ_1 protein, interacting preferably with the unoccupied TR.

2.3. Materials and Methods

2.3.A. Chemicals

T_3 was obtained from Henning GmbH (Berlin, Germany), and AM (2-butyl-3-(3, 5 diiodo-4(β-diethylamino-ethoxy) benzoyl) benzofuran) and its major metabolite DEA were a kind gift of Sanofi BV (Maassluis, The Netherlands). [^{125}I]T_3 (SA, 2200 Ci/mmol) was purchased from New England Nuclear (Boston, MA). All other reagents were of the highest grade possible.

2.3.B. Receptor expression

The rat TRβ_1 (amino acids 31-456) cDNA was cloned in the pEX vector, as previously described (17). The receptor construct contains the hormone- and DNA-binding domain. After heat shock, cells were grown at 42^{0}C for a further 1.5 h. Cells were then lysed using lysozyme and deoxycholate and fusion proteins, purified as previously described (18), and stored in incubation buffer (20 mM Tris-HCl, 0.25 M sucrose, 1 mM EDTA, 50 mM NaCl, and 5% (vol/vol) glycerol, pH 7.6) in liquid nitrogen.

2.3.C. AM solubility

AM and DEA were dissolved in ethanol to yield a stock solution of 10^{-2} M. Appropriate dilutions of both compounds in ethanol were added to incubation buffer containing ethanol (0-5%, vol/vol), Tween-80 (0.01-1%, vol/vol), or Triton X-100 (0.01-1%, vol/vol). After insoluble material was removed by centrifugation at 10, 000 x g for 5 min, AM and DEA concentrations in the supernatants were determined by HPLC (19) by comparing the peak area of the unknown sample with that of a known standard.

2.3.D. TR binding assay

Before incubation, the fusion proteins were solubilised by sonification (10 sec; 6 μm), and the nonsoluble proteins were removed by centrifugation (10, 000 x g; 5 min). Twenty to 25 μg receptor protein were incubated with [^{125}I]T_3 (10^{-11} M) for 2 h at 22°C in a shaking water bath in incubation buffer (20 mM Tris-HCl, 0.25 M sucrose, 1 mM EDTA, 50 mM NaCl, and 5% (vol/vol) glycerol, pH 7.6) containing 5 mM dithiothreitol, 0.05% Triton X-100, and 0.05% BSA. The total incubation volume was 0.5 ml. Reactions were stopped

by chilling the samples in ice-cold water. Bound and unbound hormone were separated at 4°C using a small Sephadex G-25 medium column (bed volume, 2 ml; swollen in incubation buffer with 0.05% BSA) in a Pasteur pipette. Four 0.8-ml fractions were collected from the column, using incubation buffer as eluent. The radioactivity in these fractions was taken to represent the hormonal fraction bound to the TR proteins. Specific binding was determined by calculating the difference between the counts bound in the absence and presence of an excess (10^{-7} M) of nonradioactive T_3. All incubations were performed in duplicate.

Inhibition of [^{125}I]T_3 binding by either AM or DEA was studied by adding up to 10^{-4} M of these chemicals to the incubation mixture. Scatchard analysis was performed by incubating increasing amounts of nonradioactive T_3 (1×10^{-10} to 33×10^{-10} M) with the receptor preparations. This was also done in the presence of AM and DEA at different concentrations (1×10^{-5}, 2.5×10^{-5}, and 5×10^{-5} M). In all tubes the final ethanol concentration was 1% (vol/vol), including controls without addition of the chemicals.

The time course of T_3 binding to the TRβ_1 protein was studied by incubating [^{125}I]T_3 (0.5×10^{-9} M) with the receptor protein, as described above, and stopping the reaction at the times indicated. The reaction mixtures were then immediately applied to a Sephadex G-25 medium column (2 ml) to separate bound from unbound hormone. Competitors (10^{-7} M nonradioactive T_3, 5×10^{-5} M DEA, or both) were added either at equilibrium (after 120-min incubation) or before equilibrium had been reached (after 15-min incubation). Another approach was the addition of 5×10^{-5} M DEA 5, 10, or 30 min before adding [^{125}I]T_3 (0.5×10^{-9} M) to study a possible time-dependent effect of DEA on TR binding.

2.4. Results

2.4.A. Solubility of A and DEA

AM and DEA are strong lipophilic substances. They are, therefore, easily dissolved in ethanol, but come out of solution in a hydrophylic environment. This was evident upon the addition of AM dissolved in ethanol to the incubation buffer (final ethanol concentration, 1%). We tried to overcome this by adding ethanol, Tween-80, or Triton X-100 to the incubation buffer and looked for the lowest concentration of these compounds that, on visual inspection, showed no precipitation of AM and DEA (Table 1). We found that at a concentration of 0.05% Triton X-100, no precipitation of AM and DEA could be observed up to 10^{-4}M (Table 1). Recovery of AM and DEA added to the incubation buffer with 0.05% Triton, as determined by HPLC analysis, was on the order of 80-95%, but decreased remarkably at concentrations above 10^{-4} M (Table 1). We refrained from the use of Tween-80, because the addition of 0.1% of this solution (vol/vol) strongly interfered with the binding of T_3 to nuclear TRs (Fig. 1), acting as a competitive antagonist (data not shown).

2.4.B. Binding of T_3 to the TRβ_1 protein

We next looked at the effect of 0.05% Triton X-100 on the binding of T_3 to its receptor protein. Triton significantly decreased the binding of [^{125}I]T_3. Scatchard analysis revealed an approximately 2-fold lower K_a in the presence of Triton, but the maximal binding capacities (MBCs) remained similar (Table 2). Another problem was that the protein levels used (40-50 μg/ml) were so low that non-specific effects were encountered. Addition of a 10-fold excess of BSA (0.05%) was able to overcome this problem without interfering with the binding parameters of the TRβ_1 protein (Table 2). Consequently, 0.05% Triton X-100 and 0.05% BSA were routinely added to the incubation mixtures in the following experiments.

2.4.C. DEA inhibits TR binding

The rat TRβ_1 was incubated with increasing concentrations (10^{-7}-10^{-4} M) of AM and DEA in the incubation buffer containing 5 mM dithiothreitol, 0.05% Triton X-100, and 0, 05% BSA. AM did not inhibit T_3 binding at the concentrations used, whereas DEA gave rise to a dose-dependent inhibition of the binding of T_3 to the TRβ_1; IC_{50} values ranged from 1-3 x 10^{-5} M (Fig. 2).

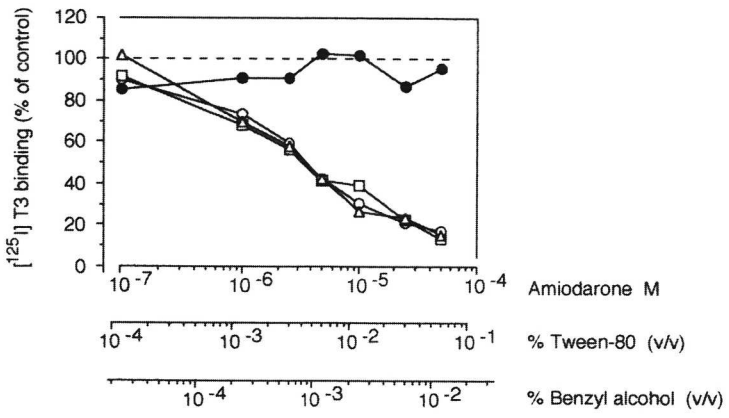

FIG. 1. The effects of AM and solvents on the specific binding of [^{125}I]T_3 to isolated rat liver nuclei Rat liver nuclei were incubated with [^{125}I]T_3 and increasing concentrations of the different constituents of the solvents (9.3% Tween-80 and 1.9% benzyl alcohol). Binding is expressed as a percentage of that in control incubations without any addition. ***open circle***: AM in solvents; ***open square***: solvents alone (9.3% Tween-80 and 1.9% benzyl alcohol); ***open triangle***: 9.3% Tween-80 alone; ***closed circle***: 1.9% benzyl alcohol alone. The final concentration of AM and (the components of) its solvents in the incubation mixtures are indicated on the *horizontal axis*. Preparation and incubation of rat liver nuclei were described previously (24).

Table 1. Solubility of AM and DEA in incubation buffer containing ethanol, Tween-80, or Triton X-100

	Ethanol (vol/vol)				Tween-80 (vol/vol)				Triton X-100 (vol/vol)				% Recovery[α]	
	0%	0.5%	1%	5%	0%	0.1%	1%	10%	0%	0.01%	0.05%	0.1%	1% Ethanol	0.05% Triton
AM														
10^{-3}	—	—	—	—	—	—	—	±	—	—	±	±	0	11-26
10^{-4}	—	—	—	±	—	—	+	+	—	—	+	+	0	91-99
10^{-5}	—	—	—	±	—	—	+	+	—	—	+	+	0	78-93
DEA														
10^{-3}	—	—	—	—	—	—	—	±	—	—	±	±	0	34-40
10^{-4}	—	—	—	±	—	—	+	+	—	—	+	+	0	90-95
10^{-5}	—	—	±	±	—	±	+	+	—	—	+	+	11-13	73-83

The minus sign indicates macroscopically visible precipitation, plus/minus an opalescent solution and plus a clear solution after the addition of A and DEA at different concentrations to the incubation buffer.

[α] Recovery of A and DEA (measured by HPLC) was determined in two experiments and expressed as a percentage of the added chemical.

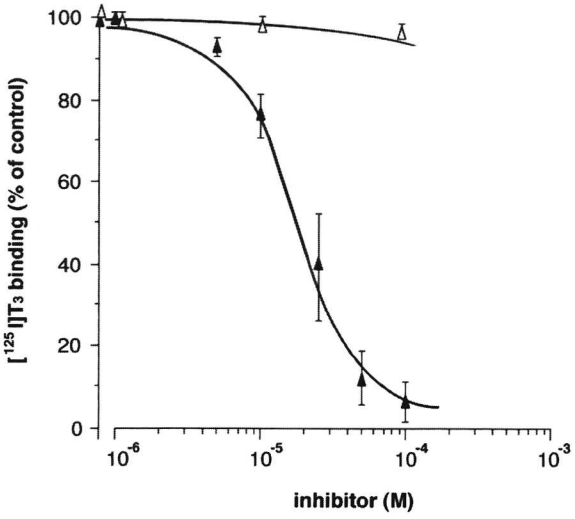

FIG. 2. Binding of T_3 to the TRβ_1 in the presence of increasing concentrations of AM and DEA. The TRβ_1 was incubated with [^{125}I]T_3 (10^{-11} M) in the presence of increasing concentrations AM (***open triangle***) and DEA (***closed triangle***) at 22 ^{0}C for 2 h. Binding is expressed as a percentage of the control value and is corrected for non-specific binding. Data are presented as the mean ± SD (n = 5).

Table 2. Effect of the addition of 0.05% Triton X-100 and 0.05% BSA on the binding of T_3 to the TRβ_1-protein, as evident from Scatchard analysis

	B_0 (%)	K_a (10^9 litres/mol)	MBC (10^{-10} M)
No addition	68 ± 6	1.5 ± 0.6	6.4 ± 1.5
+0.05% Triton	36 ± 7[α]	0.7 ± 0.2[α]	10.2 ± 4.1[α]
+0.05% Triton + 0.05% BSA	38 ± 7[α]	1.2 ± 0.6	7.2 ± 3.4

Values are the mean ± SD (n = 5).

B_0, Specifically bound [^{125}I]T_3, expressed as a percentage of the added radioactivity.

[α] $P < 0.05$ compared to no addition (by sign test on differences between pairs).

2.4.D. DEA is a noncompetitive inhibitor

We next analysed the inhibition of T_3 binding by DEA in more detail. Binding data obtained in the presence of increasing concentrations of DEA were analysed in Scatchard plots. These concentrations were chosen because the effects of these DEA concentrations in the competition curve were most pronounced. As can be seen in Fig.3A, increasing concentrations of DEA lowered the K_a, as well as the MBC, which is reminiscent of noncompetitive inhibition. Figure 3 shows representative results of two independent experiments performed in duplicate. To get a more definitive answer on the nature of the competition, the data were analysed using Lineweaver-Burke plots. As can be seen in Fig. 3B, curves were obtained with increasing slope and intercept on the y-axis and with the intersection point of the different curves on the x-axis, which is indicative of noncompetitive inhibition. We next plotted the slope and the intercept on the y-axis *vs.* the inhibitor concentration. These plots yield information on the inhibitor constants of the binding of DEA to the unoccupied (I + R$\leftrightarrow$IR; K_i) and to the occupied TR (I + TR $\leftrightarrow$ITR; K_i) respectively. As can be seen in Fig. 4, the plot of the slope *vs.* inhibitor is parabolic ($y = 3.01 + 0.06x + 0.16x^2$; r = 1.00), whereas that of the intercept *vs.* inhibitor is linear ($y = 0.334 + 0.098x$; r = 0.97; $P < 0.01$), giving a K_i of 30 μM for the binding of DEA to the occupied receptor. The parabolic curve indicates that as the DEA concentration rises, its effect on the unoccupied TR will become progressively stronger. The small slope of the curve of the intercept *vs.* inhibitor indicates that DEA is not able to influence the occupied receptor to a great extent. This is illustrated in Fig. 5, which shows the time course of [^{125}I]T_3 binding to its receptor. When DEA (5 x 10^{-5} M) was added at equilibrium (120 min), only slight competition was seen (Fig. 5A). When this concentration of DEA was added before equilibrium was reached, maximal T_3 binding occurred at a lower level (Fig. 5B). Adding an excess of cold T_3 together with DEA caused the same kinetics as adding excess T_3 alone. Furthermore, adding DEA (5 x 10^{-5} M) 5, 10, or 30 min before adding T_3 resulted in a time-dependent loss of T3 binding (Fig. 5C). Together, these results indicate that DEA interacts preferably with the unoccupied receptor, rendering it unable to bind T_3.

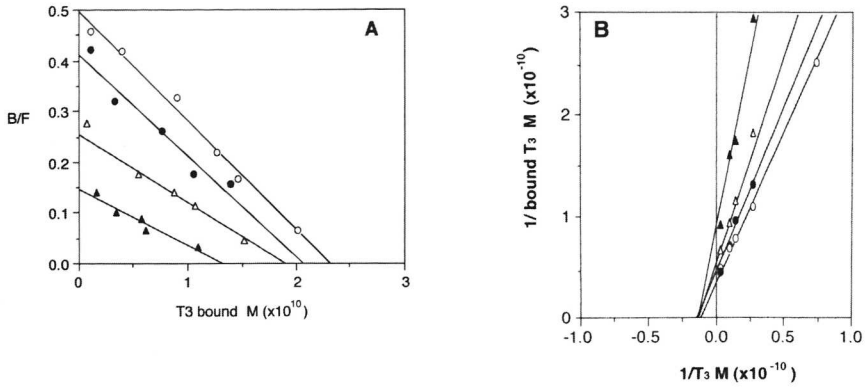

FIG. 3. Scatchard and Lineweaver-Burke plots of T_3 binding to the TR$ß_1$ in the presence of increasing concentrations of DEA. The rat TR$ß_1$ was incubated with increasing concentrations of T_3 in the presence of 0 M (***open circle***), 1.0 x 10^{-5} M (***closed circle***), 2.5 x 10^{-5} M (***open triangle***), and 5.0 x 10^{-5} M (***closed triangle***) DEA. Binding data were corrected for non-specific binding. A, Scatchard plots; the lines are described by the following functions: 0, $y = 0.50 - 0.22x$, r = 0.99; 1.0 x 10^{-5}, $y = 0.41-0.20x$, r = 0.94; 2.5 x 10^{-5}, $y = 0.25-0.13x$, r = 0.99; 5.0 x 10^{-5}; $y = 0.15-0.11x$, r = 0.92. B, Lineweaver-Burke plots; the binding data obtained in AM were used to prepare double reciprocal plots. The lines are described by the following functions: 0, $y = 0.34 + 3.05x$, r = 1.00; 1.0 x 10^{-5}, $y = 0.45 + 3.25x$, r = 1.00; 2.5 x 10^{-5}, $y = 0.54 + 4.09x$, r = 1.00, 5.0 x 10^{-5}, $y = 0.84 + 7.04x$, r = 1.00.

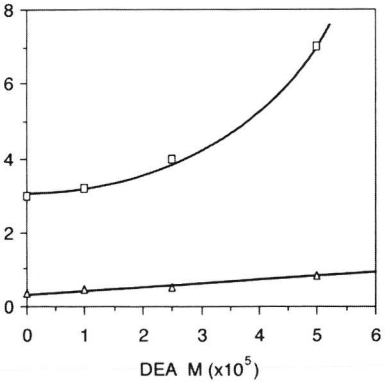

FIG. 4. Plots of the slope and intercept *vs.* inhibitor concentration. The slope (***open square***) and intercept (***open triangle***) of the different lines of the Lineweaver-Burke plots of T_3 binding to the TR$ß_1$ protein were plotted against the inhibitor concentration. These plots yield information on the inhibitor constants of the binding of DEA to the unoccupied (I+R$\leftrightarrow$IR; K_I) and occupied TR (I+ TR $\leftrightarrow$ITR; K_i), respectively.

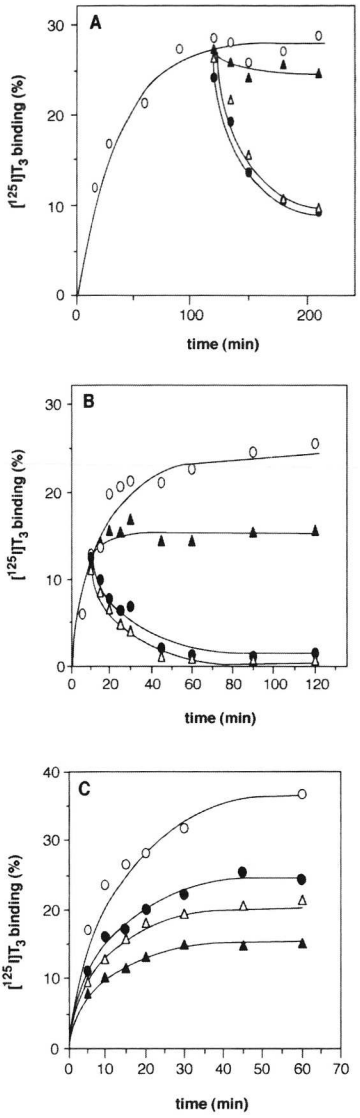

FIG. 5. Time course of [^{125}I]T_3 binding to the TRβ_1 with prior or delayed DEA addition. The rat TRβ_1 was incubated with [^{125}I]T_3 (0.5×10^{-9} M) at room temperature. At different time points the reactions were stopped, and T_3 binding was determined, as described in *Materials and Methods*. Non-specific binding was subtracted in all cases. ***open circle***: The time course of T_3 binding without any competitor. Cold T_3 (10^{-7} M; ***closed circle***), DEA (5×10^{-5} M; ***closed triangle***), or cold T_3 with DEA ($10^{-7}$and 5×10^{-5} M, respectively; ***open triangle***) were added at equilibrium (A) or before equilibrium was reached (B). C, The effect of adding 5×10^{-5} M DEA 5 min (***closed circle***), 10 min (***open triangle***), or 30 min (***closed triangle***) before the addition of [^{125}I]T_3 to the incubation mixture (time zero).

2.5. Discussion

The data on the effect of AM on the binding of T_3 to its receptor protein have been conflicting. One explanation for this could be the solubility of AM in the buffer systems used. Latham et al. (11) describe that they cannot see signs of precipitation when they add AM to their buffer containing 1% ethanol. We, on the other hand, found no soluble AM or DEA in the presence of 1% ethanol, as measured by HPLC. Moreover, we could not observe a clear inhibitory effect of A or DEA in the presence of 1% ethanol and receptor protein.

Wilson (12) dissolved AM in 50% propanol-water and found that AM precipitated upon dilution. Consequently, the experiments were carried out in a two-phase environment. In this system, coprecipitation of AM and T_3 is observed. Although we show no experiments directly assessing a possible interaction between DEA and T_3 in our buffer system, we think this is highly unlikely, as competition with T_3 alone and that with T_3 (10^{-7} M) and DEA (5 x 10^{-5} M) together show the same kinetics, whereas were DEA to interact directly with T_3 and thereby render it unable to bind to the TR, adding DEA and T_3 together should result in a slower dissociation kinetics. Furthermore, adding DEA at different times before T_3 shows a time-dependent effect on the maximal T_3 binding, which would not be expected if there were a direct interaction between DEA and T_3. Wilson (12) also describes the formation of two components from AM, the appearance of one of which, *y*, correlated with the inhibitory effect of AM. We have not been able to detect a *y*-like compound in our HPLC assays of AM or DEA. Despite the precipitation of AM, Wilson also observed a lowering of both the K_a and MBC upon addition of the drug to the binding reaction. Two additional papers (9, 20) that describe experiments looking at the effect of AM on T_3 binding to its receptor *in vitro* do not mention any solubility problems. The lack of an AM effect on TR binding in the study of Sogol *et al.* (9) may be due to the fact that they dilute AM from an ethanol stock into incubation buffer. As mentioned above, we were not able to find soluble AM when it was diluted in this way. Norman and Lavin (13), who studied the effect of AM on T_3 binding in GC cells and on solubilised TRs from different tissues, used incubations in buffers containing Tween-80 and benzyl alcohol, in which the compound remained soluble. We have shown here, however, that Tween itself appears to be a competitor of T_3 binding when the

proper control incubations are carried out. The reported action of AM as a competitive antagonist of TR binding by Norman and Lavin (13) is, thus, due to Tween-80, not to AM itself.

Our studies demonstrate that DEA behaves as a parabolic noncompetitive inhibitor and interacts preferably with the unoccupied receptor. The noncompetitive nature implies that DEA and T_3 are able to bind to the receptor at the same time on different binding sites. Our data agree with those obtained by Latham *et al.* (11) in rat liver nuclei insofar as we find a stronger competition by DEA then AM for the TRβ_1, which is the predominant receptor in liver. However, to calculate the K_d for DEA binding, they used a formula that can be derived only when linear competitive inhibition is observed (*i.e.* $K_i = K_I$). The parabolic curve implies that increasing the concentration of the inhibitor will give a more than linear effect and, consequently, a change in the binding constant, K_I. This combined with the fact that the unoccupied receptor is the main target for DEA could be part of the explanation for the late onset of AM's side effects, such as the development of hypercholesterolemia (5). AM and its metabolite DEA accumulate in tissues during treatment with the drug, and it is conceivable that an effect is only seen when their concentrations exceed a certain threshold. Furthermore, the intracellular level of T_3 is lowered during AM treatment, which may lead to lower receptor occupancy. Thus, only after a certain time span, as DEA concentrations increase (and concomitantly K_I) and more unoccupied receptors become available, will the effect of DEA become apparent. The addition of T_3 would increase the number of occupied receptors and thereby decrease the effect of the drug. This has indeed been shown by several researchers who demonstrated that changes induced by AM could be reversed by T_3 treatment (13, 16). The data presented in Fig. 5 also support this, in that adding an excess of T_3 together with DEA shows the same dissociation kinetics as adding just the excess T_3. This can be explained by the greater affinity of T_3 for the receptor than DEA, resulting in higher receptor occupancy and a decrease in the effect of DEA. In the case of mild hypothyroidism, either *in vivo* (15) or *in vitro* (13), the effect of AM is most clearly seen. This, in a way, is reflected by our experiments in which we added DEA before equilibrium was reached or before T_3 was added. In these cases, the effect of DEA was more pronounced due to the lower receptor saturation with T_3. It, therefore, appears that AM has its greatest effect when a mildly

hypothyroid condition is present. AM has a specific effect on thyroid hormone-dependent gene expression (13-16), which could arise from interference with the binding of T_3 to the TR via its major metabolite DEA (as shown in this paper). The TR is a member of the family of nuclear hormone receptors and can be divided into three major domains, the N-terminal transactivation region, the DNA-binding domain, and the C-terminal ligand-binding domain, which contains the dimerisation and ligand-inducible transactivation regions (21). As DEA competes with T_3 for binding to the receptor, it could well interfere with either dimerisation or transactivation and in that way inhibit thyroid hormone-dependent effects. Hormone binding to the TR is necessary for receptor activation. The activated receptor, in turn, influences gene expression. If DEA interferes with the binding of T_3 to its receptor protein, the receptor will not become activated and cannot influence gene expression. This means that T_3 should be present for an effect of DEA on thyroid hormone-dependent gene expression to become apparent. Evidence for this is provided by studies in GC cells, where adding AM without T_3 had no effect on rat GH expression, and studies in thyroidectomised rats that had no detectable plasma T_3, in which no effect of AM on left ventricular weight or myosin heavy chain distribution was observed (13, 16).

From our data and those of others, it is clear that DEA may act as an antagonist of the TR. The intracellular concentrations reached *in vivo* are high enough (50-500 μM/cell) (22, 23) for the drug to be able to interfere with T_3 action. The precise manner in which DEA interacts with the receptor and consequently interferes with gene activation remains to be investigated.

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CHAPTER 3

Desethylamiodarone is a competitive inhibitor of the binding of thyroid hormone to the TRα_1 protein

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3.1. Abstract

Desethylamiodarone (DEA), the major metabolite of the potent antiarrhythmic drug amiodarone (AM), is a noncompetitive inhibitor of the binding of thyroid hormone (T_3) to the β_1-thyroid hormone receptor (TRβ_1). In the present study, we investigated whether DEA acts in a similar way with respect to the TRα_1. The chicken TRα_1, expressed in an *E.coli* system, was incubated in the presence or absence of DEA with [^{125}I]T_3 in buffer containing 0.05% Triton X-100, 0.05% BSA and 1% ethanol (v/v) in order to solubilise DEA. DEA, but not AM, inhibited T_3 binding in a dose-dependent manner; the IC_{50} value was 3.5 x 10^{-5} M. Scatchard analyses in the presence of DEA demonstrated a dose-dependent decrease in K_a values, but no change in MBC. Lineweaver-Burk plots clearly indicated competitive inhibition by DEA. Pre-incubation of the TRα_1 with DEA decreased maximal [^{125}I]T_3 binding, which was independent of the duration of pre-incubation.
In conclusion, in contrast to the TRβ_1, where DEA acts as a noncompetitive inhibitor, we now report as a new finding the competitive action of DEA to the TRα_1.

3.2. Introduction

Amiodarone (AM), a potent antiarrhythmic and antianginal drug, is known to inhibit thyroid hormone-dependent gene expression. This inhibition is apparent both at the mRNA and at the protein level (2, 3). We have recently reported that not AM itself but its major metabolite desethylamiodarone (DEA) acts as a noncompetitive inhibitor of the binding of T_3 to the TRβ_1 (1). We wondered if differences between the TRβ_1 and TRα_1 (the homology between the two proteins is 72% for the hinge region and 82% for the ligand binding domain) would result in differences in inhibition of T_3 binding to these receptors by DEA. We now report that DEA (in contrast to our previous findings with respect to the TRβ_1) acts as a competitive inhibitor of T_3 binding to the TRα_1.

3.3. Materials and methods

3.3.A. Chemicals

Desethylamiodarone was a kind gift of Sanofi B.V., Maassluis, The Netherlands. 3,5,3'-Triiodothyronine (T_3) was obtained from Henning GmbH, Berlin, Germany. [^{125}I]T_3 (spec.act. 2200 Ci/mmol) was purchased from New England Nuclear, Boston, USA. All other reagents were of the highest grade possible.

3.3.B. Receptor expression

The chicken TRα_1 (amino acids 1-408) cDNA was cloned in a pEX vector as previously described (8). After heat shock at 42^{0}C for 10 min the cells were grown at 37^{0}C for 1.5 h. Cells were then lysed using lysozyme in a shaking ice-bath at 0^{0}C for 40 min. Fusion proteins were isolated as described (10) and stored in incubation buffer (20 mM Tris-HCl, 0.25 M sucrose, 1 mM EDTA, 50 mM NaCl, 5% (v/v) glycerol, pH 7.6) in liquid nitrogen. No pronounced loss of T_3 binding activity was observed during this procedure. The purity of the receptor preparation is approximately 0.5%, which is comparable to that observed by Ribeiro et.al. (7).

3.3.C. TR binding assay

The fusion proteins were defrosted on ice and solubilised by sonification (10 s, 6µm) and the non-soluble proteins were removed by centrifugation (5 min, 10,000 x g). Twenty to twenty-five µg protein was incubated with [^{125}I]T_3 (10^{-11} M) for 2h at 22^{0}C in a shaking water bath in incubation buffer containing 5 mM DTT, 0.05% Triton X-100 and 0.05% BSA. Total incubation volume was 0.5 ml. Reactions were stopped by chilling on ice water. Bound and unbound [^{125}I] T_3 were separated at 4^{0}C using a small Sephadex G25 medium column (bed volume 2 ml, swollen in incubation buffer with 0.05% BSA) in a Pasteur pipette. Four 0.8 ml fractions containing the bound hormone were collected, using incubation buffer as eluent. Specific binding was determined by calculating the difference between the counts bound in the absence and presence of an excess (10^{-7} M) of non-radioactive T_3. All incubations were done in duplicate.

Inhibition of [^{125}I]T_3 binding by desethylamiodarone (DEA) was studied by adding up to 10^{-4} M DEA to the incubation mixture. DEA was solubilised in a stock solution of 10^{-2} M in ethanol, and remained in solution under the chosen incubation conditions (1). In all tubes the final ethanol concentration was 1% (v/v), also in the controls without DEA. Scatchard analyses were performed by incubating the receptor with increasing amounts of non-radioactive T_3 (1-33 x 10^{-10} M) in the absence or presence of DEA (1 x 10^{-5}, 2.5 x 10^{-5}, 5 x 10^{-5} M).

The time course of T_3 binding to the TRα_1 protein was studied by incubating [^{125}I]T_3 (0.5 x 10^{-9} M) with the receptor protein as described above and stopping the reaction at the times indicated. The bound and unbound hormones were immediately separated using the Sephadex G-25 medium column. Competitors (non-radioactive T_3, 10^{-7} M; DEA, 5 x 10^{-5} M; or both) were added at maximal (after 120 min) and half-maximal (after 10 min) binding.

Pre-incubation experiments were performed to study a possible time-dependent effect of DEA on the unoccupied receptor by adding DEA 5 x 10^{-5} M at 5, 10 or 30 min prior to adding [^{125}I]T_3 (0.5 x 10^{-9} M) to the receptor-incubation mixture.

Table 1. Effect of desethylamiodarone (DEA) on the binding of T_3 to the TRα_1 and TRβ_1-proteins, obtained from Scatchard analysis

		DEA concentration		
	-----	1 x 10^{-5} M	2.5 x 10^{-5} M	5 x 10^{-5} M
TRα_1				
1. K_a (10^9 L/M)	1.34±0.06	1.15±0.05	0.89±0.03	0.52±0.02
2. MBC (10^{-10}M)	4.10±0.67	4.20±0.76	3.81±0.80	3.81±0.86
TRβ_1				
3. K_a (10^9 L/M)	1.22±0.25	1.03±0.21	0.97±0.27	0.87±0.21
4. MBC (10^{-10}M)	3.98±0.71	3.42±0.65	2.35±0.55	1.14±0.30

Values are mean ± S.E.M. (n = 5) ; significance of decrease is tested by two-way Anova analysis: 1. $P = 0.0002$; 2. $P = 0.43$; 3. $P = 0.03$; 4. $P = 0.0002$. Two-way Anova analysis was used, because there are two classifying variables, namely the DEA concentrations and the five independent experiments with different receptor protein batches.

3.4. Results

3.4.A. Scatchard analyses

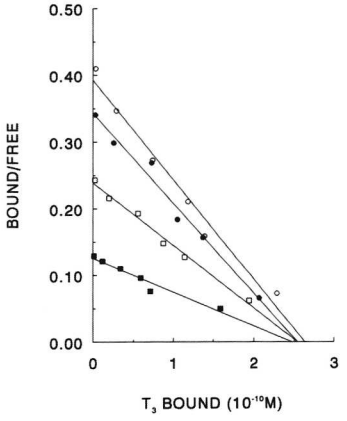
We observed a dose-dependent inhibition of T_3 binding to the chicken TRα_1 by DEA but not by AM. The IC_{50} value of DEA was 3.5 x 10^{-5} M. Scatchard analysis in the presence of 0.0, 1.0, 2.5 or 5.0 x 10^{-5} M DEA demonstrated a progressive lowering of K_a-values but MBC-values did not change with increasing DEA concentrations (Fig. 1A). These results were consistent in five separate experiments and in contrast with the effects of DEA on T_3 binding to the TRβ_1 in which both K_a and MBC decreased (Table 1). Lineweaver-Burk plots showed clearly competitive inhibition of T_3 binding to the TRα_1 by DEA (Fig. 1B). We next plotted the slope and the intercept (on the y-axis) versus the inhibitor concentration (on the x-axis). These plots yield information on the inhibition constants of the binding of DEA to the unoccupied ($I + R \leftrightarrow IR$; K_i) and to the occupied TR ($I + TR \leftrightarrow ITR$; K_i) respectively. As can be seen in Fig. 1C, the plot of the slope *vs.* inhibitor is parabolic ($y = 3.898 + 0.111x + 0.201x^2$, r = 1.00, $P = 0.005$) whereas that of the intercept *vs.* inhibitor is linear ($y = 0.072x + 0.376$, r = 0.858, NS from 0). The parabolic curve indicates that as DEA concentration rises, its effect on the unoccupied TR becomes progressively stronger. The horizontal line of the intercept *vs.* inhibitor indicates that increasing DEA concentrations have no effect on the occupied receptor.

3.4.B. Time-course experiments

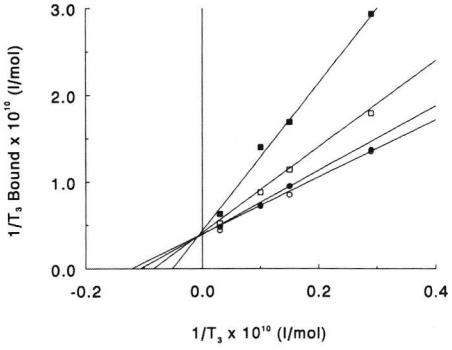
The time course of [^{125}I]T_3 binding to its receptor is shown in Fig. 2. When DEA (5 x 10^{-5} M) is added at equilibrium (120 min, Fig. 2A), only slight competition is seen. When the same concentration of DEA is added before equilibrium is reached, a lower maximal T_3 binding is observed (Fig. 2B). Adding an excess of nonradioactive T_3 together with DEA displays the same kinetics as adding the excess of T_3 alone.

Pre-incubation of the unoccupied receptor with DEA (5 x 10^{-5} M) at 5, 10 or 30 min prior to adding [^{125}I]T_3, results in the same maximal T_3 binding for the different pre-incubation times (Fig. 2C). This indicates competition between T_3 and DEA for binding sites on the α1-TR, in which T_3 effectively displaces DEA from the receptor.

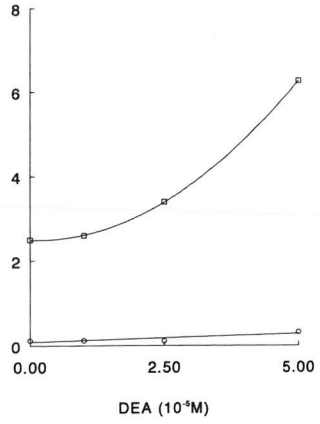
Fig. 1. Inhibition of the binding of T_3 to TRα_1 by desethylamiodarone (DEA).



A. Scatchard plots: The chicken TRα_1 was incubated with increasing concentrations of T_3 in the presence of 0 M (***open circle***), 1.0×10^{-5} M (***closed circle***), 2.5×10^{-5} M (***open square***), and 5.0×10^{-5} M (***closed square***) DEA. Binding data were corrected for non-specific binding.

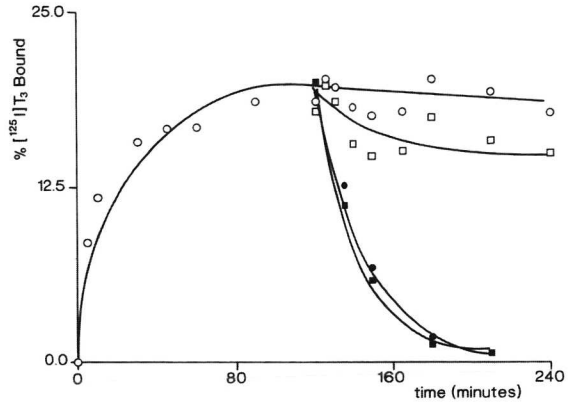


B. Lineweaver-Burk plots: the binding data obtained in panel A were used to prepare the double reciprocal plots.

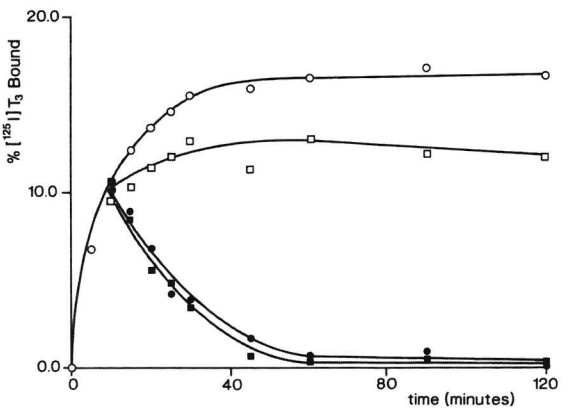


C. Plots of the slope and the intercept *vs.* inhibitor concentration: the slope (***open square***) and intercept (***open circle***) of the different lines of the Lineweaver-Burk plots of T_3 binding to TRα_1 were plotted against the inhibitor concentration. These plots yield information on the inhibitor constants of the binding of DEA to the unoccupied (I+R$\leftrightarrow$IR; K_i) and to the occupied TR (I+TR$\leftrightarrow$ITR; K_i) respectively.

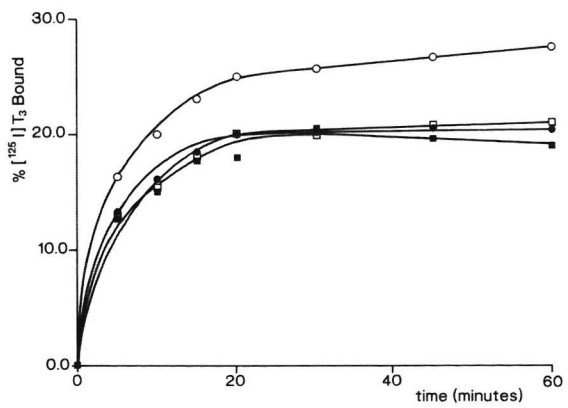
Fig. 2. Time-course of [^{125}I]T_3 binding to TRα_1 with prior or delayed addition of desethylamiodarone (DEA).



A. Addition of nonradioactive T_3 (10^{-7} M; ***closed circle***), DEA ($5x10^{-5}$ M; ***open square***) or T_3 + DEA (***closed square***) at maximal binding of T_3.



B. Addition of nonradioactive T_3 (10^{-7} M; ***closed circle***), DEA ($5x10^{-5}$ M; ***open square***) or T_3 + DEA (***closed square***) at half maximal binding of T_3.



C. Pre-incubation of TRα_1 protein with DEA (5×10^{-5} M) at 5 min (***closed circle***), 10 min (***open square***) or 30 min (***closed square***) before addition of [^{125}I]T_3 to the incubation mixture at t = 0.

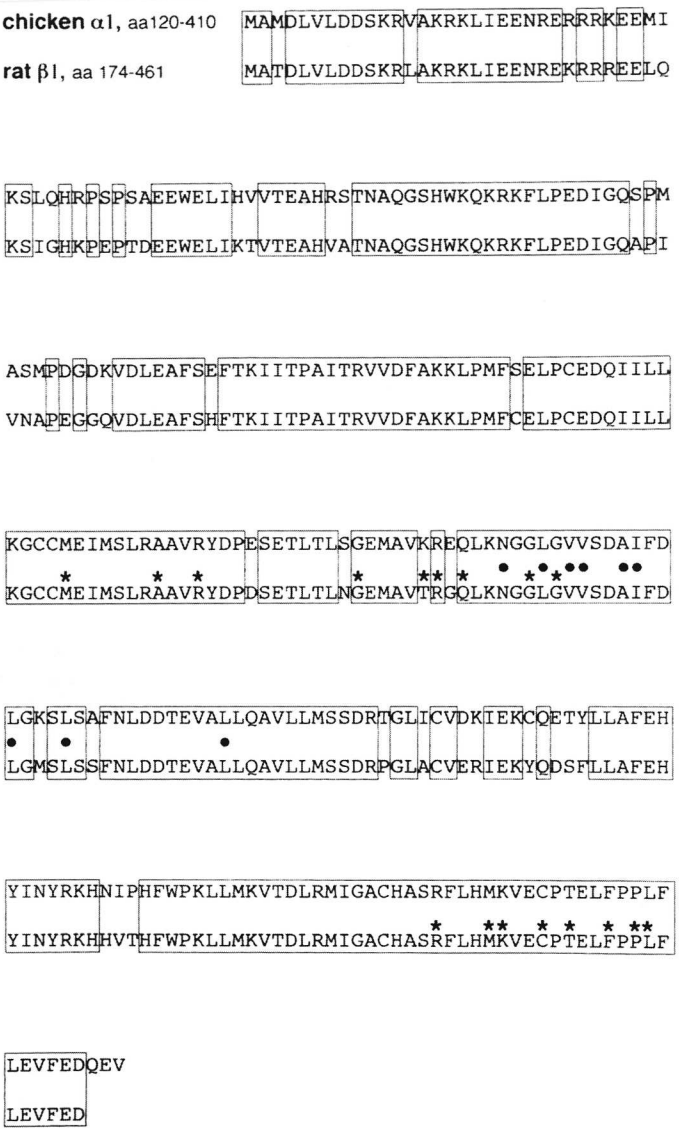


Fig. 3. Comparison between the amino acid sequences of the hormone binding domain of the chicken TRα_1- and rat TRβ_1. Identical sequences are boxed. Black dots indicate positions where point mutations of aa result in a decrease or loss of T_3 binding (9). Asterisks indicate natural occurring mutations in the human TRβ_1 gene that lead to thyroid hormone resistance (6).

3.5. Discussion

Desethylamiodarone, but not amiodarone itself, inhibited the binding of T_3 to the TRα_1, similar to our previous findings with respect to the TRβ_1. Indeed, IC_{50} values of DEA for the TRα_1 and TRβ_1 proteins were in the same order of magnitude (3.5 and 2 x 10^{-5} M respectively). The mode of inhibition of T_3 binding by DEA, however, appeared to be different for the TRα_1 and TRβ_1: DEA acted as a competitive inhibitor of T_3 binding to the TRα_1 as evident from Scatchard analyses and Lineweaver-Burk plots, whereas it is a noncompetitive inhibitor of T_3 binding to the TRβ_1. The data suggest that DEA and T_3 compete for the same binding site in case of the TRα_1 (competitive inhibition). In contrast, the noncompetitive nature of the inhibition by DEA on the TRβ_1 points to either of two possibilities, namely (1) the DEA binding site is separate from the T_3 binding site: or (2) the DEA binding site (partly) overlaps the T_3 binding site of the TRβ_1. In both cases, T_3 binding is inhibited.

A number of potentially crucial differences exist between the TRα_1 and TRβ_1 in the hormone-binding domain (aa: chicken α1 120-410; rat β1 174-461) (Fig. 3). It could therefore be postulated that the aa differences between TRα_1 and TRβ_1 in this region determine the type of inhibition by DEA. Interestingly, a number of these α/β differences are present near a region that is important for T_3 binding, because point mutations in this region decrease T_3 binding (9) (Fig. 3).

In view of the much higher affinity of T_3 for the receptor proteins as compared to DEA, it is not surprising that DEA exerts its effect on unoccupied binding sites of the TR in particular, and this is true for both the TRα_1 and TRβ_1 proteins. This suggests that it takes time before the inhibitory effect of DEA on TR binding becomes evident in patients on long-term AM medication. Clearly, tissue concentrations of DEA must exceed a certain level before they can compete effectively with T_3 for available binding sites on TRs. The fact that DEA accumulates in tissues makes it feasible that the inhibitory effects of DEA do occur in vivo. One of the side effects of amiodarone is hypercholesterolemia (presumably mediated via decreased gene expression of the LDL-receptor; (3)), which develops slowly over time and is directly related to the cumulative dosage of AM (5, 11). This clinical observation in humans might be related to our in vitro observation of inhibition of TR binding by DEA.

In conclusion, DEA acts as a competitive antagonist for the TRα_1 and as a noncompetitive antagonist for the TRβ_1. This could be reflected in differential effects of AM in different tissues because of the different distribution of the TR subtypes in the tissue (4). Future studies with amiodarone analogues or with mutated receptors might enable a more detailed analysis of the structure-function relationship of this fascinating antagonism of DEA for the binding of T_3 to its receptor proteins.

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CHAPTER 4

Structure-function relationship of the inhibition of the 3, 5, 3'-triiodothyronine binding to the TRα_1 and TRβ_1 by amiodarone analogues

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4.1. Abstract

Desethylamiodarone (DEA), the major metabolite of the potent antiarrhythmic drug amiodarone (AM), acts as a competitive inhibitor of T_3 binding to the α_1-thyroid hormone receptor (TRα_1), but as a noncompetitive inhibitor with respect to the $ß_1$-thyroid hormone receptor (TR$ß_1$). To gain insight into the structure-function relationship of the interaction between AM metabolites and TRs, we investigated the effects of several AM analogues on T_3 binding to the TRα_1 and TR$ß_1$ *in vitro*. The analogues tested were: 1) compounds obtained by deethylation of AM, DEA and desdiethylamiodarone (DDEA); 2) compounds obtained by deiodination of AM, monoiodoamiodarone (MIA) and desdiiodoamiodarone (DDIA); 3) benzofuran derivatives with various iodination grades, 2-butyl-3-(4-hydroxy-3, 5-diiodo-benzoyl)benzofuran (L3373, two iodine atoms), L6424 (L3373 with one iodine atom), and L3372 (L3373, no iodine atoms).

IC_{50} values of inhibition of T_3 binding to the TRα_1 and TR$ß_1$ respectively, were as follows (mean ± SD, expressed x 10^{-5} M): DEA 4.7 ± 0.9 and 2.7 ± 1.4 ($P < 0.001$); DDEA 3.7 ± 0.9 and 1.9 ± 0.3 ($P < 0.001$); monoiodoamiodarone, more than 20 and more than 20; DDIA 16.2 ± 5.6 and 9.1 ± 2.1 ($P < 0.01$); L3373, 3.8 ± 1.0 and 3.6 ± 0.5 (P = NS); L6424, 11.3 ± 5.7 and 10 ± 2.0 (P = NS); and L3372, no inhibition. Scatchard analyses in the presence of DDEA, DDIA, and L3373 demonstrated a dose-dependent decrease in K_a, but no change in the maximum binding capacity (MBC) of T_3 binding to the TRα_1. Langmuir plots clearly indicated competitive inhibition of T_3 binding to TRα_1 by DDEA, DDIA and L3373. In contrast, these three analogues acted differently with respect to the TR$ß_1$. DDEA and DDIA decreased both K_a and MBC in Scatchard plots using TR$ß_1$, demonstrating noncompetitive inhibition. L3373 decreased dose-dependently K_a but not MBC, values of T_3 binding to the TR$ß_1$, and clearly acted as a competitive inhibitor. K_i plots indicated that DDEA, DDIA and L3373 do not interfere significantly with occupied TRs. K_I (inhibition constant for the unoccupied receptor) plots demonstrated increasing inhibition of the T_3 binding to unoccupied receptors with increasing analogue concentrations.

In summary, 1) removal of one or two ethyl groups of AM results in compounds with strong but almost equal potency of inhibiting TR binding, whereas removal of one or two iodine atoms of AM has a lower potency in this

respect. The strong inhibitory potency of the benzofuran derivative L3373 (equalling that of the deethylated compounds) is lost upon deiodination. 2) All tested AM analogues acted as competitive inhibitors to the TRα_1. The behaviour to the TRβ_1 was different; deethylation or deiodination of AM results in noncompetitive inhibition, whereas L3373 was a competitive inhibitor. The potency of deethylated and deiodinated compounds (but not of the benzofuran derivatives) for inhibiting T_3 binding was twice as high for the TRβ_1 as for the TRα_1. 3) All tested AM analogues preferentially interfere with T_3 binding to unoccupied receptors.

The implications of these findings for the structure-activity relationship are the following: 1) the size of the diethyl-substituted nitrogen group and of the two bulky iodine atoms in the AM molecule hamper the binding of AM at the T_3 binding site of TRs; and 2) differences in the hormone-binding domain of TRα_1 and TRβ_1 are likely to account for the competitive or noncompetitive nature of inhibition of T_3 binding by AM analogues.

4.2. Introduction

Amiodarone (AM), an iodinated benzofuran derivative, is a potent antiarrhythmic and antianginal drug. It also profoundly affects thyroid hormone metabolism. The drug causes a dose-dependent decrease in serum T_3 concentrations due to diminished hepatic T_4 5'-deiodination secondary to inhibition of T_4 transport across the plasma membrane (1). It also decreases gene expression of thyroid hormone-dependent genes such as phosphoenol pyruvate carboxy kinase and the low-density lipoprotein (LDL) receptor (2, 3). The hypothesis of AM as a thyroid hormone antagonist has been further supported by the finding that desethylamiodarone (DEA) , the major metabolite of AM, inhibits the binding of T_3 to its nuclear receptor. DEA acts as a competitive antagonist with respect to binding of T_3 to the TRα_1 but, interestingly, as a noncompetitive antagonist with respect to the TRβ_1 (4, 5).
The metabolic pathway involved in *N*-dealkylation of AM can reduce tertiary amines to secondary amines (DEA) and finally to a primary amine [desdiethylamiodarone (DDEA)]. DDEA has been positively identified in plasma and myocardial tissue of AM-treated dogs, but was present in only small quantities in human plasma (6). Besides deethylation, AM also undergoes deiodination. A small peak was found in pooled plasma from patients on AM, possibly corresponding to the metabolite deiodo-desethylamiodarone (7). It is currently unknown whether biotransformation of the drug also yields monoiodoamiodarone (MIA) and desdiiodoamiodarone (DDIA).
The aim of the present study was to delineate possible inhibitory effects of AM analogues on nuclear TR binding and to evaluate any differences between α_1- and TRβ_1 in this respect. In doing so we tried to clarify the structure-function relationship of the interaction between AM analogues and TRs.

4.3. Materials and methods

4.3.A. Chemicals

Nonradioactive T_3 was obtained from Henning (Berlin, Germany). [^{125}I]T_3 (SA, 2200 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA). The AM analogues were a kind gift from Sanofi (Brussels, Belgium) and Sanofi (Maassluis, The Netherlands).

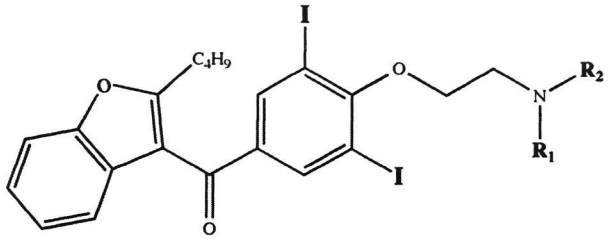
Three types of analogues were tested (Fig.1): 1) compounds obtained by deethylation of AM: DEA (L33520) and DDEA (L33530); 2) compounds obtained by deiodination of AM: MIA (L6355), DDIA (L3937); 3) benzofuran derivatives with various iodination grades: 2-butyl-3-(4-hydroxy-3, 5-diiodo-benzoyl)benzofuran (L3373, two iodine atoms), 2-butyl-3-(4-hydroxy-3-iodo-benzoyl)benzofuran (L6424, one iodine atom), and 2-butyl-3-(4-hydroxy-benzoyl)benzofuran (L3372, no iodine atoms). All reagents were of the highest grade possible.

4.3.B. Receptor expression

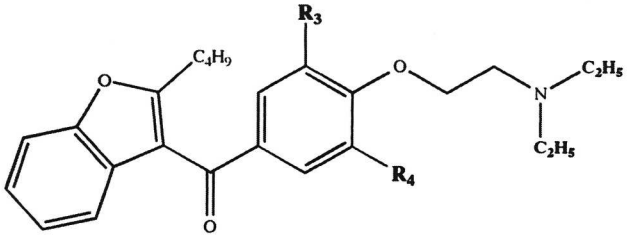
The chicken TRα_1 (amino acids 1-408) and rat TRβ_1 (amino acids 31-456) cDNA were cloned in a pEX vector, as previously described (8). After heat shock at 42^{0}C for 10 min, the cells were grown at 37^{0}C for 1.5 h. Cells were then lysed using lysozyme in a shaking ice bath at 0^{0}C for 40 min. Fusion proteins were isolated as previously described (9) and stored in incubation buffer [20 mM Tris-HCl, 0.25 M sucrose, 1 mM EDTA, 50 mM NaCl, and 5% (vol/vol) glycerol, pH 7.6] in liquid nitrogen. The purity of the receptor preparation was on the order of 0.5%.

4.3.C. TR binding assay

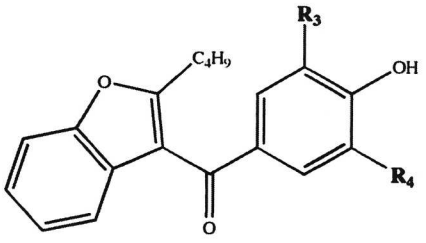
The fusion proteins were defrosted on ice and solubilised by sonification (10 s, 6 μm) and the nonsoluble proteins were removed by centrifugation (5 min, 10.000 x *g*). Receptor proteins (20-25µg protein / tube) were incubated with [^{125}I]T_3 (10^{-11} M) for 2h at 22^{0}C in a shaking water bath in incubation buffer containing 5 mM dithiothreitol, 0.025% Triton X-100, 0.05% BSA, and 1% ethanol (vol/vol). These additions to the incubation buffer were necessary to solubilise AM and its analogues in a hydrophilic environment as demonstrated previously (5). The total incubation volume was 0.5 ml. Reactions were



	R_1	R_2
amiodarone (A, L 3428)	C_2H_5	C_2H_5
desethylamiodarone (DEA, L 33520)	C_2H_5	H
desdiethylamiodarone (DDEA, L 33530)	H	H



	R_3	R_4
amiodarone	I	I
monoiodoamiodarone (MIA, L 6355)	I	H
desdiiodoamiodarone (DDIA, L 3937)	H	H



	R_3	R_4
L 3373	I	I
L 6424	I	H
L 3372	H	H

FIG. 1. The molecular constitution of AM analogues: deethylated analogues (*top*), deiodinated analogues (*middle*), and benzofuran derivates (*bottom*).

stopped by chilling on ice water. Bound and unbound [^{125}I] T_3 were separated at 4^{0}C using a small Sephadex G-25 medium column (bed volume, 2 ml; swollen in incubation buffer with 0.05% BSA) in a Pasteur pipette. Four 0.8-ml fractions, containing the bound hormone fraction, were collected using incubation buffer as eluent. Specific binding was determined by calculating the difference between the counts bound in the absence and presence of an excess (10^{-7} M) of nonradioactive T_3. All incubations were performed in duplicate. The maximal binding capacity (MBC) of TR preparations varied between 2-6 10^{-10} M/L.

The potency of AM analogues to inhibit the binding of T_3 to the TRα_1 and TRβ_1 was tested over a concentration range of 10^{-7} - 10^{-4} M. Analogues, solubilised in a stock solution of 10^{-2} M in ethanol, were incubated with receptor proteins and [^{125}I]T_3 as described above. In all tubes the final ethanol concentration was 1% (vol/vol). From these experiments the IC_{50} values of the analogues were calculated.

Scatchard analyses were performed with the most potent analogue of each group. Receptor proteins and [^{125}I]T_3 were incubated with increasing amounts of nonradioactive T_3 (1 x 10^{-10} to 33 x 10^{-10} M) in the absence or presence of DDEA, DDIA, or L3373. Three or four separate experiments were performed for each analogue; in each experiment the inhibitory effect of the analogue was tested at two concentrations and simultaneously for the TRα_1 and TRβ_1. The MBC and K_a values of the experiments presented in Table 2 were calculated using the non-linear curve-fitting computer program Ligand (Biosoft, Cambridge, UK). Changes in MBC and K_a as a function of analogue concentration were analysed by two-way ANOVA.

Langmuir plots (equivalent to Lineweaver-Burk plots) were prepared from the data of the Scatchard analyses. To delineate the type of inhibition, we analysed, by means of the *t* test, whether the intercepts on the *y*-axis differed significantly among the various analogue concentrations; differences on the *y*-axis were taken as evidence for noncompetitive inhibition, whereas the absence of such differences indicated competitive inhibition. Plotting the slopes and the intercepts of the Langmuir plots (on the *y*-axis) *vs.* the analogue concentrations (on the *x*-axis) yielded K_I (inhibition constant for the unoccupied receptor) and K_i plots, providing information on the inhibition constants of the binding of the analogues to unoccupied TR ($I + R \leftrightarrow IR$; K_I) and occupied TR ($I + TR \leftrightarrow ITR$; K_i) respectively.

4.4. Results

4.4.A. Inhibitory potency of analogues

Whereas AM itself had almost no inhibitory effect on the binding of T_3 to its receptors, deethylation of AM resulted in compounds that greatly inhibited the binding of T_3 to its receptors (Table 1 and Fig. 2). The inhibitory potency was similar upon the removal of one or two ethyl groups. Deiodination of AM resulted in compounds with slightly increased inhibitory potency relative to that of AM. The inhibition became stronger with progressive deiodination, but IC_{50} values were smaller than those of deethylated compounds. The compound L3373, in which the (diethylamino)ethoxy side-chain of AM is replaced by a hydroxyl group, inhibited the binding of T_3 with approximately the same potency as the deethylated compounds. Monodeiodination of L3373 greatly reduced the inhibitory potency, which was completely lost when both iodine atoms were removed. The observed inhibitory effects of AM analogues were in the same order of magnitude for both TRα_1 and TRβ_1, although IC_{50} values of deethylated and deiodinated analogues of AM were twice as high for the TRα_1 as for the TRβ_1.

TABLE 1. Inhibition of the binding of [^{125}I]T_3 to the TRα_1 and TRβ_1 by amiodarone and its analogues

	n	IC_{50} values of amiodarone analogues (10^{-5} M)[a]		*P* value[b]
		TRα_1	TRβ_1	
Amiodarone	5	>20	>20	
Desethylamiodarone	7	4.7 ± 0.9	2.7 ± 1.4	<0.001
Desdiethylamiodarone	8	3.7 ± 0.9	1.9 ± 0.3	<0.001
Monoiodoamiodarone	3	>20	>20	
Desdiiodoamiodarone	8	16.2 ± 5.6	9.1 ± 2.1	<0.01
L3373 (two iodine atoms)	6	3.8 ± 1.0	3.6 ± 0.5	NS
L6424 (one iodine atom)	3	11.3 ± 5.7	10 ± 2.0	NS
L3372 (no iodine atom)	3	No inhibition	No inhibition	

n, Number of experiments.
[a] Values are the mean ± SD
[b] Differences between TRα_1 and TRβ_1, determined by *t* test.

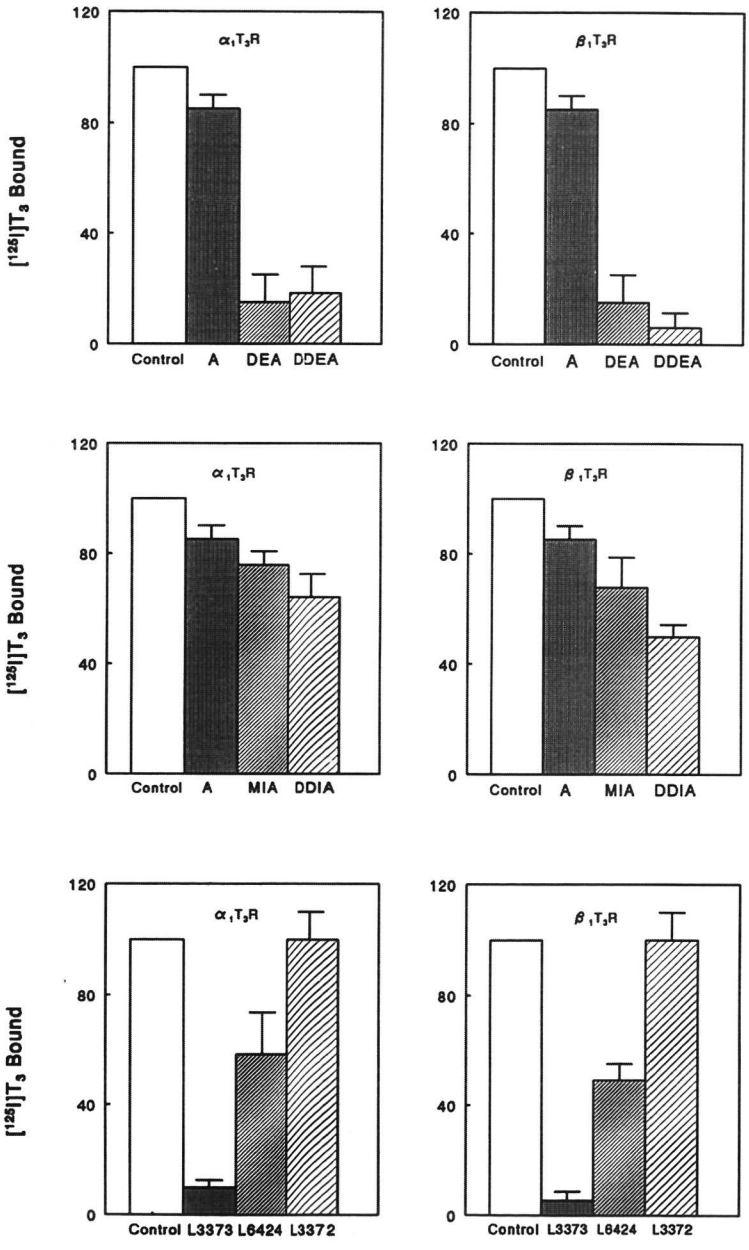


FIG. 2. Inhibition of the in vitro binding of [^{125}I]T_3 to the TRα_1 (*left panels*) or TRβ_1 (*right panels*) by AM and its analogues (final concentration, 10-4 M). Values are given as mean ±SD (n=3) and expressed as percentage of specifically bound [^{125}I]T_3 in the absence of AM or its analogues.

4.4.B. Competitive and noncompetitive inhibition by analogues

Representative Scatchard plots and Langmuir analysis of the effect of DDEA, DDIA, and compound L3373 are depicted in Figs. 3 and 4, respectively. The statistical analysis of the experimental results is given in Table 2.

TABLE 2. Characteristics of the inhibition of the binding of T_3 to TRα_1 and TRβ_1 by desdiethylamiodarone (DDEA), desdiiodoamiodarone (DDIA) and 2-butyl-3-(4-hydroxy-3, 5-diiodobenzoyl)benzofuran (L3373), as evident from Scatchard plots and Langmuir analyses

	TRα_1		TRβ_1	
	MBC	K_a	MBC	K_a
DDEA				
Scatchard plots				
0.0 x 10^{-5} M	3.46 ± 0.56	2.19 ± 0.18	4.57 ± 0.77	1.03 ± 0.12
1.0 x 10^{-5} M	3.32 ± 0.65	1.86 ± 0.05	3.64 ± 0.61	0.76 ± 0.04
2.5 x 10^{-5} M	3.12 ± 0.89	1.18 ± 0.18	2.28 ± 0.63	0.66 ± 0.03
P (by two-way ANOVA)	NS	0.0001	0.0002	0.0004
Langmuir analysis	Competitive inhibition (*P* = 0.46)		Noncompetitive inhibition (*P* = 0.004)	
DDIA				
Scatchard plots				
0.0 x 10^{-5} M	3.34 ± 1.53	2.02 ± 0.72	3.13 ± 0.95	1.21 ± 0.60
3.0 x 10^{-5} M	3.17 ± 1.37	1.60 ± 0.30	2.57 ± 1.25	1.12 ± 0.49
6.0 x 10^{-5} M	3.13 ± 1.71	1.32 ± 0.34	2.22 ± 0.98	0.86 ± 0.48
P (by two-way ANOVA)	NS	0.04	0.02	0.045
Langmuir analysis	Competitive inhibition (*P* = 0.46)		Noncompetitive inhibition (*P* = 0.008)	
L3373				
Scatchard plots				
0.0 x 10^{-5} M	2.99 ± 0.32	1.98 ± 0.52	3.87 ± 0.83	0.90 ± 0.27
1.0 x 10^{-5} M	2.85 ± 0.11	1.69 ± 0.30	3.72 ± 0.75	0.78 ± 0.18
2.5 x 10^{-5} M	2.76 ± 0.30	1.01 ± 0.06	3.83 ± 1.08	0.51 ± 0.18
P (by two-way ANOVA)	NS	0.02	NS	0.002
Langmuir analysis	Competitive inhibition (*P* = 0.62)		Competitive inhibition (*P* = 0.52)	

MBC, 10^{-10} M/litre; K_a, 10^9 litre/M; values are the mean ± SD (DDEA, n = 4; DDIA, n = 4; L3373, n = 3). *P* values determined by Langmuir analyses indicate whether the intercepts on the *y*-axis differ significantly between the various analogue concentrations.

With respect to the binding of T_3 to the TRα_1, none of the three analogues affected the T_3 MBC of the receptor, but all decreased the affinity constant of T_3 binding in a dose-dependent manner. Langmuir analysis confirmed the competitive nature of the inhibition of T_3 to the TRα_1 by these analogues. A

different picture emerged for the TRβ_1. DDEA decreased both MBC and K_a dose-dependently in the Scatchard plots and was shown to be a non-competitive inhibitor by Langmuir analysis. DDIA also decreased MBC and K_a, indicating noncompetitive inhibition. This was also evident from the Langmuir plots, although the intercepts on the *y*-axis were just marginally different among the various concentrations of the drug (P = 0.08). The compound L3373 only affected the affinity constant and clearly was a competitive inhibitor of the TRβ_1.

4.4.C. K_i and K_I plots of analogues

K_i plots (intercept *vs.* inhibitor) were linear, with a negligible slope for both TRα_1 and TRβ_1, indicating lack of interference of DDEA, DDIA, and L3373 with occupied receptors. In contrast, K_I plots (slope *vs.* inhibitor) were linear with a significant slope for both TRα_1 and TRβ_1, indicating stronger inhibition of T_3 binding to unoccupied receptors with increasing concentrations of DDEA, DDIA, and L3373. K_I values of DDEA were 3.1 x 10^{-5} M (TRα_1) and 1.5 x 10^{-5} M (TRβ_1), those of DDIA were 9.4 x 10^{-5} M (TRα_1) and 6.2 x 10^{-5} M (TRβ_1), and those of L3373 were 2.2 x 10^{-5} M (TRα_1) and 3.1 x 10^{-5} M (TRβ_1). The K_I plots appear to become parabolic at analogue concentrations exceeding 2.5 x 10^{-5} M (DDEA and L3373) or 6 x 10^{-5} M (DDIA; data not shown), suggesting a progressively stronger inhibition of T_3 binding to unoccupied receptors.

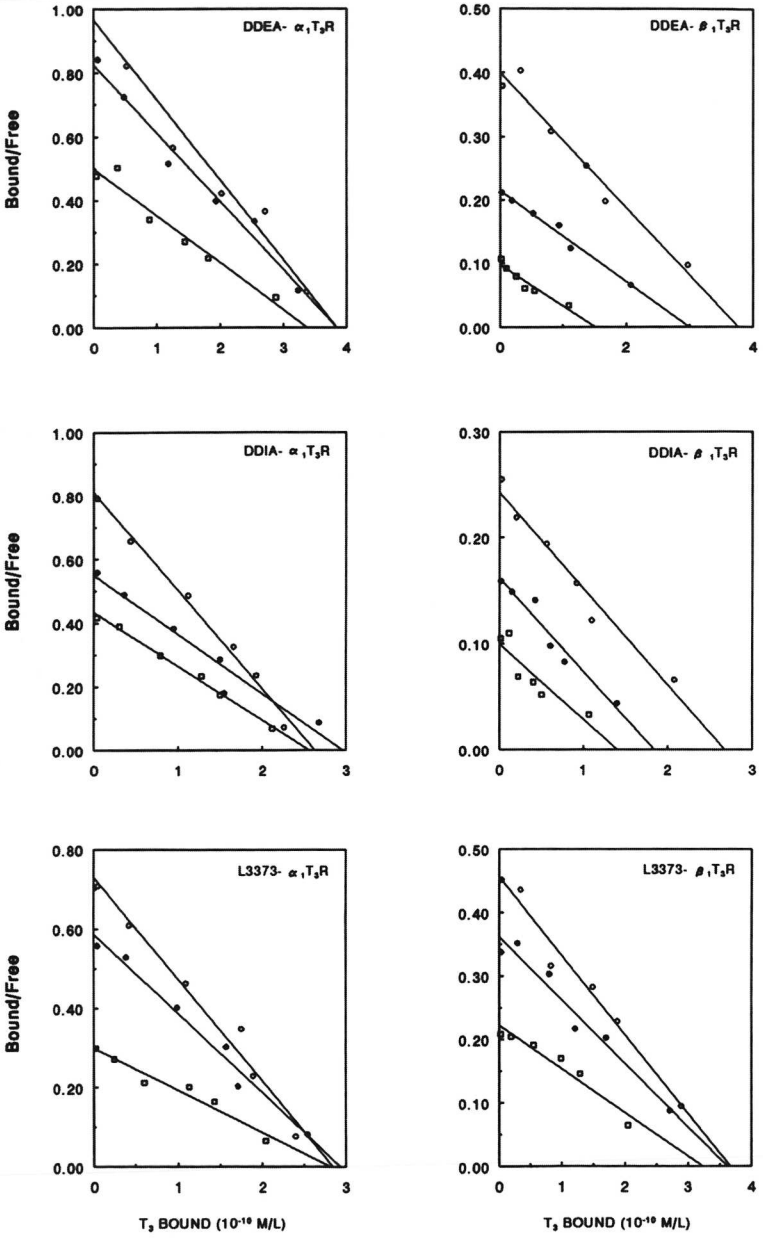


FIG. 3. Scatchard analyses of the binding of T_3 to TRα_1 (*left panels*) or TRβ_1 (*right panels*) in the absence (open circles) or presence of AM analogues. *Top panel*: DDEA, 1.0 x 10^{-5} M (closed circles), and 2.5 x 10^{-5} M (open squares). *Middle panel*: DDIA, 3 x 10^{-5} M (closed circles), and 6 x 10^{-5} M (open squares). *Bottom panel*: L3373, 1, 0 x 10^{-5} M (closed circles), and 2, 5 x 10^{-5} M (open squares).

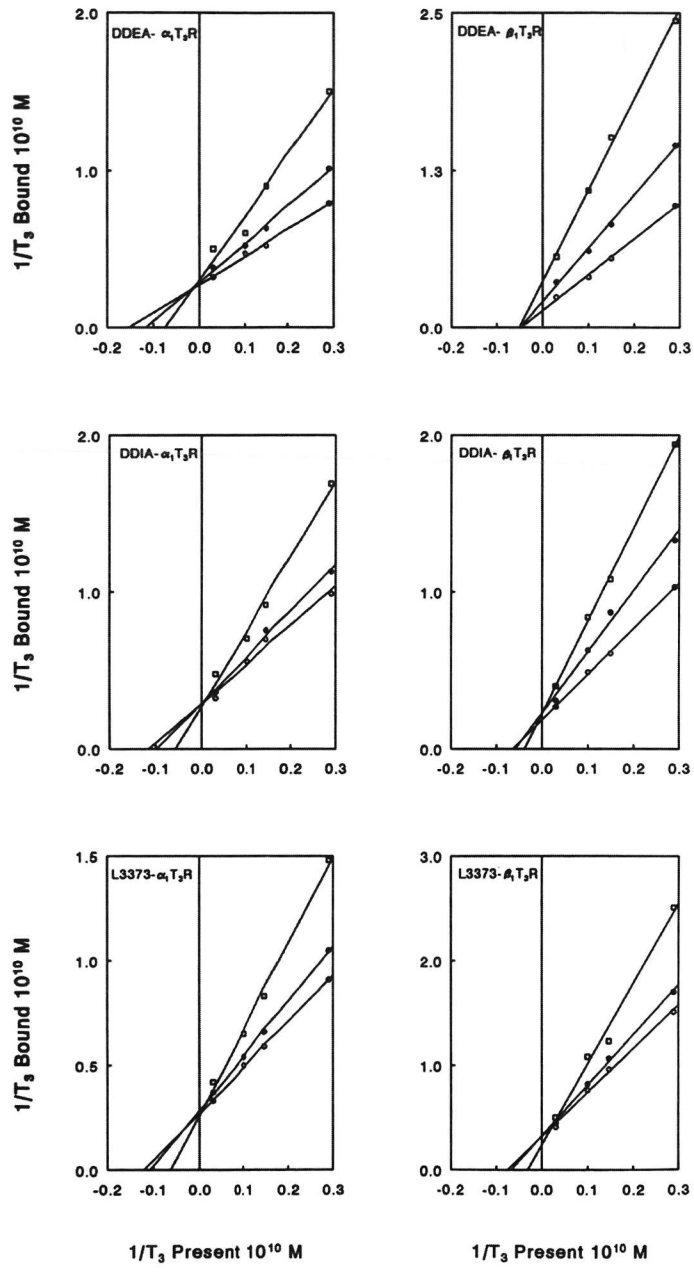


FIG. 4. Langmuir plots of the binding of T_3 to TRα_1 (*left panels*) or TR$ß_1$ (*right panels*) in the absence (open circles) or presence (closed circles and open squares) AM analogues constructed from the data obtained in the Scatchard analysis.

4.5. Discussion

Our results indicate that structural changes in the AM molecule affect the potency of the drug to compete with T_3 for binding to TRs. The strongest competitive behaviour is seen with deethylated AM and the compound L3373, which has a phenolic OH. A weaker, but still measurable, competition is seen with deiodinated AM. The data support the hypothesis that one of the mechanisms of action of AM is induction of a hypothyroid-like condition by interference with the binding of T_3 to the nuclear receptors (1). The likelihood of this mechanism to be operative *in vivo* is underlined by various pharmacological studies. Firstly, the main metabolite of AM is DEA (7), which slowly accumulates in body tissues, reaching concentrations capable of inhibiting T_3 nuclear binding (5). The preference of DEA to interfere with unoccupied TR is in line with clinical data indicating a gradual development of its thyroid hormone antagonistic effects *in vivo* (10). Furthermore, administration of DEA itself has similar effects as the parent drug (11). Secondly, although not much is known on the occurrence of deiodinated AM *in vivo*, one tablet of 200 mg of AM generates during its biotransformation 6 mg free iodine (1), indicating that the deiodinated forms may occur. Lastly, the compound L3373 is much like benziodarone (the difference being that the 2-*n*-butylgroup in benziodarone is replaced by an ethyl group in L3373), which exerts similar actions as AM (12).

The action of AM can be explained by structural similarity between AM and iodothyronines (Fig. 5). Important functional groups, identified in studies with iodothyronine analogues (13), in the T_3 molecule for the binding to the nuclear receptor are 1) the 4'-OH group for the formation of hydrogen bonds, 2) the I atoms for hydrophobic interactions (the I atoms can be replaced by small alkyl groups, roughly the size of iodine, without great impairment of receptor binding), 3) the diphenyl ether linkage for controlling conformation, and 4) the alanine side-chain amino acid and carboxylate group for the formation of an ion pair with a charged part of the receptor. A number of studies compared the structural similarity between AM and iodothyronines by superimposing the iodophenyl ring of A to either the inner or outer ring of the iodothyronines (15, 16). We propose that the iodophenyl ring of AM and its analogues is equivalent to the T_3 inner ring, and that the 4'-side group is able to position

itself well into the receptor-binding pocket, as this model is most compatible with the competitive behaviour of the analogues tested in this study.

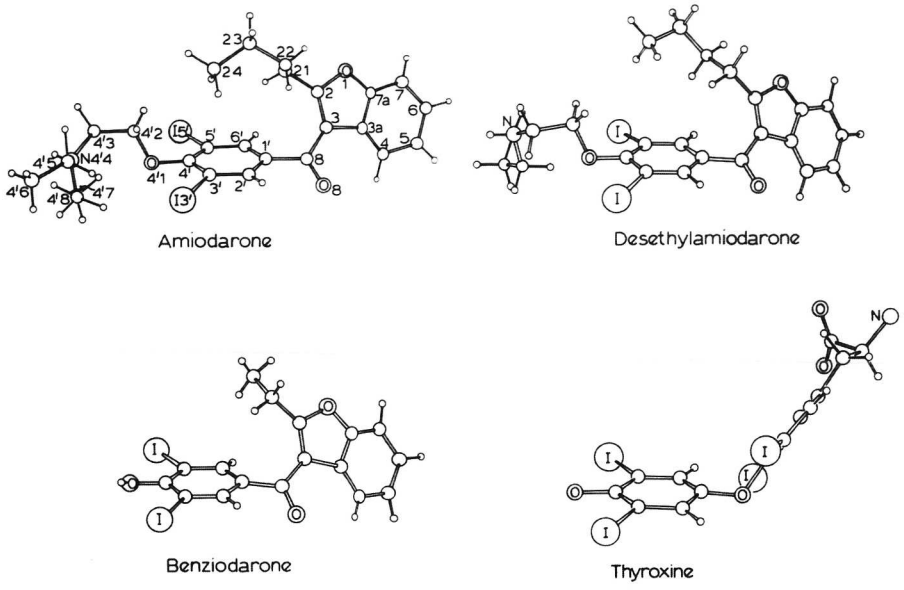


FIG. 5. Molecular conformation of AM, DEA, benziodarone, and T_4. The conformation of T_3 is similar to that of T_4 (14). (Figures are by courtesy of Dr. V. Cody (Buffalo, NY) (15).)

Removing the bulky I groups from AM results in an increase in competitiveness. It may allow a better fit of AM in the binding pocket of the TR, probably by releasing the constraints put on the molecule by the two I atoms. Deiodinated AM is, therefore, better able to wriggle itself into the pocket. However, there is not enough space to alleviate the hindrance caused by the two *N*-ethyl groups, which is reflected in the fact that the IC_{50} of DDIAM is at least 4-fold higher then that of DEA or L3373.

Deethylation of AM leads to an increase in competitive behaviour, probably as a result of both a change in size and in conformation (15) of the 4'-side group and a change in the charge of this side group (less basic). Because the 4'-side group is probably positively charged, it may be able to interact with a charged side-chain of the TR molecule, stabilizing the interaction. The finding that the L3373 compound is as effective in competing for T_3 binding as DEA shows that it is not just the size of the 4'-side group that matters. The I atoms are

important, as deiodination of L3373 results in total loss of competitive potency, and the benzofuran group may be involved as well.

It can be envisaged that not only the size and conformation of the 4'-side group, but also the position of the benzofuran group and its 2-side-chain play a role in the competitive ability of AM and its analogues. It has been shown using x-ray crystallography that there is a change between AM *vs.* DEA and benziodarone in the conformation of the 2-side chain, which results in a different overall conformation (15). Charged side groups, equivalents of the alanine side-chain, are not present on the benzofuran moiety, which may also influence competing ability. This can explain why L3373, even though it has a phenolic OH like T_4, does not compete as well as might have been expected on the basis of its structural similarity to T_4 (15).

The differential behaviour (competitive *vs.* noncompetitive) of the α_1- and β_1-subtypes of the receptor toward DDEA and DDIA could be explained by differences in the amino acid sequences of these two receptor proteins. One example is Tyr^{395} in TRβ_1, which is a Ser in the TRα_1-subtype. Interestingly, this position 395 would end up on the top of helix 6 in the α/β barrel model near the putative binding pocket (18, 19) so it could well have an influence on AM binding. Our future studies will be aimed at using other AM analogues and mutants of the TR to further elucidate the mechanism of inhibition of AM, with respect to the binding of T_3 to TRα_1 and TRβ_1.

Acknowledgements

We thank Sanofi Recherche (Montpellier, France) for their generous gifts of AM and its analogues. We especially thank Dr. Vivian Cody for very helpful discussions and providing us with graphic illustrations of the conformational structures of AM and the analogues. Marianne Platvoet-ter Schiphorst and Nel de Jong are acknowledged for their technical assistance.

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CHAPTER 5

Effect of mutations in the TRβ_1 on the inhibition of T_3 binding by desethylamiodarone

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5.1. Abstract

Desethylamiodarone (DEA) acts as a competitive inhibitor of T_3 binding to the α_1-thyroid hormone receptor (TRα_1) but as a noncompetitive inhibitor with respect to the ß$_1$-thyroid hormone receptor (TRß$_1$). To gain insight into the position of the binding site of desethylamiodarone on the TRß$_1$ we investigated the naturally occurring mutants Y321C, R429Q, P453A, P453T and the artificial mutants L421R and E457A in the ligand binding domain of the human TRß$_1$. The IC_{50} values (in µM) of DEA for P453A (50 ± 11) and P453T (55 ± 16) mutant TRß$_1$ are not different from that for the wild type TRß$_1$ (56 ± 15), but the IC_{50} values of R429Q (32 ± 7; $P < 0.001$) and E457A (17 ± 3; $P < 0.001$) are significantly lower than of the wild type. Scatchard plots and Langmuir analyses indicate a noncompetitive nature of the inhibition by DEA of T_3 binding to all four mutant TRß$_1$s tested. Mutants P453A and P453T do not influence overall electrostatic potential, and also do not influence the affinity for DEA compared to wild type. Mutant E457A causes a change from a negatively charged amino acid to a hydrophobic amino acid, enhancing the affinity for DEA. Mutant R429Q, located in helix 11, causes an electrostatic potential change from positive to uncharged, also resulting in greater affinity for DEA. We therefore postulate that amino acids R429 and E457 are at or close to the binding site for DEA, and that DEA does not bind in the T_3 binding pocket itself, in line with the noncompetitive nature of the inhibition of T_3 binding to TRß$_1$ by DEA.

5.2. Introduction

Amiodarone, an iodinated benzofuran derivative, is a potent antiarrhythmic and antianginal drug. It also profoundly affects peripheral thyroid hormone metabolism. The drug causes a dose-dependent decrease of serum T_3 concentrations due to diminished hepatic T_4 5'-deiodination secondary to inhibition of T_4 transport across the plasma membrane (1). It also decreases gene expression of thyroid hormone-dependent genes such as α-myosin heavy chain and the low-density lipoprotein receptor (2, 3). The hypothesis that amiodarone is a thyroid hormone antagonist has been further supported by the finding that desethylamiodarone (DEA), the major metabolite of amiodarone, inhibits the binding of T_3 to its nuclear receptor. DEA acts as a competitive antagonist with respect to binding of T_3 to the TRα_1 isoform but interestingly as a noncompetitive antagonist with respect to the TRβ_1 isoform (4, 5). Further insight in this antagonistic effect of DEA on TR binding can be obtained by evaluating the changes in the molecular constitution of either the drug or the receptor. We have previously reported the results of competition studies with amiodarone analogues: the bulky iodine atoms, the hydrophobicity, the electric charge and the overall size of the analogues markedly influenced the nature and potency of their inhibition on TR binding (6). These studies, however, did not provide further information on the localization of the DEA binding site on the receptor. Therefore we studied the effect of naturally occurring and artificial mutations in the TRβ_1 that are known to decrease T_3 affinity (R429Q, P453A and P453T), hormone-dependent transactivation (E457A), homodimerisation (R429Q) or heterodimerization (L421R, 9th heptad mutant).

5.3. Materials and methods

5.3.A. Chemicals

Nonradioactive 3, 5, 3'-triiodothyronine (T_3) was obtained from Henning GmbH, Berlin, Germany. [^{125}I]T_3 (spec. act. 2200 Ci/mmol) was purchased from New England Nuclear, Boston, USA. Desethylamiodarone was a kind gift of Sanofi Recherche (Montpellier, France). All reagents were of the highest grade possible.

5.3.B. Receptor expression

Receptors were expressed in *Escherichia coli* as a GST fusion protein using pGEX-2TK vector (Pharmacia Biotech, Sweden) containing either wild type human TRß$_1$, naturally occurring mutant human TRß$_1$ Y321C, R429Q, P453A, P453T, or artificial mutant human TRß$_1$ L421R and E457A (residues 174-461) (7, 8). The cells were grown overnight in 2 x YT-G medium (yeast extract 10 g/L, tryptone 16 g/L, NaCl 5 g/L and 10% (w/v) glucose) and than diluted 1:10 in pre-warmed (37°C) medium until an optical density of 1-1.2 was reached. After IPTG (isopropyl-ß-D-thiogalactoside) 1 mM F.D. was added the cells were grown for another 1.5 h. Cells were then lysed by sonification in PBS (2 x 20 s, 50 W on ice). Each receptor protein preparation was purified using glutathione-Sepharose 4B affinity resin (Pharmacia Biotech, Sweden) according to the manufacturer's instructions with the following modifications (9-11): Triton X-100 was used in a final concentration of 0.5%, PBS used as a wash solution contained 2 mM DTT and 0.2 mM PMSF. The proteins were stored at a high concentration in incubation buffer (20 mM Tris-HCl, 0.25 M sucrose, 1 mM EDTA, 50 mM NaCl, 5% (v/v) glycerol, 5 mM DTT pH 7.6) in liquid nitrogen, thawed on ice and diluted to the desired concentration just before use.

5.3.C. TR binding assay

The receptor proteins were incubated with [^{125}I]T_3 (10^{-11} M) for 30 min at 22°C in a shaking water bath in incubation buffer containing 0.025% Triton X-100, 0.05% BSA and 1% ethanol (v/v). These additions to the incubation buffer were necessary to solubilise DEA in an hydrophilic environment as demonstrated previously (5). Total incubation volume was 0.5 ml. Reactions

were stopped by chilling on ice-water. Bound and unbound [^{125}I]T_3 were separated at 4^{0}C using a small Sephadex G25 medium column (bed volume 2 ml, swollen in incubation buffer with 0.05% BSA) in a Pasteur pipette. Four 0.8 ml fractions, containing the bound hormone fraction, were collected using incubation buffer as eluent. Specific binding was determined by calculating the difference between the counts bound in the absence and presence of an excess (10^{-7} M) of nonradioactive T_3. All incubations were done in duplicate. The proteins were diluted to provide a maximal binding capacity (MBC) of the TR preparations between 3 and $10x10^{-10}$ M.

The potency of DEA to inhibit the binding of T_3 to the wild type and the mutated TRß$_1$s was tested over a concentration range of 10^{-7} to 10^{-4}M. DEA solubilised as a stock solution of 10^{-2}M in ethanol was incubated in various concentrations with receptor proteins and [^{125}I]T_3 as described above. In all tubes the final ethanol concentration was 1% (v/v). From these experiments the IC_{50} values of DEA for the wild type and the mutated TRß$_1$s were calculated.

Scatchard analyses were performed with DEA concentrations around the IC_{50} values. Receptor proteins and [^{125}I]T_3 were incubated with increasing amounts of non-radioactive T_3 (1×10^{-10} to 33×10^{-10}M) in the absence or presence of DEA. Four to six separate experiments were done for wild type and mutant receptor proteins; in each experiment the inhibitory effect of DEA was tested at two or three concentrations. MBC and K_a values were calculated using the non-linear curve-fitting computer program Ligand (Biosoft, Cambridge, UK). Changes in MBC and K_a as a function of DEA concentration were analysed by two-way ANOVA. Langmuir plots were prepared from the data of the Scatchard analyses.

5.4. Results

5.4.A. Inhibitory potency of DEA for wild type and mutant TRß$_1$

The relative affinities for T_3 of all TRß$_1$-GST fusions (wild type and mutants) are similar to results obtained before (7, 8). The IC_{50} value of DEA for the wild type human TRß$_1$ is 56 ± 15 µM (Table 1), in good accordance with the figure reported for rat TRß$_1$ (5). No reliable IC_{50} values for the mutant TRß$_1$s Y321C and L421R could be determined because the initial binding of [^{125}I]T_3 was too low. The IC_{50} values of DEA for the mutant receptors P453A and P453T are similar to those of the wild type, implying that there is no effect of P435 mutants, which however do influence T_3 binding, on DEA binding. The IC_{50} values for the mutant receptors R429Q and E457A are significantly lower indicating that their affinity for DEA is increased compared to wild type (Table 1, Fig. 1). The inhibitory potency (IC_{50}) of DEA on T_3 binding bears no relation to the affinity (K_a) of the different mutants for T_3 binding (Table 2).

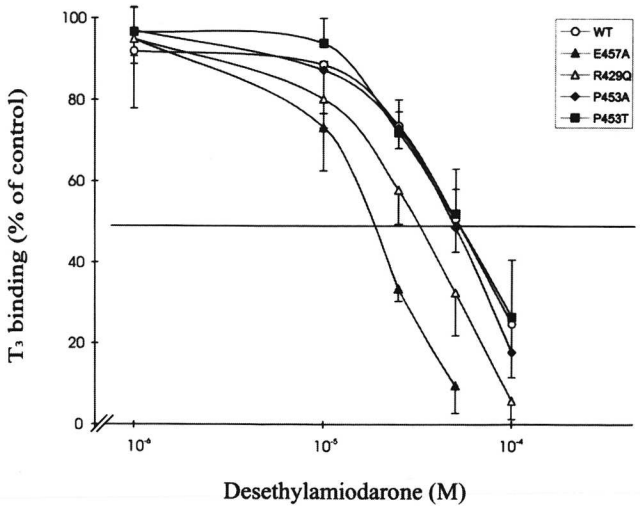

FIG. 1: Binding of T_3 to the wild type and human mutant TRß$_1$. Wild type (***open circle***), R429Q (***open triangle***), P453A (***closed diamond***), P453T (***closed square***) and E457A (***closed triangle***) were incubated with [^{125}I]T_3 in the presence of increasing concentrations DEA. Binding is expressed as a percentage of the control value without DEA. Data are presented as the mean ± S.D.

Table 1. Inhibition of the binding of [^{125}I]T_3 to wild type and mutant human TRß$_1$ by desethylamiodarone (DEA)

Human TRß$_1$	no of exp.	IC_{50} µM (mean±sd)
Wild type TRß$_1$	8	56±15
Y321C mTRß$_1$	B_o too low to determine IC_{50}	
L421R mTRß$_1$	B_o too low to determine IC_{50}	
L454A mTRß$_1$	B_o too low to determine IC_{50}	
P453A mTRß$_1$	7	50±11
P453T mTRß$_1$	9	55±16
R429Q mTRß$_1$	9	32±7*
E457A mTRß$_1$	4	17±3*

Bo, initial binding

* $p<0.001$ vs. wild type, determined by *t*-test.

Table 2. Characteristics of the inhibition of the binding of T_3 to the wild type and mutant human TRß$_1$ by desethylamiodarone (DEA), as evident from Scatchard plots

DEA	–	10^{-5} M	$2.5.10^{-5}$ M	5.10^{-5} M	p-value
MBC 10^{-10} M					
Wild type	5.86±1.0		5.21±0.73	4.57±0.66	0.03
R429Q	5.60±0.41	4.57±0.59	2.96±1.00		0.0003
P453A	7.47±1.94		6.34±1.58	5.06±1.82	0.03
P453T	8.82±1.24	8.78±1.22	7.83±1.23	6.32±2.39	0.007
E457A	3.96±.38	2.94±0.16	1.62±0.30		0.002
Ka 10^9 L/M					
Wild type	1.17±0.18		0.84±0.11	0.56±0.10	0.002
R429Q	0.99±0.30	0.83±0.21	0.82±0.19		0.15
P453A	0.50±0.09		0.40±0.07	0.31±0.04	0.005
P453T	0.36±0.09	0.33±0.05	0.27±0.07	0.21±0.05	0.005
E457A	1.00±0.12	0.90±0.15	0.72±0.22		0.03

Values are given as the mean ±SD (wt n=4; R429Q n=4; P453A n=4; P453T n=6; E457A n=4).

5.4.B. Nature of inhibition of T3 binding by DEA on wild type and mutant TRß$_1$

Representative Scatchard plots of the effect of DEA on T_3 binding to the different TRß$_1$s, are depicted in Fig. 2. DEA decreased both MBC and K_a in a dose-dependent manner as evident from the Scatchard plots for the binding of T_3 to the wild type and the mutant TRß$_1$ (P453A, P453T and E457A) (Table 2). Langmuir plots demonstrated noncompetitive inhibition by DEA for the wild type and these three mutated receptors. In the case of mutant R429Q, DEA decreased MBC in a dose-dependent manner, but the observed decrease in K_a did not reach statistical significance. After plotting the data in a double reciprocal plot (Langmuir plot), it was however clear that DEA is a noncompetitive inhibitor for T_3 binding to this mutant as well (Fig. 3).

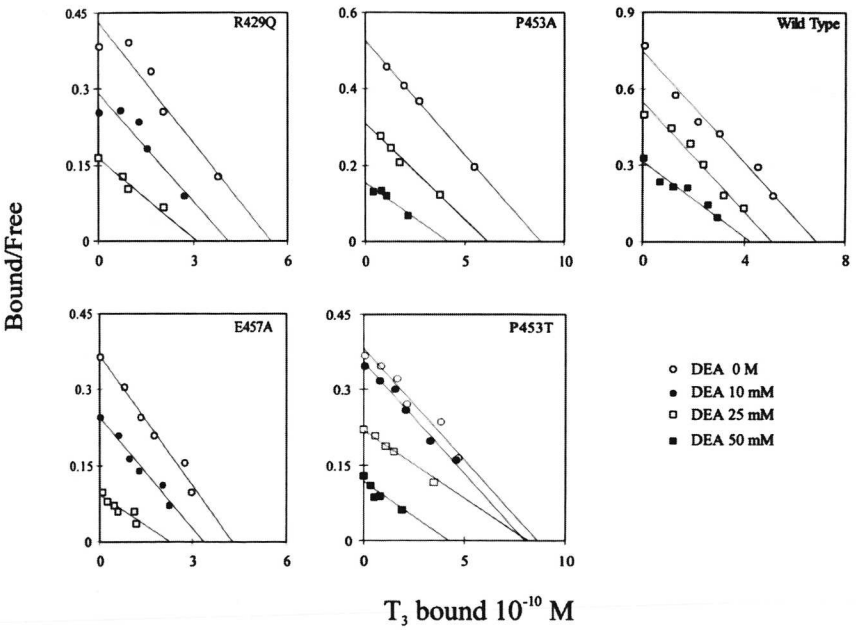

FIG. 2: Scatchard analyses of the binding of T_3 to wild type and human mutant TRß$_1$ in the absence (***open circle***) or presence of DEA10µM (***closed circle***), DEA 25µM (***open square***) and DEA 50µM (***closed square***).

5.5. Discussion

Whereas amiodarone itself has almost no inhibitory effect on the binding of T_3 to its nuclear receptors, the major metabolite DEA does inhibit the binding of T_3 (4, 5). The results of the present study indicate that some mutations in the ligand binding domain of human TRß$_1$ change the potency of DEA to inhibit T_3 binding to the TR. Whereas no change in inhibitory potency was observed for the naturally occurring mutants P453A and P453T relative to wild type TRß$_1$, a stronger inhibitory potency of DEA was observed for the mutant TRß$_1$s R429Q and E457A. This differential behaviour of mutant TRß$_1$ with respect to the antagonistic (inhibitory) effect of DEA allows us to postulate a putative location of the DEA binding site in the ligand-binding domain of TRß$_1$.

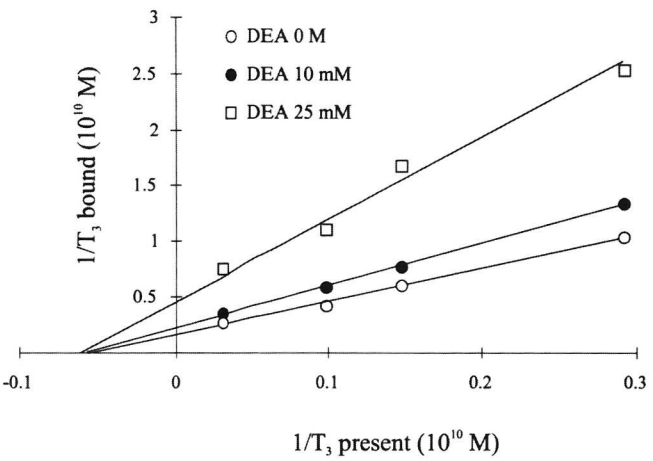

FIG. 3: Langmuir plot of the binding of T_3 to mutant TRß$_1$ R429Q in the absence (***open circle***) or presence (***closed circle*** and ***open square***) of DEA, constructed from the data obtained in the Scatchard analysis.

The amino acid sequences of the hormone binding domain of the rat TRα$_1$ and the human TRß$_1$ show 88% identity and are identical from the start of helix 11 (H362 in rTRα$_1$ and H416 in hTRß$_1$), except for the last three amino acids of rTRα$_1$ which are lacking in hTRß$_1$ (Fig. 4). It is assumed that the three-dimensional structure of the hormone-binding domain of rTRα$_1$ and hTRß$_1$ is comparable (12). Therefore the position of amino acids R429, P453 and E457 in hTRß$_1$ reflects the position of R375, P399 and E403 in rTRα$_1$ (Fig. 4).

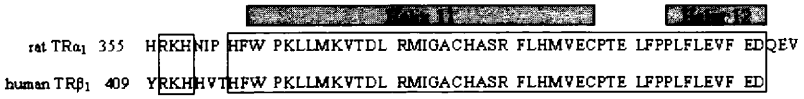

FIG. 4: Amino acid sequences of the rTRα1 (H355-V410) and the hTRß1 protein (Y409-D461) from the start of helix 11 until the C-terminal end.

Inhibition of T_3 binding by DEA was noncompetitive in nature for the wild type and all four mutant TRß$_1$s tested, indicating that the DEA binding site on the receptor is not equivalent to the T_3 binding site of TRß$_1$. The hormone appears to fit tightly in the binding pocket, and is completely buried inside the receptor molecule (12). Its fit is such that there is no apparent extra room inside the binding pocket. This, combined with the fact that DEA is larger and that its ring structures are not perpendicular, supports the notion that the DEA binding site may be different from that of T_3.

The results with mutant hTRß$_1$ presented in this paper permit a more precise delineation of the DEA binding site. Amino acid P453 is situated at the beginning of the amphipathic helix 12 in a hydrophobic region at the outer surface of the receptor protein. The change from proline to threonine (P453T) or from proline to alanine (P453A), although decreasing T_3 binding (Table 2), does not grossly affect the hydrophobicity on the outside of the TRß$_1$. The structure of the protein could differ, however, because proline is thought to be important in angle formation. There is no influence of these mutations on the affinity for DEA, suggesting that it may not be the precise receptor structure that is important for DEA binding but its surface hydrophobicity.

Amino acid E457 is a negatively charged spot within a hydrophobic surface facing outward into the solvent (12). Mutant E457A, which may be deficient in co-activator interaction (13), gives a change from negative charged glutamate to hydrophobic alanine, resulting in an increased inhibitory potency of DEA. This again is compatible with a greater affinity of DEA for a more hydrophobic outer surface of the TRß$_1$. To further prove that hydrophobicity is important in DEA binding, 9th heptad (14) mutant L421R, also facing outward into the solvent, was tested. A decrease in inhibitory potency of DEA for this mutant was expected on the basis of the change from the hydrophobic amino acid leucine to the positively charged arginine. Unfortunately the initial binding of T_3

was too low to determine the IC_{50} values of DEA for this mutant hTRß$_1$ and perform the other experiments.

In our study the ligand binding affinity of mutant R429Q was not affected but the inhibitory potency of DEA was almost two times higher compared to wild type (IC_{50} 32 µM *vs.* 56 µM). Despite the normal ligand binding affinity and co-activator recruitment (15) the RTH mutant R429Q (16, 17) is impaired in co-repressor release giving rise to increased negative transcription regulation (15). The arginine residue 429 (TRß$_1$) participates in a hydrophilic interaction with the charged pair arginine 383 (TRß$_1$) and glutamic acid 311 (TRß$_1$) forming a polar invagination (15). We recently argued that (part of) the mechanism of action of DEA could be an increase in co-repressor binding by the TR (18). An interesting parallel is therefore present between the supposed mechanisms of action behind R429Q and DEA.

In conclusion, these findings lead us to postulate that amino acids R429 and E457 are at or close to the binding site for DEA. This putative DEA binding site on hTRß$_1$ thus differs from the T_3 binding site, in accordance with the noncompetitive nature of the inhibition of T_3 binding to TRß$_1$ by DEA. The involvement of the amino acids R429 and E457 in cofactor binding (13, 15) combined with their apparent role in DEA binding (this study) may help to clarify the mechanism of action of the drug amiodarone.

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CHAPTER 6

Interaction between nuclear hormone receptors and co-activators analysed using a nonradioactive "pull-down" assay

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6.1. Abstract

Many transcription factors signal their presence to the transcription initiation complex via protein-protein interactions (1). Among these factors are the family of the nuclear hormone receptors that repress transcription by interaction with co-repressors like N-CoR and SMRT and increase transcription via co-activators like SRC-1, and GRIP-1. The interaction between these proteins can be studied *in vitro* by binding one of them to a support which can be easily separated from the incubation mixture (usually glutathione-Sepharose beads) and labelling the other so that it can be detected after electrophoretic separation (usually ^{35}S label) (2). In this paper we describe a novel non-radioactive method for studying these protein-protein interactions (pull-down assay) using the interaction between the thyroid hormone receptor $ß_1$ ($TRß_1$) and the nuclear receptor interaction domain (NID) of the co-activator GRIP-1 (3) as a model. The required sensitivity is reached by using a hemagglutinin (HA) tag and a high-affinity monoclonal anti-hemagglutinin antibody (clone 3F10) conjugated with peroxidase in combination with a long-acting peroxidase substrate.

6.2. Cloning and expression of proteins

We cloned the HA-tag at the N-terminus of the TRß$_1$ hormone binding domain by first adding appropriate restriction enzyme sites using PCR to the 5' and 3' end so that the TRß$_1$ insert could be cloned in-frame with the HA-tag in plasmid pBH6 (Roche Molecular Biochemicals, Germany). The 6-His tag also present in this vector at the C-terminus is not incorporated into the expressed protein because the TRß$_1$ stop codon is 5' of it. The HA-tagged TR was then expressed in *E.coli* using a standard procedure (manufacturer's protocol). After the bacteria were lysed (by sonification 2x20 s, 50 W on ice) and debris was removed by centrifugation, the supernatant containing the HA-TR was stored in aliquots at –70 °C. The HA-TRß$_1$ bound T_3 with wild-type affinity (K_d, $0.3x10^{-9}$ M). The GST-GRIP1-NID fusion containing the nuclear receptor interaction domain (NID) of the co-activator GRIP-1 attached to gluthathione-S-transferase (GST) was expressed in *E.coli* as described before (3).

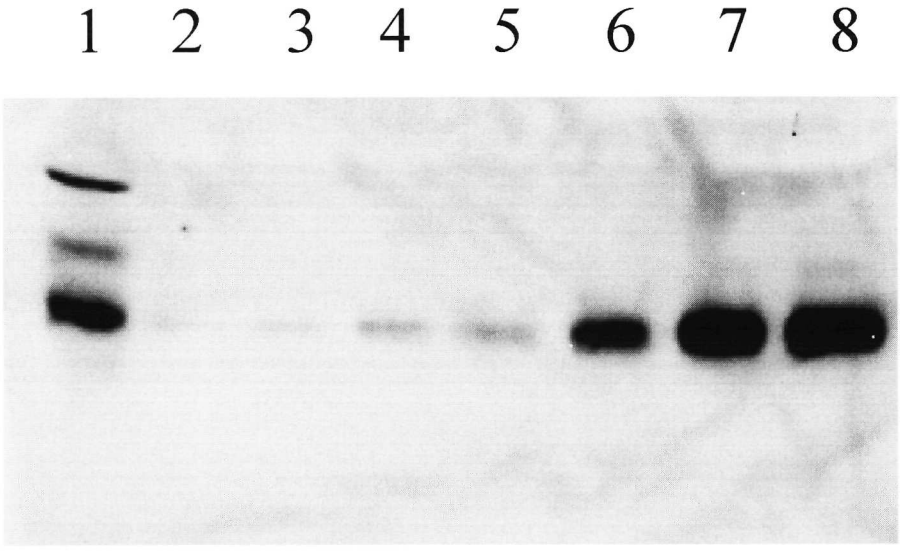

Fig 1. Interaction between the TRß$_1$ hormone binding domain and the NID of GRIP-1. HA-tagged TRß$_1$ was incubated with GST-GRIP1-NID coupled to glutathione beads as described. After elution, samples were Western blotted and the presence of the HA-tag was visualized using a peroxidase chemiluminescent substrate. The figure shows the dose-dependent effect of T_3 on the binding between the receptor and the co-activator. Lane 1, one-quarter input; lane 2, no GST-GRIP1-NID; lane 3-8 increasing concentration of T_3 was added to the binding reaction (0, 0.6, 1, 3, 30 and 100 nM, respectively).

6.3. Binding experiment conditions

GST-GRIP1-NID was bound to glutathione-Sepharose beads as described (3). GST-NID beads were resuspended in binding buffer (20 mM Hepes, pH 7.9; 80 mM KCl; 10 mM $MgCl_2$; 10% glycerol; 1 mM DTT; 0.1% NP-40; 0.1% Triton X-100; Complete (EDTA free) protease inhibitor (Roche Molecular Biochemicals, Germany)). HA-TR containing lysate (8 µl) was diluted to 20 µl with binding buffer containing 20 µg/ml BSA and preincubated with or without T_3 for 30 min at 4 °C. Thereafter an equal volume of GST-NID beads was added (total reaction volume 40 µl) and the incubation continued for 90 min at 10 °C whilst shaking. The samples were then spun for 20 s in a microfuge and the beads were washed three times with 250 µl cold binding buffer. The beads were then dried in a Speedvac and resuspended in 20 µl protein loading buffer. After boiling for 2 min the 20 µl samples were applied to a 10% SDS-PAGE gel and run at 4 mA/cm. When the bromophenol blue had reached the bottom of the gel, it was blotted onto PVDF membrane (Roche Molecular Biochemicals, Germany) with a Hoeffer SE70 semidry blotter (Pharmacia, Sweden) using a transfer buffer without SDS and lowered methanol (15%) (4).

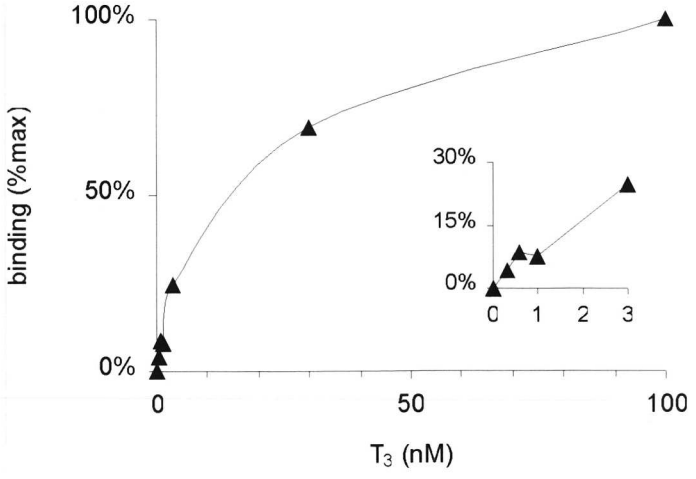

Fig. 2. Dose-response curve of the binding of TRß$_1$ and GRIP-1 NID. Graphical representation of the dose-dependent effect of T_3 on the binding between TRß$_1$ and GRIP-1 NID. Data are expressed as percentage of maximum binding and plotted as mean of two independent experiments. The insert shows an enlargement of the start of the curve (between 0 and 3 nM T_3).

6.4. Detection of the HA-tag with 3F10

The blot was blocked for at least 1 h at 37 °C with 1% Blocking Reagent (Roche Molecular Biochemicals, Germany) in phosphate-buffered saline (PBS), where after the high affinity monoclonal anti-HA antibody conjugated with peroxidase (anti-HA-3F10-POD) was added in the same buffer and incubation continued for 1 h at room temperature. The blot was washed four times 5 min with 0.1% Triton/PBS and then LumiLight^{plus} substrate (Roche Molecular Biochemicals, Germany) was added. After 5 min the chemiluminescent signal was detected and quantified using a Lumi-Imager (Roche Molecular Biochemicals, Germany). Average exposure time was 2 min.

As can be seen from the image of the gel (Fig. 1), it is possible to detect the HA-tagged TR after its interaction with the GRIP1-NID. No specific binding was seen without either HA-TR (not shown) or hormone (Fig. 1, lane 3). The interaction between the TR and a co-activator is hormone-dependent which is comparable to previous publications (3,5). The intensity of the bands was quantified using the LumiAnalyst software and yielded the graphical representation depicted in Fig. 2. The maximum binding was on average 20% of input, a result which is less than that found in another paper dealing with the TR/NID interaction (3). This can be explained by the fact that we use less T_3 (10^{-7} instead of 10^{-5} M) and we have Triton X-100 that is known to lower T_3 binding affinity (6), in the binding buffer. Furthermore, the actual relative concentrations of both proteins may differ between this study and that published previously. The shape of the dose-response curve is however comparable to that of the interaction of the TR with SRC-1 in the sense that the start of the curve and its plateau are at similar T_3 concentrations (5). We therefore offer this non-radioactive adaptation of the pull-down assay as a reliable alternative to the radioactive one.

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CHAPTER 7

Desethylamiodarone interferes with the binding of co-activator GRIP-1 to the β_1-thyroid hormone receptor

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7.1. Abstract

Ligand binding to the thyroid hormone nuclear receptor β_1 (TRβ_1) is inhibited by desethylamiodarone (DEA), the major metabolite of the widely used anti-arrhythmic drug amiodarone. Gene expression of TR-regulated genes can therefore be affected by amiodarone due to less ligand binding to the receptor. Previous studies have indicated the possibility of still other explanations for the inhibitory effects of amiodarone on T_3-dependent gene expression, probably via interference with receptor / co-activator and co-repressor complex. The binding site of DEA is postulated to be on the outside surface of the receptor protein overlapping the regions were co-activator and co-repressor bind. Here we show the effect of a drug metabolite on the interaction of TRβ_1 with co-activator GRIP-1 (glucocorticoid receptor interacting protein-1). The T_3-dependent binding of GRIP-1 to the TRβ_1 is disrupted by DEA. A DEA dose experiment showed that the drug metabolite acts like an antagonist under "normal" conditions (at 10^{-7} M T_3 and $5 \times 10^{-6} \rightarrow 10^{-3}$ M DEA), but as an agonist under extreme conditions (at 0 and 10^{-9} M T_3 and > 10^{-4} M DEA). To our knowledge, these results show for the first time that a metabolite of a drug which was not devised for this purpose, can interfere with nuclear receptor / co-activator interaction.

7.2. Introduction

Hormones can influence transcription via binding to their specific nuclear receptors. Occupancy of binding sites with ligand results in conformational changes of the receptor protein, allowing dissociation of co-repressors and association of the hormone-receptor complex with nuclear co-activators. This sequence of events ultimately leads to modulation of the transcription rate of the hormone-responsive genes (1). The role of nuclear co-repressors and co-activators in transcriptional regulation has only recently become apparent. Their biological relevance is highlighted by the finding that particular mutations in the human thyroid hormone receptor ß$_1$ (hTRß$_1$) disturb the interaction between co-activator and co-repressor interactions and TR, resulting in thyroid hormone resistance (2). Desethylamiodarone (DEA), the major metabolite of the powerful anti-arrhythmic and antianginal drug amiodarone, is a noncompetitive inhibitor of the binding of T_3 to the TRß$_1$ protein (3), its binding site is postulated to be on the outside surface of the receptor protein overlapping the regions where co-activator and co-repressor bind (4). Here we report that DEA can also affect the binding of co-activator glucocorticoid receptor interacting protein-1 (GRIP-1) to the ligand-receptor complex.

Amiodarone is widely used in the treatment of a variety of cardiac diseases. It is a peculiar drug in view of its extensive tissue accumulation (notably in the liver, lung and adipose tissue) and very long elimination half-life. Characteristic structural features are its resemblance to thyroxine and its high iodine content. The release of pharmacological quantities of iodine during biotransformation of the drug results in iodine-induced thyrotoxicosis and hypothyoidism in a minority (about 16%) of patients. In contrast, in almost every patient treated with amiodarone hypothyroid-like effects are observed in the expression of various T_3-responsive genes. Examples are the decrease in ß-adrenoceptor density and Na, K - ATPase activity in the heart, and the decrease of low-density lipoprotein (LDL) receptor density in the liver as evident from studies in humans and in experimental animals (5, 6, 7, 8). These hypothyroid-like effects can be reversed by the administration of T_3, and are explained from a decreased transcription of involved genes (9). For example the increase in serum LDL cholesterol observed in amiodarone-treated patients (10) is caused by a decreased expression of the hepatic LDL receptor protein, which is due to

a decreased transcription of the gene encoding for the LDL receptor. The decrease in transcription might well be caused by a lower TR occupancy as amiodarone substantially decreases the generation of T_3 out of T_4 in the liver by inhibition of type 1 deiodinase (11). Furthermore DEA has been shown to be a competitive inhibitor of the binding of T_3 to TRα_1 (12) and a noncompetitive inhibitor with respect to TRβ_1 (3). Further insight into this antagonistic effect of DEA on TR binding was obtained by evaluating the changes in the molecular constitution of either the drug or the receptor. We have previously reported the results of competition studies with amiodarone analogues: the bulky iodine atoms, the hydrophobicity, the electric charge and the overall size of the analogues markedly influenced the nature and potency of their inhibition on TR binding (13). These studies, however, did not provide further information on the localization of the DEA binding site on the receptor. Therefore we studied the effect of naturally occurring and artificial mutations in TRβ_1 that were known to decrease T_3 affinity. The results of these studies demonstrated that amino acids E457 and R429 are at or near the DEA binding site on the receptor (4). Since these residues may be involved in co-activator (E457) (14) or co-repressor (R429) (15, 16) interaction, this finding led us to hypothesize a possible disruption of the TRβ_1 / co-activator interaction by DEA. The aim of the present study was to verify this hypothesis by studying the influence of DEA on the T_3-dependent binding of co-activator GRIP-1 to hTRβ_1.

7.3. Materials and Methods

7.3.A. Chemicals

T_3 was obtained from Henning (Berlin, Germany). DEA from Sanofi Recherch (Montpellier, France) was used as a 10^{-2} M stock solution in ethanol.

7.3.B. Protein expression

Co-activator GRIP-1 was expressed as a glutathione-*S*-transferase (GST)-GRIP-1-nuclear receptor interacting domain (NID) fusion protein containing the NID of the co-activator GRIP-1 attached to GST. This fusion protein was expressed in *E.coli* as described previously (17).

TRβ_1 (amino acids 153-461) was cloned with a hemagglutinin epitope-tag (HA-TRβ_1) at the N-terminus, which did not interfere with the hormone-binding domain at the C-terminus. The HA-TRβ_1 expressed in *E.coli* and isolated as described before (17, 18).

7.3.C. Pull-down assay

To study the receptor co-activator interaction in the presence of DEA, we used a recently developed non-radioactive 'pull-down' assay (18). In short, GST-GRIP-1-NID bound to glutathione-Sepharose beads was resuspended in binding buffer (20 mM HEPES, pH 7.9; 80 mM KCl; 10 mM $MgCl_2$; 10% glycerol; 1 mM DTT; 0.1% NP-40; 0.1% Triton X-100; Complete (EDTA free) protease inhibitor (Roche)). HA-TRβ_1-containing lysate (8µl) was diluted to 20 µl with binding buffer containing 20 µl/ml bovine serum albumine and preincubated with or without T_3 and in the absence or presence of DEA for 30 min at 4° C. Thereafter GST-GRIP1-NID beads were added and the incubation continued for 90 min at 4° C whilst shaking. The samples were washed, dried and resuspended in protein loading buffer containing 5 mM DTT. They were run on a 10 % SDS-PAGE gel blotted onto PVDF membrane (Roche) using a transfer buffer without SDS and lowered methanol (15%).

For detection of the HA-tag, the blot was incubated with a high affinity monoclonal anti-HA antibody conjugated with peroxidase (POD) (anti-HA 3F10-POD). Using Lumi-Light ^{plus} substrate (Roche) the chemiluminescent signal was detected and quantified using a Lumi-Imager (Roche). Average exposure time was 2 min.

To investigate whether DEA had an effect on this protein-protein interaction two sets of experiments were performed. First the dose effect of T_3 was studied in the absence and presence of a constant amount of DEA. TRß$_1$ was preincubated with thyroid hormone (T_3) over a concentration range of 3×10^{-10} up to 10^{-7} M T_3 in the absence and presence of 10^{-5} M DEA. In the second set of experiments, the dose-dependent effect of DEA was studied. TRß$_1$ was preincubated with DEA over a concentration range of 10^{-6} up to 10^{-3} M DEA in the absence of T_3 and in the presence of 10^{-9} M and 10^{-7} M T_3.

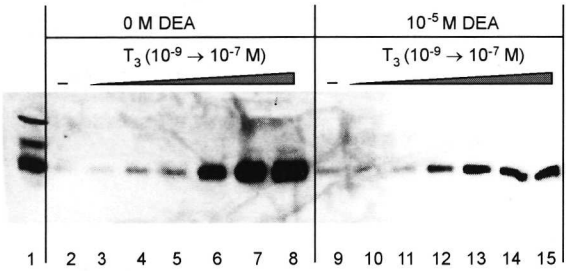


Fig. 1a. Western blots of HA-TRß$_1$, depicting the dose-dependent effect of T_3 on the binding of TRß$_1$ to GRIP-1 in the absence and presence of 10^{-5} M DEA. Lane 1: 10% of HA-TRß$_1$ input. Lanes 2-8: binding in the presence of 0; 3×10^{-10}; 6×10^{-10}; 10^{-9}; 3×10^{-9}; 3×10^{-8}; 10^{-7} M T_3, respectively, in the absence of DEA. Lanes 9-15: binding in the presence of 0; 3×10^{-10}; 6×10^{-10}; 10^{-9}; 3×10^{-9}; 3×10^{-8}; 10^{-7} M T_3, respectively, in the presence of 10^{-5} M DEA. HA-TRß$_1$ was detected by anti-HA-POD and chemiluminescent substrate.

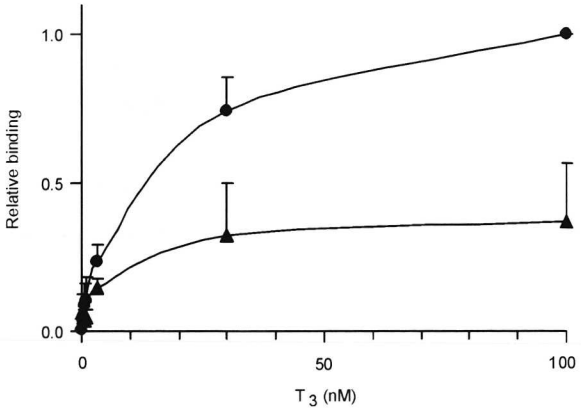
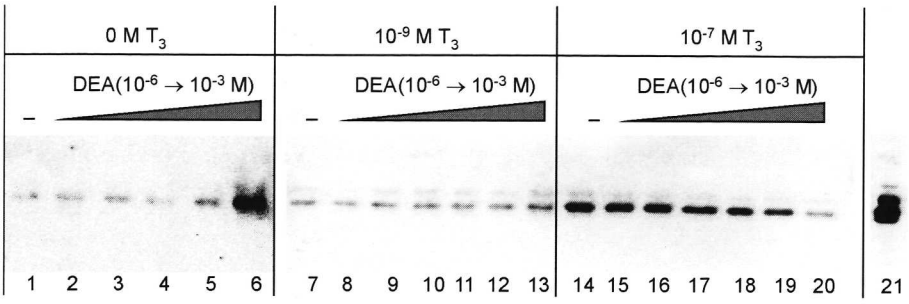


Fig. 1b. Dose-response effect of T3 on the binding of TRß1 to GRIP-1 in the absence (***closed circle***) and presence (***closed triangle***) of 10^{-5} M DEA. Quantification and plot of the light units from Western blot (a), measured by the Lumi Imager. The relative binding is the ratio of the observed binding of TRß1 to GRIP-1 to the (maximal) binding at 10^{-7} M T3 in the absence of DEA.

7.4. Results

The results of the T_3 dose effect experiments indicate a T_3 dose-dependency of the binding of GRIP-1 to the TRβ_1, which was observed previously (19). The binding of GRIP-1 to the TRβ_1 increased at higher T_3 and was lower in the presence of 10^{-5} M DEA at all T_3 concentrations tested (Fig. 1a) ($P < 0.0001$; ANOVA, two way with replication; $n = 3$). Individual points of the binding curves differ significantly at 3×10^{-8} M ($P < 0.01$; $n = 5$) and at 10^{-7} M ($P < 0.05$; $n = 4$) (Fig. 1b). The HA-TRβ_1 protein binds T_3 with wild type affinity (K_D 0.3×10^{-9} M).

A DEA dose-dependent effect of the binding of GRIP-1 to the TRβ_1 was also observed. In the absence of T_3 the binding of GRIP-1 to TRβ_1 remains very low at DEA concentrations up to 10^{-5} M (Fig. 2a); however, a 10-fold increase is observed in the presence of 10^{-3} M DEA. It appears therefore that DEA acts as an agonist with respect to the unoccupied TRβ_1. In the presence of 10^{-9} M T_3 the DEA-induced increase in binding is less pronounced and significantly different from that obtained in the absence of T_3 ($P < 0.001$; ANOVA, two-factor with replication; $n = 4$) (Fig. 2b). Interestingly, at 10^{-7} M T_3, a reversal of the dose-response pattern is seen and the binding curve differs significantly from the other two ($P < 0.001$; ANOVA, two-factor with replication; $n = 4$). At a T_3 concentration of 10^{-7} M the highest binding of GRIP-1 to TRβ_1 is observed in the absence of DEA, and the binding decreases in the presence of DEA in a dose-dependent manner (Fig. 2a, 2b).



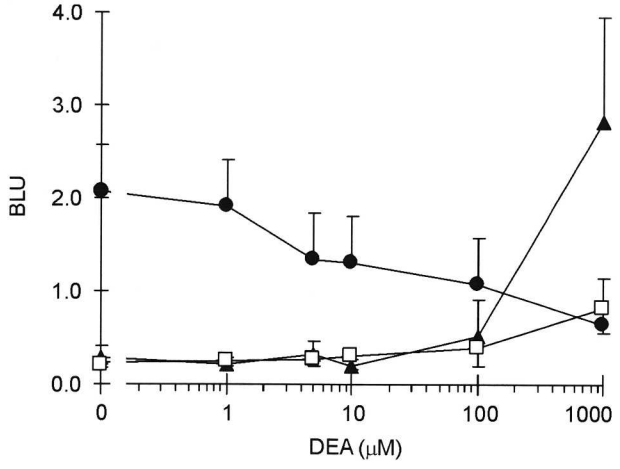


Fig. 2. **a**: Western blots of HA-TRß$_1$, depicting the dose-dependent effect of DEA on the binding of TRß$_1$ to GRIP-1 in the presence of 0, 10^{-9} or 10^{-7} M T_3. Lanes 1-6: binding in the absence of T_3 and in the presence of 0; 10^{-6}; 5 x 10^{-6}; 10^{-5}; 10^{-4}; 10^{-3} M DEA, respectively. Lanes 7-13: binding in the presence of 10^{-9} M T_3 and in the presence of 0; 10^{-6}; 5 x 10^{-6}; 10^{-5}; 5 x 10^{-5}; 10^{-4}; 10^{-3} M DEA, respectively. Lanes 14-20: binding in the presence of 10^{-7} M T_3 and in the presence of 0; 10^{-6}; 5 x 10^{-6}; 10^{-5}; 5 x 10^{-5}; 10^{-4}; 10^{-3} M DEA, respectively. Lane 21: 10% of HA-TRß$_1$ input.

Fig 2. **b**: Dose-response effect of DEA on the binding of TRß$_1$ to GRIP-1 in the absence of T_3 (***closed triangle***) and in the presence of 10^{-9} M T_3 (***open square***) and 10^{-7} M T_3 (***closed circle***). Quantification and plot of the light units from Western blot (a), Measured by Lumi Imager. The light units are expressed as BLUs (Boehringer Light Units). The crossing of the lines at high and low T_3 concentration is indicative for partial antagonism.

7.5. Discussion

From the above results we conclude that DEA can act as a partial antagonist: the actual mode of action depends on the ambient T_3 concentration. This phenomenon can be explained from the noncompetitive nature of the inhibition of T_3 binding to TRβ_1 by DEA. This noncompetitiveness allows the formation of both the complexes DEA / TRβ_1 and DEA / T_3 / TRβ_1, since the binding sites of T_3 and DEA are not the same. Only in the case of DEA/T_3/TRβ_1 will there be antagonism, i.e. there must be some T_3 present for DEA to act as an antagonist.

In order to relate these *in vitro* findings to the *in vivo* situation, T_3 and DEA concentrations in our experiments must be compared with tissue levels *in vivo*. In human serum, T_3 values are in the order of 10^{-9} M and DEA levels in the order of 2×10^{-6} M (20) in patients on long-term amiodarone treatment. Rat liver T_3 and DEA levels are reported to be about 10^{-8} mol/kg (11) and 1.2×10^{-5} mol/kg (21) respectively. In human liver, DEA concentration can reach 4×10^{-3} mol/kg (22). A thousand times higher DEA concentration in liver and other tissues compared to T_3, is therefore possible. The actual intracellular T_3 and DEA levels are not known, but taking into account the above-mentioned serum and tissue concentrations, our *in vitro* model may well represent the *in vivo* situation in the human cell. DEA is a very lypophilic drug and accumulates substantially in tissues during amiodarone treatment. The drug also inhibits T_4-5'-deiodination into T_3 resulting in a substantial decrease of intracellular T_3 levels and lower TR occupancy (11). At a "normal" receptor occupancy of about 50 %, the effect of DEA will be limited. However, at higher DEA concentrations and especially in the case of a decreased T_3 occupancy of the binding sites, DEA will act as an antagonist, resulting in lower co-activator binding.

Simplified, T_3-dependent gene regulation can be seen as an on/off switch (with or without ligand), whereas in the fine-tuning of this process many other proteins are involved. This complex regulation system thus forms a balance between hormone, receptor, co-activators, co-repressors and other proteins involved in the transcription machinery. DEA interferes in this balance in three ways. First, by inhibiting deiodination of T_4 to T_3 in the liver (11). Second, by a

noncompetitive inhibition of the binding of T_3 to the TRβ_1 (3). And third, by disrupting the binding of co-activator GRIP-1 to the TRβ_1 (this study).
Overall we postulate that not amiodarone itself, but its stable major metabolite DEA interferes with T_3-dependent gene expression (23) by interfering with the binding of T_3 to the TR and disrupting the co-activator / TR binding. This mechanism of action would explain the T_3 antagonistic effects of DEA observed in patients on long-term amiodarone treatment.

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CHAPTER 8

Dronedarone acts as a selective inhibitor of 3,5,3'-triiodothyronine binding to TRα_1: *in vitro* and *in vivo* evidence

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8.1. Abstract

Dronedarone (Dron), without iodine, was developed as an alternative to the iodine-containing antiarrhythmic drug amiodarone (AM). AM acts, via its major metabolite desethylamiodarone (DEA), *in vitro* and *in vivo* as a thyroid hormone receptor α_1 (TRα_1) and TRβ_1 antagonist. Here we investigate whether Dron and/or its metabolite debutyldronedarone inhibit T_3 binding to TRα_1 and TRβ_1 *in vitro* and whether dronedarone behaves similarly to amiodarone *in vivo*.

In vitro, Dron had an inhibitory effect of 14% on the binding of T_3 to TRα_1 but not on TRβ_1. Desethylamiodarone inhibited T_3 binding to TRα_1 and TRβ_1 equally. Debutyldronedarone inhibited T_3 binding to TRα_1 by 77%, but to TRβ_1 only by 25%.

In vivo, AM increased plasma TSH and rT_3, and decreased T_3. Dron decreased T_4 and T_3, rT_3 did not change, and TSH fell slightly. Plasma total cholesterol was increased by AM but remained unchanged in Dron-treated animals. TRβ_1-dependent liver low density lipoprotein receptor protein and type 1 deiodinase activities decreased in AM-treated, but not in Dron-treated animals. TRα_1-mediated lengthening of the QTc interval was present in both the AM- and Dron-treated animals.

The *in vitro* and *in vivo* findings suggest that dronedarone via its metabolite debutyldronedarone acts as a TRα_1-selective inhibitor.

8.2. Introduction

Dronedarone [n-(2-butyl-3-(4-(3-dibutylaminopropoxy)-benzoyl)-benzofuran-5-yl)-methanesulfonamide; SR 33589 B; Dron; Fig. 1] is a new antiarrhythmic drug devoid of iodine, developed to replace the widely used, very potent, antiarrhythmic drug amiodarone (AM; Fig. 1), which releases pharmacological quantities of iodine during its biotransformation. Consequently, treatment with AM gives rise to iodine-induced hypothyroidism or thyrotoxicosis in approximately 15 % of patients (1). Apart from the effect of iodine excess on the thyroid gland induced by AM, it profoundly affects extrathyroidal metabolism of thyroid hormones. AM decreases the 5'-deiodination of T_4 into T_3 in the liver, mediated by inhibition of cellular T_4 uptake. The main metabolite of AM, desethylamiodarone (DEA; Fig. 1) was found to inhibit the binding of T_3 to its nuclear receptors. Indeed, AM treatment causes a dose-dependent decrease in the expression of several T_3 -dependent genes. For instance, the AM-induced increase in plasma low-density lipoprotein (LDL) cholesterol is explained by a decrease in the hepatic expression of the LDL receptor gene at both the mRNA and protein levels (2,3). Interestingly, the type of inhibition differs for α_1- and β_1–thyroid hormone receptors (TRα_1 and TRβ_1, respectively). DEA is a competitive inhibitor of T_3 binding to TRα_1 (4), whereas it is a noncompetitive inhibitor of T_3 binding to TRβ_1 (5). Protein-protein binding studies with the hTRβ_1 and the co-activator glucocorticoid receptor interacting protein (GRIP-1) showed an inhibitory effect of DEA on the T_3 dependent binding of the co-activator to the TRβ_1 (6). This mechanism of action further supports the idea that DEA has a T_3 antagonistic effect. It is thus of much interest to evaluate whether the new related drug, Dron, has the same effect as AM on extrathyroidal hormone metabolism and T_3 receptor binding. Dron is now under active study intended to determine its usefulness in patients with cardiac arrhythmias (7). The aim of the present study was to investigate a putative inhibitory effect of Dron and its metabolite debutyldronedarone (DBDron), on the binding of T_3 to TRα_1- and TRβ_1. To this end we performed *in vitro* binding studies with expressed TRα_1 and TRβ_1 proteins and *in vivo* studies in rats where we concentrated on a number of post receptor effects mediated by TRα_1 and TRβ_1. In both studies we compared the effect of Dron with that of AM.

8.3. Materials and methods

8.3.A. Materials

Nonradioactive T_3 was obtained from Henning (Berlin, Germany). [^{125}I]T_3 (specific activity, 2200 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA). Dron, DBDron, AM and DEA were gifts from Sanofi Pharmaceuticals, Inc.-Synthélabo (Montpellier, France). The goat polyclonal LDL receptor (LDL-r; C20) IgG (sc-11824, lot J161) was obtained from Santa Cruz Biotechnology, Inc. (San Diego, CA). All reagents were of the highest grade possible.

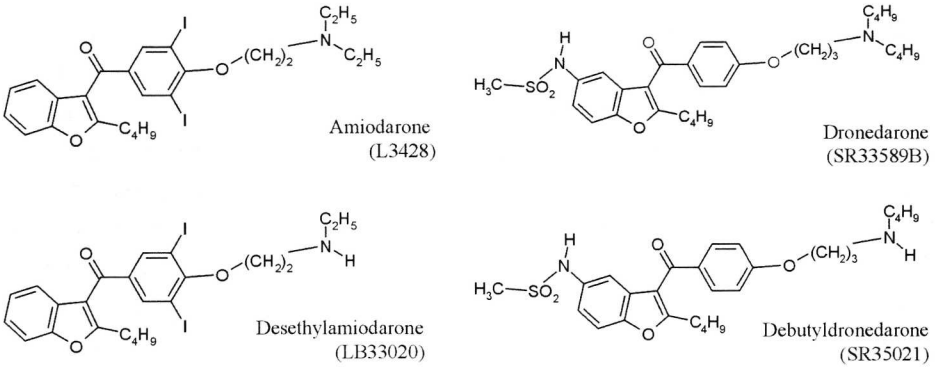


FIG. 1. Chemical structures of AM and Dron (*top*) and their metabolites DEA and DBDron (*bottom*).

8.3.B. In vitro receptor binding assay

The chicken α_1 (amino acids 1-408) and the human $ß_1$ (amino acids 153-461) TRs were expressed in *Escherichia coli*, isolated as described previously (8,9), and stored in incubation buffer [20 mM Tris-HCl, 0.25 mM sucrose, 1mM EDTA, 50 mM NaCl, and 5% (vol/vol) glycerol, pH 7.6] containing 5mM dithiothreitol (DTT) in liquid nitrogen. Receptor proteins were thawed on ice (20-25µg protein /tube) and were incubated with [^{125}I]T_3 (10^{-11} M) for 30 min at 22^0C in a shaking water bath in incubation buffer with 5 mM DTT, 0.025% Triton X-100, 0.05% BSA and 1% ethanol (vol/vol). The total incubation volume was 0.5 ml. Reactions were stopped by chilling on ice water. Bound and unbound [^{125}I] T_3 were separated at 4^0C using a small Sephadex G-25 medium column (bed volume, 2 ml; swollen in incubation buffer with 0.05%

BSA) in a Pasteur pipette. Four 0.8-ml fractions, containing the bound hormone fraction, were collected using incubation buffer as eluent. Specific binding was determined by calculating the difference between the counts bound in the absence and presence of an excess (10^{-7} M) of nonradioactive T_3. All incubations were performed in duplicate.

The potency of Dron, DBDron and DEA to inhibit the binding of T_3 to TRα_1 and TRβ_1 was tested over a concentration range of 10-100 μM. The compounds solubilised in a stock solution of 10^{-2}M in ethanol, were incubated with receptor proteins in the presence of [^{125}I]T_3 as described above. In all tubes the final ethanol concentration was 1% (vol/vol). Binding is expressed as the percentage of specifically bound [^{125}I]T_3 in the absence of the compounds. In each experiment the effect on T_3 binding to TRα_1 and TRβ_1 was assayed simultaneously.

Scatchard analyses were performed with DBDron to investigate the type of inhibition. TRα_1 or TRβ_1 proteins in the presence of [^{125}I]T_3 were incubated with increasing amounts of nonradioactive T_3 ($1x10^{-10}$ to $33x10^{-10}$M) in the absence or presence of DBDron (0, 25, and 50 μM). Changes in maximum binding capacity (MBC) and K_a as a function of DBDron concentration were tested by one-way ANOVA.

Langmuir plots were prepared from the data of the Scatchard analyses. To delineate the type of inhibition, we analysed the data by one-way ANOVA to determine whether the intercepts on the y-axis differed significantly among the various drug concentrations. Evidence for competitive inhibition is one intercept on the y-axis and dose-dependent increasing intercepts on the x-axis, whereas noncompetitive inhibition is characterized by one intercept on the x-axis and dose-dependent increasing intercepts on the y-axis.

8.3.C. In vivo studies

Male Wistar rats (220-260 g), housed under normal conditions with free access to standard laboratory chow and tap water, were divided into four groups (eight rats per group). They received water (controls), an aqueous suspension of 50 mg/kg body weight Dron (Dron50), 100 mg/kg body weight Dron (Dron100), or 100 mg/kg body weight AM (AM100) by gastric tube daily for 2 wk. Twenty-four hours after the last administration, electrocardiographs (ECGs) were made. During ECG recording, body temperature was kept

constant (36.6-37.4°C) by a custom-made, water-heated, warming pad and monitored by a rectal probe (Ellab, Roedovre, Denmark). Rats were kept under anaesthesia and could freely breathe under 100% oxygen-supplemented air (0.2 litre/min) via a cone (density,0.7 mm) 1 cm from their nose. AgCl-coated silver needles were used to record ECG (Einthoven lead I). Output signals were amplified by a custom-made amplifier, sampled at 1 kHz (Data Acquisition Card AT-MIO-16E-10, National Instruments, Austin, TX, USA), band pass-filtered at 100 Hz-50 Hz direct current, and analysed using AcqKnowlegde software version 3.2.6 (MP 100 Manager, Biopac Systems, Inc., Santa Barbara, CA, USA). Thereafter, blood was collected by cardiac puncture, and plasma was stored at -20°C. The liver was removed and stored in liquid nitrogen. All animal experiments were approved by our local animal welfare committee.

8.3.D. Plasma assays

Plasma cholesterol and triglycerides were determined by using a fully enzymatic dye method (Modular P analyser, Roche Molecular Biochemicals, Mannheim, Germany). Quantification of plasma LDL cholesterol and high density lipoprotein (HDL) cholesterol were carried out by precipitation as described previously (3). Total T_4, total T_3 and rT_3 were measured by in-house RIAs (10), using rat null plasma as diluent. Free T_4 (FT4) and TSH were determined by Delfia fluoroimmunoassay (PerkinElmer, Wallac Inc., Freiburg Germany). FT_4 and FT_3 indexes were calculated as the product of the total T_4 or total T_3, respectively, and T_3 resin uptake. The latter was determined using the Immulite chemiluminescent immunoassay T_3 uptake kit (Diagnostic Products, Los Angeles, CA, USA). All samples were measured within one run. Data are expressed as mean ± SD. Differences between the thyroid hormone parameters were tested using *t* test, differences between the lipid parameters were tested by Mann-Whitney rank-sum test.

8.3.E. LDL-r protein expression and type 1 deiodinase (D1) activity in liver

Liver whole cell extracts were prepared in homogenisation buffer [10 mM HEPES, 10% glycerol, 0.25 M sucrose, 25 mM KCl, 1 mM EDTA, 5 mM DTT, and protein inhibitor mix (Complete, Roche Molecular Biochemicals), pH 7.6].

Thirty-five micrograms of protein were applied to a 7.5% SDS-PAGE gel, transferred to PVDF membrane using a wet electro blotting apparatus, and probed with a specific LDL-r goat polyclonal antibody (diluted 1:1000). A polyclonal rabbit antigoat horseradish peroxidase-conjugated secondary antibody (DAKO Corp., Copenhagen, Denmark; diluted 1:3000) was used to reveal primary antibody binding using the Western Light kit and Lumi-Imager software (Roche Molecular Biochemicals). D1 activity in liver was measured as previously described (11).
Values are expressed as the mean ± SD. Differences were determined by *t* test.

8.4. Results

8.4.A. In vitro studies: Inhibitory potencies of Dron, DBDron and DEA

The inhibitory effects of 10, 25, 50 and 100 μM DEA, Dron and DBDron, respectively, on T_3 binding to both TRα_1 and TRβ_1 are presented in FIG. 2A and 2B. Dron at 100 μM showed a slight, but significant, inhibition of 14 ± 3% ($P < 0.01$) of T_3 binding to TRα_1, but no inhibition of the binding of T_3 to TRβ_1. For the metabolites DEA and DBDron, a dose-dependent inhibition of T_3 binding to both receptor isoforms was observed. DEA at 100 μM inhibited the binding of T_3 to the TRα_1 by 94 ± 3 % ($P < 0.01$) and that to TRβ_1 by 82 ± 4% ($P < 0.01$) compared with that when no DEA was present. DBDron at 100 μM strongly inhibited the binding of T_3 to TRα_1 by 77± 3 % ($P < 0.01$) but that to the TRβ_1 by only 25 ± 4 % ($P < 0.01$) compared with no DBDron (by Mann-Whitney nonparametric rank-sum test, values are mean ± SEM ; $n = 5$). DEA concentrations causing 50% inhibition of T_3 binding (IC_{50} values) were 30 ± 4 and 71 ± 3 μM for the TRα_1 and TRβ_1, respectively (Table 1), in good accordance with earlier studies (4,9). The IC_{50} values of DBDron were 59 ± 4 and 280 ± 30 μM (Table 1) for TRα_1 and TRβ_1 respectively.

TABLE 1. Concentrations required for 50 % inhibition of binding of [^{125}I]T_3 to the TRα_1 and TRβ_1 by DEA and DBDron

	IC_{50} values (μM)		*P*
	DEA	DBDron	
TRα_1	30 ± 4	59 ± 4	< 0.01
TRβ_1	71 ± 3	280 ± 30	< 0.01
P	< 0.01	< 0.01	

Values are the mean ± SEM (n = 5). *P* values were determined by paired *t* test.

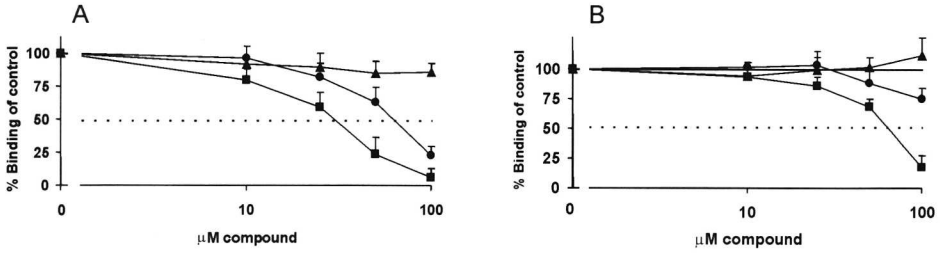


FIG. 2. Inhibition of the *in vitro* binding of [^{125}I]T_3 to the TRα_1 (A) or TRβ_1 (B) by DEA (*squares*), Dron (*triangles)* and DBDron (*circles*). Values are given as mean ± SD (n = 5) and are expressed as the percentage of specifically bound [^{125}I]T_3 in the absence of the compounds.

8.4.B. In vitro studies: Type of inhibition of DBDron

A representative Scatchard plot and Langmuir analysis of the effect of DBDron on TRα_1 are depicted in Fig. 3. The MBC of TRα_1 was not affected by DBDron (P = 0.21), but the affinity constant (K_a) of T_3 binding was decreased in a dose-dependent manner (P < 0.01), as shown in Table 2. Langmuir analyses confirmed the competitive nature of the inhibition of binding of T_3 to TRα_1 by DBDron. The intercept on the y-axis did not change from 0 - 50 μM DBDron (P = 0.25), whereas the intercept on the x-axis increased in a dose-dependent way (P < 0.03).

The results of Scatchard analysis of DBDron incubated with TRβ_1 are also presented in Table 2. The inhibitory effect of DBDron on TRβ_1, however, was too low to perform reliable kinetic studies. MBC values did not show a dose-dependent relation ($P = 0.82$) with DBDron concentration, and the K_a values did not decrease dose-dependently ($P = 0.34$).

TABLE 2. Characteristics of inhibition of binding of T_3 to TRα_1 and TRβ_1 by DBDron as evident from Scatchard plots

Scatchard plots	MBC (10^{-10} M/litre)	K_a (10^9 litres/M)
TRα_1		
0 μM DBDRON	1.53 ± 0.55	0.28 ± 0.15
25 μM DBDRON	1.34 ± 0.42	0.25 ± 0.13
50 μM DBDRON	1.36 ± 0.55	0.19 ± 0.11
P	0.21	<0.01
TRβ_1		
0 μM DBDRON	2.34 ± 0.50	0.15 ± 0.01
25 μM DBDRON	1.89 ± 0.53	0.15 ± 0.01
50 μM DBDRON	2.08 ± 0.50	0.14 ± 0.01
P	0.82	0.34

Values are the mean ± SEM TRα1, $n = 9$; TRβ1, $n = 7$. *P* values were determined by one-way ANOVA.

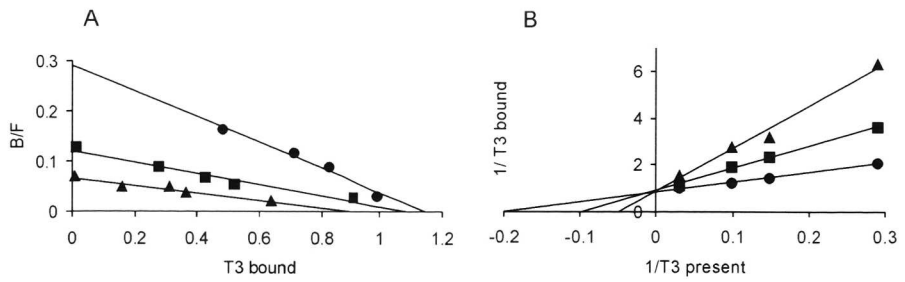


FIG. 3. Scatchard analyses (A) and Langmuir plot (B) of the binding of T_3 to TRα_1 in the absence (*circles*) or presence of DBDron 10μM (*squares*) and 25 μM (*triangles*).

8.4.C. In vivo studies: rat plasma parameters

Body weights were similar in the four groups at baseline. In the Dron100 group two rats died before the end of the study due to breathing problems. The gain in body weight during the experiment was lower in the Dron- and AM- treated animals than in controls (Table 3).

Plasma T_3 and FT_3 index in the Dron50, Dron100, and AM100 groups were lower than control values. Plasma rT_3 and TSH were significantly higher in the AM100 group relative to control values. Plasma FT_4 did not differ among groups, but T_4 and FT_4 index were lower in the Dron100 group than in controls (Table 3).

Total plasma cholesterol was not affected in the Dron50 and Dron100 groups compared with controls, nor were LDL cholesterol and HDL cholesterol affected by Dron. However, total cholesterol, LDL cholesterol, and HDL cholesterol increased in the AM group (Table 3).

TABLE 3. Effect of Dron or AM treatment in rats on ΔBW, plasma thyroid hormones, and plasma lipids

	Controls (n = 8)	Dron 50 (n = 8)	Dron 100 (n = 6)	AM 100 (n = 8)
Δ BW (g)	87 (63-93)	70 (50-90)[a]	65 (-25-75)[b]	62 (51-75)[b]
TSH (ng/ml)	1.02 ± 0.24	1.05 ± 0.54	0.87 ± 0.36[c]	1.87 ± 0.88[b]
T_4 (nmol/litre)	74 ± 9.6	62 ± 13	57 ± 12[d]	72 ± 15
FT_4 index	115 ± 15	97 ± 20	89 ± 18 [d]	113 ± 23
FT_4 (pmol/litre)	39.5± 7.3	33.4 ± 7.1	33.7 ± 8.1	40.8 ± 9.0
T_3 (nmol/litre)	1.38 ± 0.10	1.17 ± 0.16[d]	1.09 ± 0.19 [b]	1.03 ± 0.17[b]
FT_3 index	2.21 ± 0.16	1.84 ± 0.25[b]	1.71 ± 0.30 [b]	1.61 ± 0.26[b]
rT_3 (nmol/litre)	0.10 ± 0.01	0.09 ± 0.02	0.10 ± 0.01	0.15 ± 0.02[b]
Total chol (mmol/litre)	2.05 ± 0.18	1.86 ± 0.25	1.81 ± 0.50	2.27 ± 0.29[b]
LDL-chol (mmol/litre)	0.93 ± 0.05	0.95 ± 0.13	0.86 ± 0.37	1.15 ± 0.42[e]
HDL-chol (mmol/litre)	0.96 ± 0.10	0.85 ± 0.18	1.05 ± 0.11	1.20 ± 0.29[a]
Heart rate (bpm)	382 ± 35	365 ± 26	390 ± 26	351 ± 23[a]
QTc interval (msec)	0.114 ± 0.013	0.125 ± 0.009[f]	0.142 ± 0.023[a]	0.141 ± 0.018[a]

Values are the mean ± SD, Δ BW, The difference between d 1 and d 15 body weights (median and range).
[a]P < 0.05 *vs.* controls; [b]P < 0.01 *vs.* controls; [c]P = 0.1 *vs.* controls; [d]P < 0.02 *vs.* controls; [e]P = 0.07 *vs.* controls; [f]P = 0.055 *vs.* controls

8.4.D. In vivo studies: post receptor effects in rat heart and liver

Heart rate, expressed as beats per minute, was not different among the groups. However, QTc intervals showed a dose-dependent prolongation in both Dron- and AM-treated groups (Table 4).

D1 activity was not changed in both Dron-treated groups, but decreased by 70 % in the AM100 group ($P < 0.0001$; Fig. 4). LDL-r protein levels in both Dron-treated groups did not differ from the LDL-r protein levels in the livers of control animals. However, in the AM100 group LDL-r protein expression was decreased ($P < 0.004$); Fig. 4).

8.5. Discussion

8.5.A. In vitro studies

From our results it is clear that Dron itself has only a slight inhibitory effect on the binding of T_3 to TRα_1 and has no effect on T_3 binding to TRβ_1. In contrast, the metabolite DBDron was a very potent inhibitor of T_3 binding to TRα_1, but a weak inhibitor of T_3 binding to TRβ_1. Dron and DBDron are, like AM and DEA, strong lypophilic compounds, not easily dissolved in an aqueous environment. To keep the compounds in solution, we added 0.025% Triton X-100 to the assay buffer, which has no effect on affinity (12). The solubility of Dron and DBDron was tested at OD_{400}. No opacity (precipitation) was observed at concentrations up to 100 µM, but OD_{400} rose in an exponential manner at higher concentrations. The IC_{50} value of DBDron with respect to TRβ_1 could therefore not be measured directly, but was estimated by extrapolation from the dose-response curve. It appears that the inhibitory effect of DBDron is about 4.7 times greater for T_3 binding to TRα_1 than to TRβ_1. From the Scatchard plots and Langmuir analysis it is evident that the inhibitory effect of DBDron on T_3 binding to TRα_1 is competitive in nature. We were unable to obtain reliable data on the type of inhibition by DBDron to TRβ_1 because we could not add sufficient amounts of DBDron to reach concentrations around the IC_{50} value of 280 µM, as the drug came out of solution at concentrations of approximately 100 µM.

The effect of Dron is similar to that of AM in so far that not the parent drug, but its metabolites DBDron and DEA, respectively, are the potent inhibitors of T_3 binding to the TR. Although DBDron is less potent than DEA in this respect by a factor 2 for TRα_1 and 4 for TRβ_1 the two metabolites differ markedly from each other in that DBDron inhibits T_3 binding to TRα_1 but much less to TRβ_1, whereas DEA clearly inhibits T_3 binding to both TRα_1 and TRβ_1 within the same order of magnitude.

The quantitative differences between DEA and DBDron with respect to inhibiting T_3 binding to TRα_1 and TRβ_1 are most likely the result of differences in structures between the two compounds and/or between the two receptors. The ligand binding domain of the cTRα_1 and hTRβ_1 are 89% homologous. The differences in the amino acid sequences between cTRα_1 and hTRβ_1 are mainly located in strand 3/helix 3, in strand 7/helix 7 and in helix 10. Studying the three-dimensional structure of the hTRβ_1 (PDB Id: 1BSX, studied using Cn3D v. 3.0 via the NCBI website), it becomes apparent that some of the differences between TRα_1 and TRβ_1 in helix 10 are on the same surface side of the receptor as amino acids R429 and E457, which we demonstrated in earlier studies (9) to be involved in DEA interaction to TRβ_1. These differences in amino acid sequences can lead to subtle changes in the three-dimensional structure for TRα_1 compared with TRβ_1. The chemical structures of DEA and DBDron (Fig. 1) have great similarity, but there are some important differences. DEA has two bulky iodine atoms, which DBDron has not. The amino side-chain of DBDron is longer and has a butyl group instead of an ethyl group. It is possible that the extra methanesulfonamide group in the DBDron molecule, which carries a positive charge, is of importance in explaining the decreased affinity to TRβ_1 compared with TRα_1.

The competitive nature suggests competition between T_3 and DEA or DBDron at the same binding site on the TRα_1. However, it is also possible that T_3 and DEA or DBDron do not bind in the same binding pocket, but that the binding of DEA or DBDron to the receptor will change the structure of the TRα_1 binding pocket in such a way that T_3 can no longer bind to TRα_1 . This conformational change could *in vivo* result in a reduction of the available binding sites for T_3 binding on the TRα_1 in tissues when DEA or DBDron is bound. The binding of T_3 and DEA or DBDron to the TRs is a reversible process, and its equilibrium is dependent on the concentrations of the compounds present. When the

concentrations of DEA or DBDron in tissue rises because of the accumulation of the drug metabolites, the binding of T_3 to the TRs will decrease, resulting in a decrease in T_3-dependent gene expression.

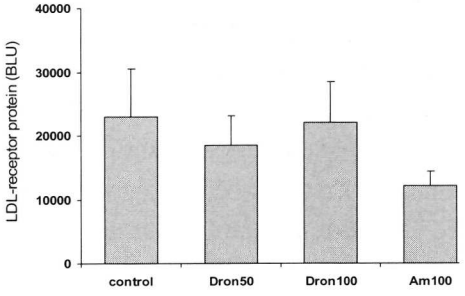
8.5.B. In vivo studies

The *in vitro* data suggest that DBDron could act as a selective antagonist to TRα_1. Such an effect *in vivo* would require tissue concentrations of DBDron remaining below the IC_{50} value of 280 µM. In dogs treated orally with Dron (20 mg/kg.d) for 4 wk, the myocardial content of Dron is 23 µmol/kg and that of DBDron 6 µmol/kg (13). In the same study dogs treated with AM (40 mg/kg.d) for 4 wk had myocardial concentrations of 28 µmol/kg AM and 21 µmol/kg DEA. Dron and DBDron concentrations in human tissues are not known, but AM and DEA concentrations are 62 and 274 µmol/kg, respectively, in human hearts obtained from autopsies (14). The human tissue concentrations are higher than in the dog studies, probably related to the accumulation of AM and DEA in tissues upon long-term treatment of patients with AM. The DEA concentrations in the human heart are much higher than the IC_{50} values of DEA for TRα_1 and TRβ_1 and allow for an antagonistic effect *in vivo* on T_3-dependent gene expression mediated via both receptor isoforms. The few available data on DBDron content in the heart are compatible with the idea that DBDron concentrations will not be sufficiently high to exert an antagonistic effect on TRβ_1, but will allow antagonism to TRα_1. If so, this could be of clinical relevance. The heart has an abundance of TRα_1 relative to TRβ_1, whereas tissues such as the liver express TRβ_1 more abundantly than TRα_1 (15). AM and Dron have similar electrophysiological effects on the heart (13,16-18), and their antiarrhythmic effect might well be mediated at least partly via TRα_1 by inducing a local hypothyroid-like condition (19). One of the side-effects of AM is an increase in plasma cholesterol caused by a down-regulation of the T_3-dependent LDL-r gene in the liver (2,3).

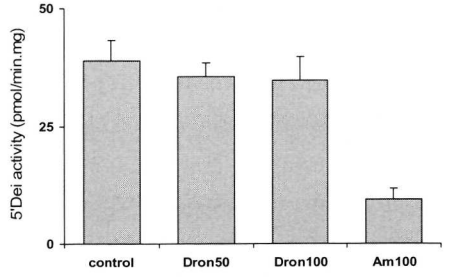
Because of the higher affinity of DBDron to TRα_1, we hypothesized that the effect of Dron on heart rate and QTc interval would be similar to that of AM, but that Dron, in contrast to AM, will not cause hypercholesterolemia and changes in the liver LDL-r expression, as T_3-induced changes in cholesterol metabolism are mainly mediated via TRβ_1 (20).

TABLE 4. Effect of Dron or AM treatment in rats on heart rate, QTc intervals, liver D1 activity and LDL-r protein expression, compared with controls

	Dron	AM
TRα_1-mediated effects		
Heart rate	Same	Same
QTc interval	Up	Up
TRβ_1-mediated effects		
D1	Same	Down
LDL-r	Same	Down



A, Levels of LDL-r protein in livers of control rats (control) and rats treated with Dron (50 or 100 mg/kg body weight) or AM (100 mg/kg body weight). LDL-r protein was visualized using Western blotting with a specific polyclonal antibody, and signals were quantified using a Lumi-Imager. Data are expressed as the mean ± SD, *, $P < 0.004$.



B, Activity of D1 in livers of control rats (control) and rats treated with Dron (50 or 100 mg/kg body weight) or AM (100 mg/kg body weight). D1 activity was measured as described in *Materials and Methods*. Data are expressed as the mean ± SD, **, $P < 0.0001$.

FIG. 4. Effect of Dron and AM on LDL-r protein expression and D1 activity in rat liver.

Our *in vivo* experiments support this hypothesis. First, Dron and AM had differential effects on thyroid hormone metabolism. AM, but not Dron, caused a decrease in the activity of liver D1, the enzyme responsible for the generation of T_3 from T_4 and the degradation of rT_3 into 3,3'-diiodothyronine. As the liver is the main site for production of T_3 and the degradation of rT_3, the decrease in plasma T_3 and the increase in plasma rT_3 upon AM treatment are explained by

the fall in liver D1 activity in agreement with previous studies (Ref.21 and references therein). The gene encoding D1 is under thyroid hormone control, an effect mainly mediated via TRβ_1 (22). The absence of an effect of Dron on liver D1 activity could thus be taken as evidence in favour of Dron not interfering with T_3 binding to TRβ_1, but the available evidence suggests another mechanism. AM treatment is not associated with a fall in liver D1 mRNA (Refs.2 and 21 and references therein), but the decrease in liver D1 activity is due to less availability of the substrate T_4 caused by inhibition of cellular T_4 uptake and direct inhibition of D1 by amiodarone (21), which frequently results in higher T_4 and FT_4 levels.

Dron treatment tends to decrease plasma TSH, whereas AM, in contrast, gives rise to higher TSH levels. The TSH increase by AM is explained by the iodine excess generated by the drug, as has been reported previously (Ref.1 and references therein). Dron treatment is associated with a decrease in total and free T_4 and T_3 concentrations. The decreases in T_4 and T_3 cannot be explained from changes in plasma thyroid hormone-binding proteins, because the T_3 uptake measurements in plasma remained unchanged, nor from changes related to nonthyroidal illness, because plasma rT_3 remained unaltered. Although an effect of diminished food intake cannot be excluded, the lower T_4 and T_3 production by the thyroid gland might be the result of diminished TSH secretion.

TSH secretion is under negative thyroid hormone control, an effect mediated mainly by TRβ_1, but the pituitary thyrotrophs also contain TRα_1 (23-25). In TR$\alpha_1^{-/-}$ mice, lower plasma TSH levels are observed as a result of a decreased expression of the TSHα–subunit (26). Therefore, our *in vivo* data showing decreased T_4 and T_3 levels and slightly lower TSH in Dron-treated rats might be interpreted as resulting from a TRα_1-selective inhibition of T_3 binding by Dron.

Secondly, we observed a lengthening of the QTc interval in rats treated with either AM or Dron, a typical effect of class III antiarrhythmic drug. It has been suggested in the case of AM that part of this increase can be explained by its inhibition of the binding of T_3 to TR in the heart (19). Recently, it was shown that the TRα_1 is the isoform involved in setting the heart rate (15, 26). Deleting TRα_1 not only lowers the heart rate, but also increases the QTc interval. This fits with our data for Dron and AM. When we combine the results of our *in vitro*

and *in vivo* studies, the lengthening of the QTc interval would be the result of a selective inhibition of T_3 binding to the TRα_1 in the heart.

Thirdly, we did not observe a change in plasma cholesterol or liver LDL-r expression in rats treated with Dron. AM-induced hypercholesterolemia cannot be explained by the rise in TSH, because even higher TSH levels in mildly hypothyroid animals do not result in an increase in plasma cholesterol (2). The increase in plasma cholesterol by AM, which acts on both TRα_1 and TRβ_1, can be explained by a decreased expression of LDL-r in the liver at both mRNA and protein levels (3). It has recently become clear that most of the regulation of cholesterol metabolism and LDL receptor expression in liver is TRβ_1 dependent (20). Thus, the lack of effect of Dron on plasma cholesterol and LDL-r expression can be explained by our *in vitro* data showing that Dron is a TRα_1-selective inhibitor.

In conclusion, the *in vitro* and *in vivo* data presented in this paper indicate that Dron, via its metabolite DBDron, is a TRα_1-selective inhibitor of T_3 binding to its receptor. This isoform selectivity can explain the effects of Dron on the heart (a mainly TRα_1 organ) and the lack of effect on the liver (a mainly TRβ_1 organ) (Table 4) and may also point the way to designing TR isoform-specific antagonists.

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CHAPTER 9

General discussion

9.1. Inhibition by amiodarone of the binding of T_3 to TRs

The in vitro effect of the lypophilic compounds AM and DEA on the binding of T_3 to its nuclear receptors has generated several conflicting reports. These discrepancies are probably due to the fact that AM and DEA are easily dissolved in ethanol, but come out of solution in the aqueous environment of the incubation buffer. To keep AM and DEA in solution different lypophilic solvents have been used like benzyl alcohol, ethanol, propanol, Tween 80 and Triton X-100 (Table 1). Strong inhibition by AM was reported by Franklyn (1), Norman (2), Wilson (3) and Drvota (4), but from our study (5) it became evident that the inhibitory effect by AM reported in the first two papers (1;2) was not due to AM, but to the inhibitory effect of benzyl alcohol and Tween 80 itself. Wilson (3) used a two-phase incubation system with 50% propanol/water (v/v) in which AM came out of solution upon dilution, resulting in non-reliable measurements. The cause of the strong inhibition by AM described in the study of Drvota is still unclear (4).

Table 1. Reported discrepancies on the in vitro inhibitory effect of AM on the binding of T3 to TR, as explained from the applied solvent in the incubation medium

Year	Author	Inhibition by AM	Inhibition by DEA	TR from	Solvens % Final Dilution
1985	Franklyn (1)	+	n.t.	rat anterior pituitary	1% benzyl alcohol
1985	Eil (62)	-	n.t.	human skin fibroblasts	2% ethanol
1987	Latham (6)	minimal	+	human lymphocytes, bovine heart, rat liver	1% ethanol, nuclei washed with 0.5% Triton X-100
1988	Topliss (63)	-	n.t.	rat liver	1-10% ethanol
1989	Wilson (3)	+	n.t.	rat liver	50% propanol/water
1989	Norman (2)	+	n.t.	rat pituitary tumour	9.3% Tween 80 1.9% benzyl alcohol
1993	Gøtzsche (7)	minimal	+	rat heart	0.2% ethanol, nuclei washed with 0.5% Triton X-100
1994	Bakker (5)	minimal	+	bacterial expressed TR$ß_1$ protein	1% ethanol 0.05% Triton X-100
1994	Barlow (64)	-	n.t.	Macaca liver	acidified assay buffer
1995	Drvota (4)	+	n.t.	baculovirus-produced hTR$ß_1$	0.6% ethanol

+ = strong inhibition, - = no inhibition, n.t.= not tested

In contrast to the results described in the above mentioned papers, the studies of Latham (6) and Gøtzsche (7) report only a slight inhibition by AM. In these studies AM was dissolved in ethanol and since in both studies nuclei were washed with 0.5% Triton X-100, the remaining Triton X-100 was probably enough to keep AM in solution. In our studies (Chapter 2) and (Chapter 3) 0.05% TritonX-100 was added to the incubation buffer to keep AM and DEA in solution. The other papers listed in Table 1 did not show an inhibitory effect by AM on the binding of T_3 to TRs. This lack of effect is probably due to the fact that in these experiments AM was diluted from the ethanol stock into the incubation buffer without any precaution to keep AM in solution, resulting in insoluble AM in the aqueous incubation buffer.

It is clear from the above discussion that the strong lypophilic compounds AM and DEA are easily dissolved in ethanol, but precipitate in an aqueous environment. In order to solve this problem and to get more insight in the physicochemical behaviour of AM and DEA the solubility of the compounds was tested in a hydrophylic incubation buffer as described in chapter 2. Ethanol, Tween-80, or Triton X-100, at various concentrations, were added to the incubation buffer containing AM or DEA from 10^{-3} to 10^{-5}M. Macroscopically visible precipitation, opalescent or clear solution were scored and in the clear solutions AM and DEA recovery was measured by HPLC. A clear solution was only obtained in the presence of 1% and 10% Tween-80 or in the presence of 0.05% and 0.1% Triton X-100 at AM and DEA concentrations up to 10^{-4}M. Recovery was not measured in the samples with Tween-80, because this solvent, like benzyl alcohol itself, interferes strongly with the binding of T_3 to thyroid hormone receptors. No soluble AM and DEA were detected in the presence of 1% ethanol. However when 0.05% Triton X-100 was added, recovery in the order of 80-95% was found as measured by HPLC, but recovery decreased markedly at AM and DEA concentrations above 10^{-4}M. From these findings it became clear how to keep AM and DEA in solution without interference of the solvent itself in the binding of T_3. This made it possible to study the interaction of AM and DEA on the binding of T_3 to its receptors.

Further studies with AM and DEA, the major metabolite of AM, in both our group and by others (6;7) showed that DEA, but not AM, had a clear inhibitory effect on the binding of T_3 to its receptors. This inhibition was about 80% at a

concentration of 10^{-4}M DEA. The same inhibitory potency of 80% at a concentration of 10^{-4}M DEA was reported by Bakker (5), and van Beeren (8) using the chicken TRα_1 and the rat TRβ_1 expressed in *E.coli*. A review from the group of Baxter (9) in 1998 stated: "The cardiac anti-arrhythmic agent amiodarone has TR antagonist activity (Norman and Lavin, 1989) but it has a very low affinity for TR and it becomes toxic to the cells at the concentrations required for its "antagonist" actions (unpublished data). Therefore, we cannot be certain about amiodarone as a true TR antagonist." Their doubt is understandable taking in account the discrepancy in the reports on this subject; however the choice of the reference was unfortunate, because the effect of AM in the paper of Norman and Lavin (2) was due to the solvents used. On the other hand, they were right in doubting the antagonism of AM. Not AM, but its major metabolite DEA is the antagonist with respect to binding of T_3 to TRs as is clearly evident from the experimental results described in chapter 2 and 3.

9.2. Structure-function relationship in the interaction between amiodarone and the TR isoforms

9.2.A. Competitive *vs.* noncompetitive inhibition: differences between TRα_1 and TRβ_1 isoforms

In chapter 2 and 3 we described the inhibitory effect of DEA on the binding of T_3 to the rat TRβ_1 and chicken TRα_1. Surprisingly the type of inhibition differs for the two receptor isoforms. DEA acts as a competitive inhibitor of the binding of T_3 to TRα_1 as evident from the concentration-dependent decrease in K_a but not in MBC, indicating that DEA and T_3 compete for the same binding site on the TRα_1-isoform. In contrast, DEA acts as a noncompetitive inhibitor of T_3 binding with respect to TRβ_1: the K_a as well as the MBC decreases in a concentration-dependent manner. DEA and T_3 are thus likely to bind to the receptor on different or partly overlapping binding sites.

The different types of inhibition of T_3 binding to TRα_1 and TRβ_1 by DEA can be due to differences in amino acid sequences in the LBD of these isoforms (Fig. 1). These differences can give rise to discrepancies in the structure of TRα_1 compared to that of TRβ_1, resulting in isoform specific actions. Before discussing differences between the amino acid sequences of the LBD of the human TRα_1 and TRβ_1 in detail, the human sequences will be compared to that of the chicken TRα_1 and the rat TRβ_1, because the cDNA used in our studies is from chicken and rat origin (for TRα_1 and TRβ_1 expression respectively).

The LBD of the human and chicken TRα_1 are 95% homologous. From the eleven amino acids that are different, nine change to a residue with the same physicochemical properties. Only the differences of isoleucine 201 (I201) situated in H5 into methionine (M) and serine 341 (S341) situated in H10 into cysteine (C) from human to chicken TRα_1 respectively, introduce two residues containing an S atom, one of which is potentially able to form di-sulfide bridges with other residues in the protein. However, these residues are situated in non-conserved regions and will probably not result in significant differences between the human and chicken TRα_1 protein.

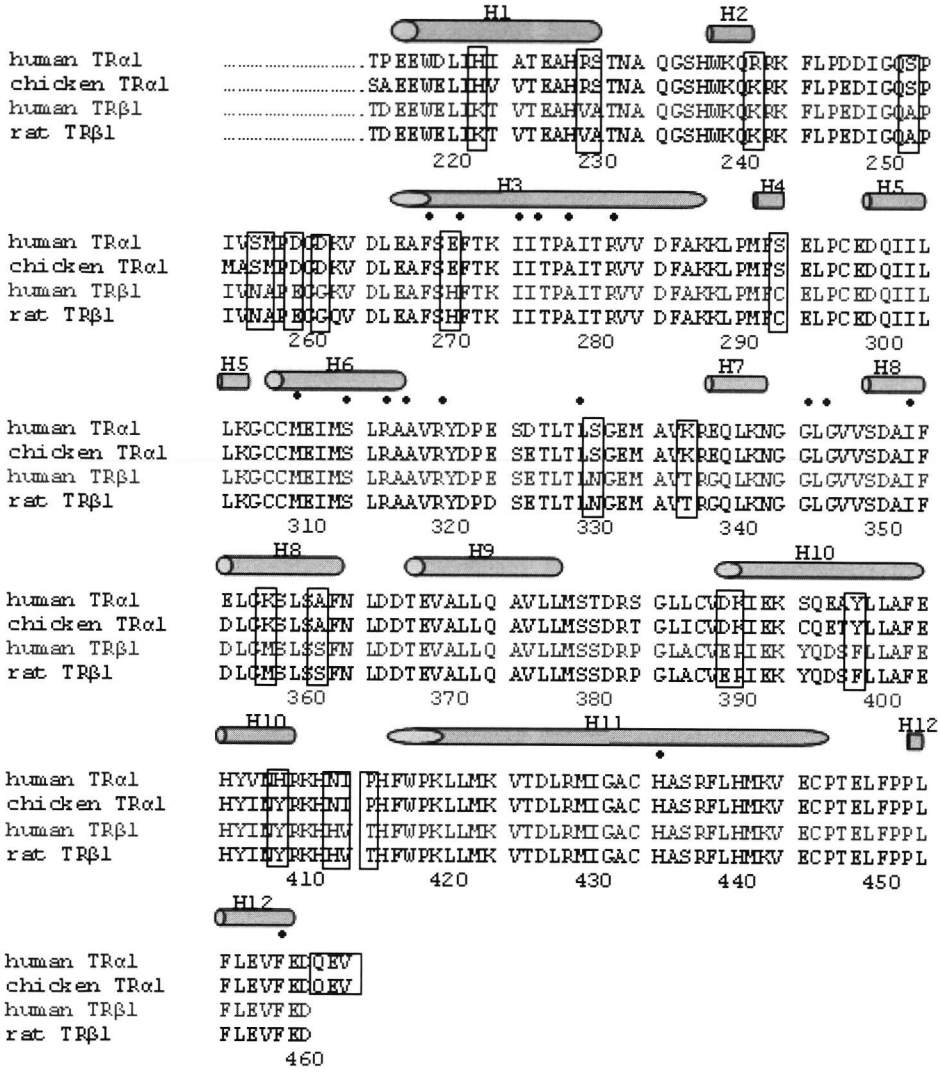
There is an extremely high sequence identity between human and rat TRβ_1 LBD with only two residues that are different. Lysine 263 (K263) situated between H2 and H3 changes into glutamine (Q), and aspartic acid 324 (D324)

situated between H6 and H7 changes into glutamic acid (E) from human to rat TR$ß_1$. These differences between rat and human will probably have no effect on the charge of the surface of the protein or to the protein folding. Both K and Q can have a positive charge, dependent on pH, and the charge of the acidic amino acids D and E is the same. The difference in size is only one CH_2-group in both cases.

```
human TRα1                TPEEWDLIHI ATEAHRSTNA QGSHWKQRRK FLPDDIGQSP
human TRβ1                TDEEWELIKT VTEAHVATNA QGSHWKQKRK FLPEDIGQAP
                                220        230        240        250

human TRα1      IVSMEDGDKV DLEAFSEFTK IITPAITRVV DFAKKLPMFS ELPCEDQIIL
human TRβ1      IVNAFEGGKV DLEAFSHFTK IITPAITRVV DFAKKLPMFC ELPCEDQIIL
                      260        270        280        290        300

human TRα1      LKGCCMEIMS LRAAVRYDPE SDTLTLSGEM AVKREQLKNG GLGVVSDAIF
human TRβ1      LKGCCMEIMS LRAAVRYDPE SETLTLNGEM AVTRGQLKNG GLGVVSDAIF
                      310        320        330        340        350

human TRα1      ELGKSLSAFN LDDTEVALLQ AVLLMSTDRS GLLCVDKIEK SQEAYLLAFE
human TRβ1      DLGMSLSSFN LDDTEVALLQ AVLLMSSDRP GLACVERIEK YQDSFLLAFE
                      360        370        380        390        400

human TRα1      HYVNHRKHNI PHFWPKLLMK VTDLRMIGAC HASRFLHMKV ECPTELFPPL
human TRβ1      HYINYRKHHV THFWPKLLMK VTDLRMIGAC HASRFLHMKV ECPTELFPPL
                      410        420        430        440        450

human TRα1      FLEVFEDQEV
human TRβ1      FLEVFED
                      460

>gi|825639|emb|CAA38899.1| thyroid hormone receptor alpha 1 [Homo sapiens]
>gi|586092|sp|P10828|THB1_HUMAN Thyroid hormone receptor beta-1
```

Fig. 1. Amino acid sequences of the human TR$α_1$ and human TR$ß_1$ LBD. Subtype specific differences are boxed. Numbering is for TR$ß_1$.

The amino acid (aa) sequences of the hTR$α_1$ and hTR$ß_1$ LBD starting at helix 1 are 85% homologous, which however does not mean that the other 15% are all sub-type specific differences. Sub-type specific differences are defined on the basis of the consensus sequence found in three of four species human, rat, chicken and xenopus laevis (h, r, c, xl) (10). When for instance the TR$α_1$ specific sequence is found to be conserved in at least three out of four species (h, r, c, xl) but differs between TR$α_1$ and TR$ß_1$, it is viewed as a genuine subtype-specific difference. An amino acid residue that differs between all four species within one isoform is therefore thought to be of less importance simply




```
>gi|825639|emb|CAA38899.1| thyroid hormone receptor alpha 1 [Homo sapie
>gi|135710|sp|P04625|THA_CHICK THYROID HORMONE RECEPTOR ALPHA
>gi|586092|sp|P10828|THB1_HUMAN Thyroid hormone receptor beta-1
>gi|586094|sp|P18113|THB1_RAT Thyroid hormone receptor beta-1
```

Fig. 2. Amino acid sequences of the human and chicken TRα_1 and the human and rat TRβ_1 LBD. H1-H12 indicate the helices. Black dots indicate residues that make contact to the ligand, α *vs.* β isoform specific differences are boxed. Numbering is for TRβ_1.

because its nature does not seem to be that relevant for receptor function. Looking at the differences between TRα_1 and TRβ_1 LBD in this way 10% genuine subtype specific differences are found. None of them is seen in residues that make contact with the ligand (Fig. 2, indicated with black dots) (10). The largest part of the twenty-six different isoform-specific residues in the hTRα_1 and hTRβ_1 LBD are concentrated in clusters: they are situated in H1 (3aa), in the loop between H2 and H3 (5aa), in the loop between H6 and H7 (2aa), H8 (2aa), in H10 (4aa), and in the loop between H10 and H11 (3aa). Most of the differences are changes into residues with other physicochemical properties, like a change of charge of the residue to positive, negative or uncharged or to changes of electron clusters, for instance when aromatic amino acids are exchanged. Distinct differences in interaction by synthetic analogues to TRα_1 and TRβ_1 LBD have been reported (11) and are probably due to these changes on the outer- and inner-surface of the LBDs. The crystal structures of the rTRα_1 and hTRβ_1 LBD have been solved and both LBDs consist of 12 α-helices and 4 β-strands organized in three layers. Structural differences are found in two loops that link conserved α-helices: the loop between H1 and H3 and the loop between H9 and H10. Structural differences are also seen in the final two turns of H11, where hTRβ_1 bends more inward compared to rTRα_1 producing a shift between H11 and H12 (10) (Fig. 3). Although the position of the binding site of DEA on TRα_1 and TRβ_1 LBD is still unknown, it is feasible that the discrepancy in the type of inhibition on the binding of T_3 to TRα_1 and TRβ_1 by DEA is caused by the differences in the amino acid sequences described above and resulting structural changes of the TRα_1 and TRβ_1 LBD.

9.2.B. Determinants of (non) competitive inhibition: effect of changes in the amiodarone compound or in the receptor protein

The structure of the TR LBD was not yet elucidated when we performed the experiments described in chapter 2 and 3. To get more insight in the structure-function relationship between amiodarone and the two isoforms (chapter 4 and 5), we performed TR binding studies changing either the compound amiodarone or the TR protein.

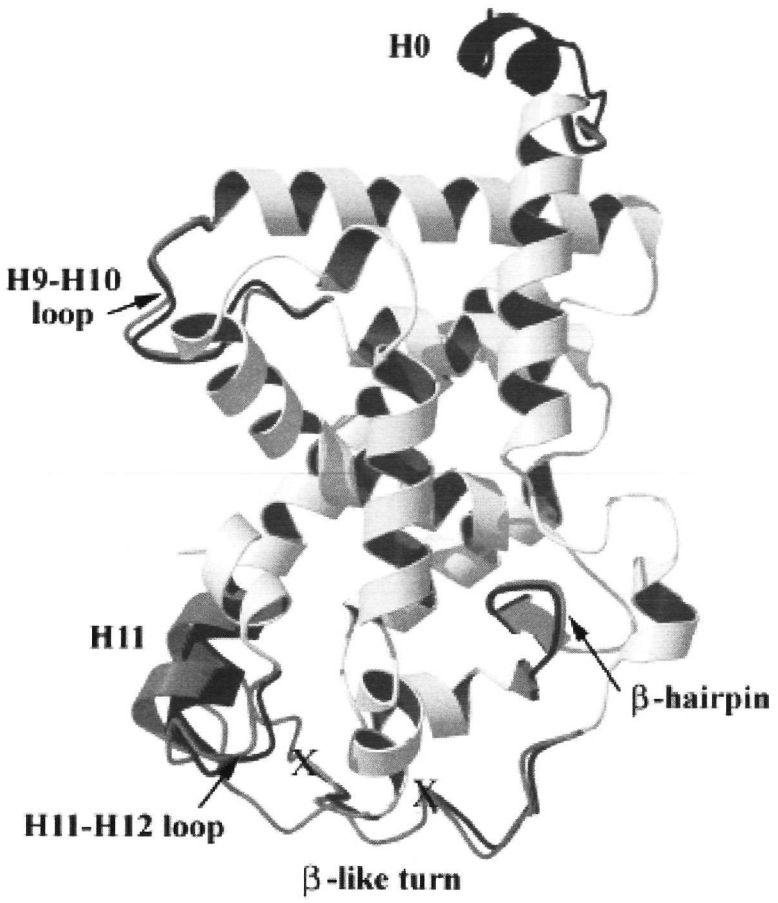


Fig. 3. Superposition of the crystal structures of rTRα_1 and hTRβ_1 LBD in ribbon drawing. Differences between the structures are marked in *green* for TRα_1 and *blue* for TRβ_1 (From Wagner, Mol Endo 15(3): 398-410).

First we looked from the side of the compound (chapter 4). Several AM metabolites and analogues were tested to evaluate the influence of changes in the make up of these molecules on their T_3 binding inhibitory potency on the TRα_1 and TRβ_1. The results of these TRα_1 and TRβ_1 isoform specific differences in the inhibition of T_3 binding by other AM analogues besides DEA are described in chapter 4. The effect of AM analogues on the binding of T_3 to the TR isoforms were evaluated as the potency of inhibition and the type of inhibition. Although AM itself has a slight inhibitory effect on the binding of T_3 binding to TRα_1 and TRβ_1, deethylation results in compounds with a strong

inhibitory effect. There is no major difference in the inhibitory potential upon the removal of one or two ethyl groups. Monodeiodination of AM results in a compound with a slightly increased inhibitory potential relative to that of AM, but the inhibition becomes stronger with complete deiodination. Compound L3373, in which the side-chain of AM is replaced by a hydroxyl group, inhibits the binding of T_3 to TRα_1 and TRβ_1 with the same potency as the deethylated compounds. The inhibitory potency is greatly reduced upon monodeiodination, and is completely lost when both iodine atoms are removed.

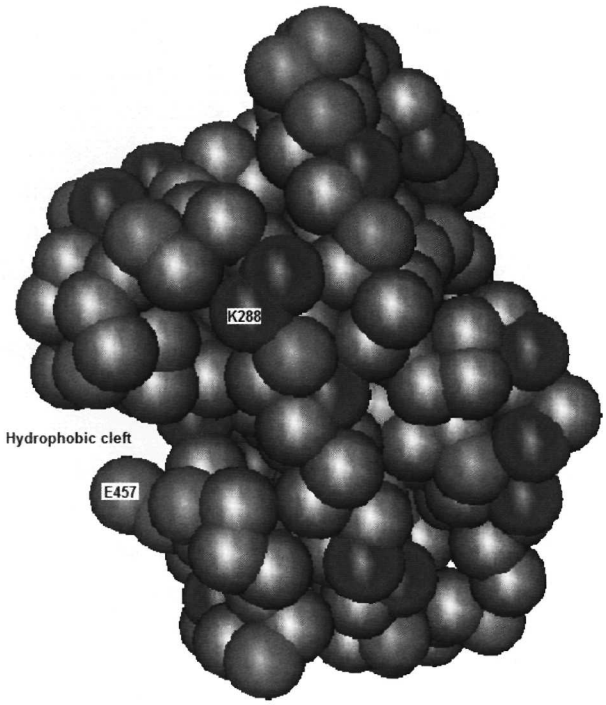


Fig. 5. Electrostatic surface representation of the hTRβ_1 LBD bound to T_3 (*orange*). The positively charged amino acids are marked in *blue*, the negatively charged amino acids are marked in *red*. The hydrophobic cleft is indicated. Amino acids E457 (red) and K288 (blue) form a "charged clamp" for co-activator binding.

Deethylation and deiodination of AM result in compounds that inhibit the binding of T_3 binding to TRα_1 in a competitive fashion, whereas the inhibition with respect to TRβ_1 is of a noncompetitive nature. In contrast to these

deethylated and deiodinated AM metabolites, compound L3373 shows the same competitive behaviour to both TRα_1 and TRβ_1 subtypes.
In order to understand how these drugs might act as inhibitors T_3-receptor binding, computer graphics modelling studies have been carried out to compare these molecular structures with those of T_3 and T_4. The molecular conformations of AM, DEA and benziodarone (similar to L3373, except the change from butyl to ethyl side chain) are similar (Fig. 4). The iodophenolic ring of amiodarone can be matched with either the tyrosyl or the phenolic ring of T4. The best fit of AM and its analogues with T_4 was obtained by superposition of the phenolic (outer) ring of T_4 (12). Another computational analysis also reports that superimposing the benzoylbenzofuran moiety of AM on the inner ring of T_3 and the diiodophenyl group on the outer ring provides the largest overlap of molecular volumes and gives the closest match of functional groups between AM and T_3 (13). These calculations do not give a clear answer, but are nice approaches in the process leading to the elucidation of the mechanism of action of AM and its analogues. From the results of our inhibition studies on AM analogues it is likely that: [1] the size of the diethyl substituted nitrogen-group and of the two bulky iodine atoms in the AM molecule hamper the binding of AM at or near the T_3-binding site of T_3 receptors, [2] differences in the LBD of TRα_1 and TRβ_1 are likely to account for the competitive or noncompetitive nature of inhibition of T_3 binding by AM analogues, and [3] compound L3373 does not discriminate between TRα_1 and TRβ_1 in type of inhibition to these TRs isoforms. However these findings did not give further indications to localize the DEA-binding site.

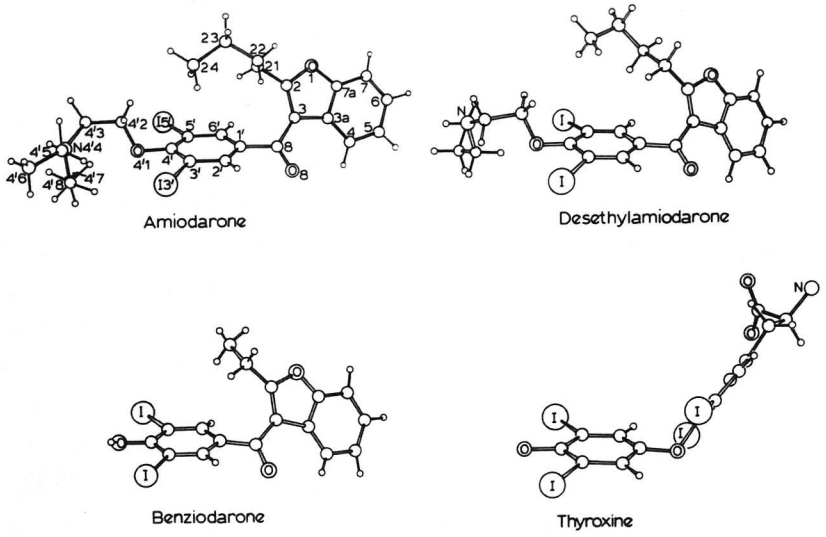


Fig. 4. Molecular conformation of AM, DEA, benziodarone, and T4. The conformation of T_3 is similar to that of T_4. [Figures are by courtesy of Dr. V. Cody (Buffalo, NY) (12).]

Next we looked from the protein side (chapter 5). The inhibitory effect of T_3 binding by DEA was studied in TR$ß_1$ proteins with mutations in the LBD, but which could still bind T_3. The potency of DEA to inhibit T_3 binding to the TR indeed changed in some of these LBD mutants. No change in inhibitory potency was observed for the naturally occurring mutants P453A and P453T relative to wild type TR$ß_1$, but a stronger inhibitory potency was observed for the mutants R429Q and E457A. This differential behaviour of mutant TR$ß_1$s with respect to the inhibitory effect of DEA allowed us to postulate a putative binding site in the LBD of TR$ß_1$. Inhibition of T_3 binding is noncompetitive in nature for the wild type and all mutants tested, indicating that the DEA binding site on the receptor is not equivalent to the T_3 binding site of TR$ß_1$. Furthermore, from the crystal structure of the rTR$α_1$ (14) and TR$ß_1$ (15) it is known that E457 is situated in helix 12 as a charged residue in the hydrophobic cleft on the outside of the receptor (Fig. 5). This hydrophobic cleft of the TR-LBD, composed of residues from helices (H) 3,5,6 and 12, also has some positive and negative charged residues, as can be seen in Fig. 5.
Co-repressors can bind to the TR using two or three interaction domains (IDs) which contain the core consensus amino acid sequence I/LXXI/VI (16);17).

Co-activators also bind to the same hydrophobic cleft of the LBD. Molecular studies have established that an LXXLL amino acid motif within the co-activators mediates this interaction with the T_3 activated receptor (17). This is confirmed by the crystal structure of the TR/GRIP NR box peptide complex (18) (Fig. 6).

In mutant E457A the physicochemical property of the residue changes from very charged to non-charged, resulting in an even more hydrophobic cleft on the LBD of TRβ_1. Keeping in mind that DEA shows a stronger inhibitory potency for mutant E457A compared to wild type, it could well be that this hydrophobic cleft is the binding site for DEA. With respect to TRα_1 we have to speculate upon the DEA binding site, because we did not perform studies with mutations in the TRα_1 protein.

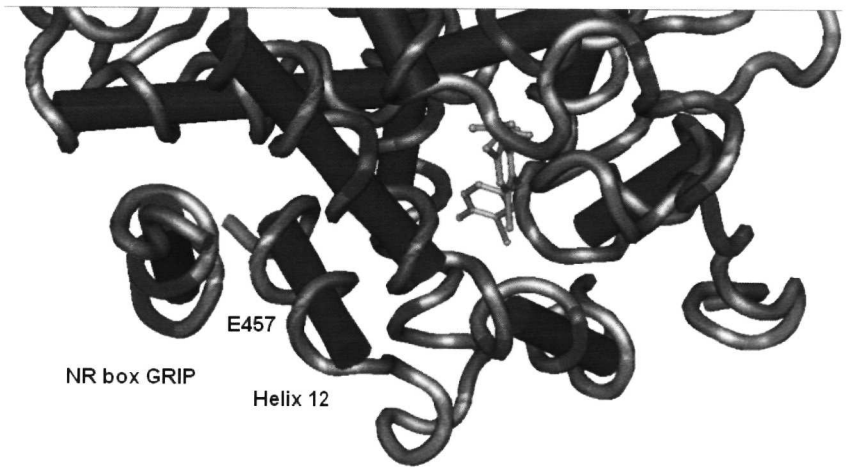


Fig. 6. Model of the interaction of the NR box on co-activator GRIP with the hydrophobic cleft formed by H12 on the hTRβ_1 bound to T_3 (orange).

It is likely that the binding sites for DEA on the TRα_1 and TRβ_1 are not the same, due to the fact that DEA and other deethylated and deiodinated AM metabolites inhibit the binding of T_3 to TRα_1 in a competitive fashion, whereas the inhibition with respect to TRβ_1 by these metabolites is of a noncompetitive nature, indicating distinct binding sites for DEA on TRα_1 and TRβ_1. The competitive type of inhibition for the TRα_1 suggests competition of DEA and T_3 for binding in the binding pocket, or a binding site for DEA close to the T_3 binding pocket.

9.3. Interference of amiodarone with co-factor binding

To achieve transcriptional activity of T_3-dependent target genes, DNA-bound TRs have to recruit general transcription factors (GTFs) to the preinitiation complex, but before that other proteins, the so-called co-activators are required. Two distinct groups of co-activators have emerged: ligand-dependent co-activators that bind to activation function (AF)-2 in the LBD, and co-activators that are associated with the N-terminal AF-1 region. The structural basis of the AF-2 interaction has been resolved, but the structural basis for the AF-1 interaction is not well understood (19).

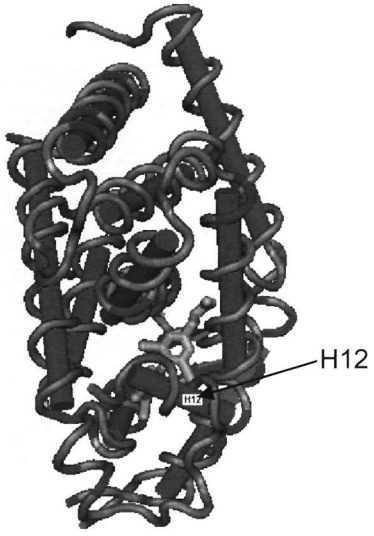


Fig. 7: The α-helical model of the crystal structure of the hTRß$_1$ LBD in the presence of T_3 (*orange*). The positively and negatively charged amino acids are marked in *blue* and *red* respectively. Helix 12 is indicated as H12.

Since the structure of the TR LBD is solved, our knowledge about structure and function of the LBD has increased enormously. The three-dimensional structure of the hTRß$_1$ LBD consists of 12-α helices and 4-β-strands arranged in a three-layered sandwich, as observed for rTRα_1 (10) (Fig. 7) this structure T_3 binds to a hydrophobic cavity buried within the core of the LBD. Important in the understanding of co-activator binding is the observation that in the ligand bound structure, helix 12 is folded against the protein, creating a 'lid' across the ligand-binding pocket. The structural data, together with the transcriptional

activation data, imply that the position of helix 12 is crucial for receptor activation, creating a binding surface for the co-activator binding. This hydrophobic groove within the LBD of the TR interacts with an LXXLL motif containing α-helix of the co-activator glucocorticoid receptor interacting protein 1 (GRIP-1) (18) (Fig. 6). GRIP-1 contains three copies of an LXXLL motif in its central region, called the nuclear receptor box (NR Box). This LXXLL motif is able to bind to the hydrophobic cleft of nuclear receptors, which includes E457 on helix 12.

When glutamine 457 (E457) is mutated to alanine DEA shows a stronger inhibitory potency. Interestingly this aa is situated in the same hydrophobic region where GRIP-1 interacts. Later on it became clear that the positively charged TR residue lysine 288 (K288) in H3 and the negatively charged TR residue glutamic acid 457 (E457) in H12 are involved in GRIP-1 binding to the TR. These TR residues form a "charged clamp" (20) and are completely conserved between different nuclear receptors (17;21). The observations with the TRs were confirmed by structural studies of the estrogen receptor (ER) in the presence of co-activator GRIP-1 show interaction of lysine 362 (K362), leucine 372 (L372) and glutamic acid 542 (E542) with the co-activator nuclear receptor (NR) box (22).

Because of the possible co-localization of the DEA and GRIP-1 binding sites on the LBD of the TR, we decided to study the effect of DEA on the binding of GRIP-1 to the hTRβ_1. The binding of co-activator GRIP-1 to hTRβ_1, as described in chapter 7, is T_3 dependent in a concentration responsive manner, supporting the above-mentioned mechanism that co-activator can only bind to liganded receptors.

When DEA is present the binding of GRIP-1 to hTRβ_1 strongly decreases concentration dependently. This diminished binding suggests competition between DEA and GRIP-1 for the same binding site on the hTRβ_1, but can also be due to the effect of lower T_3 binding to the receptor caused by the inhibitory effect of DEA on the binding of T_3 to TRβ_1. Although there are no structural or mutational studies reported on the binding of GRIP-1 to the TRα_1 isoform, it is well accepted that co-activators like GRIP-1 bind to the same hydrophobic cleft in the LBD of TRα_1 and TRβ_1, taking into account that the residues in the TR that bind to GRIP-1 are identical for TRα_1 and TRβ_1. For TRβ_1 it is clear, that DEA inhibits the binding of the ligand T_3 and that DEA

inhibits the binding of co-activator GRIP-1 to the receptor. We did not perform the TR/GRIP-1 binding studies in the presence of DEA with the TRα_1 isoform. It is likely that DEA also inhibits the binding of GRIP-1 to the TRα_1, although it is possible that there is an other mechanism of action because of the differential type of inhibition by DEA for the binding of T_3 to TRα_1 (competitive) and TRβ_1 (noncompetitive).

Since the coactivator-receptor interaction is essential for gene activation it can be concluded, that DEA acts as an antagonist in the presence of T_3.

9.4. Dronedarone

Amiodarone (AM), a drug developed for the treatment of angina pectoris but nowadays mainly used in the treatment of cardiac arrhythmias, has many side effects as described in the introduction of this thesis. The thyroidal side effects are due to a cytotoxic effect of the drug on thyroid follicular cells and to iodine excess caused by the release of pharmacological quantities of iodine during biotransformation of the drug. Therefore an analogue without iodine was developed: Dronedarone (Dron). This new drug, just like amiodarone, reduces heart rate and prolongs the QTc interval in dogs and rabbits (23;24). By analogy to AM, we evaluated the effect of Dron on the binding of T3 to the TRα_1 and TRβ_1. In *in vitro* binding studies, using receptor proteins expressed in a bacterial expression system, Dron itself had only a slight inhibitory effect on T_3 binding to TRα_1 and no effect on T_3 binding to TRβ_1. In contrast, the major metabolite debutyldronedarone (DBDron) was a very potent inhibitor of T_3 binding to TRα_1, but a weak inhibitor of T_3 binding to TRβ_1. Comparing AM to Dron several similarities and discrepancies can be found. Similarities are that not the parent drugs themselves, but their major metabolites desethylamiodarone (DEA) and debutyldronedarone (DBDron) are inhibitors of T_3 binding to TRα_1 and to TRβ_1. Discrepancies are that DEA is a very potent inhibitor of T_3 binding to both TRα_1 and TRβ_1, whereas DBDron is a strong inhibitor of T_3 binding to TRα_1, but a weak inhibitor of T_3 binding to TRβ_1. The *in vitro* results suggest that Dron might act as a TRα_1 selective antagonist.
The heart has an abundance of TRα_1 mRNA (25) and protein (26) relative to TRβ_1, whereas TRβ_1 is the predominant isoform in liver (27). If Dron is a TRα_1 selective antagonist, Dron could have considerable clinical advantages compared to AM besides the lack of thyroidal side effects due to iodine excess. Thus, Dron will have the same beneficial electrophysiological effects on the heart like AM which are TRα_1 mediated, but will lack TRβ_1 mediated side effects caused by AM like increased plasma cholesterol (28). By down-regulation of the TRβ_1 mediated synthesis of the LDL-receptor in liver during AM treatment, plasma cholesterol will rise, just as in hypothyroidism. If Dron is a really selective TRα_1 antagonist, the rise of plasma cholesterol and other TRβ_1 mediated effects will not occur during Dron treatment.

We tested this hypothesis in rats treated with AM or Dron. As specific TRα_1 mediated effects heart rates and QTc intervals were determined. As TRβ_1 mediated effects plasma cholesterol, the enzymatic activity of liver deiodinase 1 (D1) and the protein expression of the liver LDL-receptor gene were measured (29;30). AM and Dron indeed had similar effects on the prolongation of the QTc interval in the hearts of rats treated with AM or Dron compared to controls, whereas heart rate did not change (chapter 8). Prolongation of the QTc interval is also seen in the atrial muscle of rabbits (24) and in the hearts of dogs treated orally with AM or Dron (23). As expected AM and Dron do have differential effects on plasma cholesterol clearance. Only AM but not Dron increased total plasma cholesterol, LDL cholesterol and HDL cholesterol, in agreement with earlier studies in rats (31). The gene expression of the LDL-r was likewise decreased in the livers of AM but not in the livers of Dron treated rats. AM decreases the TRβ_1 regulated liver D1 enzyme activity (32); In contrast liver D1 activity was not affected in the Dron treated animals. These findings support the hypothesis that AM but not Dron inhibits TRβ_1 mediated gene expression. Whereas Dron thus appears to act as a TRα_1 selective antagonist, another thyroid hormone analogue GC-1 shows TRβ_1-selective agonistic actions. This agonist, like T_3 decreases plasma triglyceride and plasma cholesterol in rats (33) and primates (34) whereas T_3 but not GC-1 induced tachycardia.

A recent study has been published on the efficacy of Dron in humans for the prevention of atrial fibrillation (35). The aim of the study was to select the most appropriate concentration of Dron for prevention of recurrent atrial fibrillation after successful cardioversion. It was designed as a multicentre double-blind, randomised, placebo controlled trail. One hundred and ninety-nine patients from 50 centres and 11 countries were included in the primary analyses; patients were randomised into 4 groups: placebo, 800 mg, 1200 mg or 1600 mg Dron per day. Gastro-intestinal side effects were the most frequent cause for discontinuation of the drug. No evidence of thyroid, hepatic, neurological, ocular or pulmonary complications was found. In addition, no Dron-associated proarrhythmic reactions side effects were observed. In particular, torsade de pointes did not occur, although it happened in dogs treated with Dron (23). From this study it was concluded: "Dronedarone, at a 800 mg daily dose, appears to be effective and safe for the prevention of AF relapses after

cardioversion. The absences of thyroid side effects and of proarrhythmia are important features of the drug."

Ongoing studies like the Australian-American-African Trial with Dronedarone in Atrial Fibrillation or Flutter for the Maintenance of Sinus Rhythm (ADONIS) and the European Trial in Atrial Fibrillation or Flutter Patients Receiving Dronedarone for the Maintenance of Sinus Rhythm (EURIDIS), and other dronedarone trials will delineate the antiarrhythmic profile of Dron and its side effects compared to AM; also the effect of Dron on mortality will be determined. The outcome of these studies will have crucial impact on the drug's commercial viability (36).

9.5. Amiodarone and dronedarone as TR antagonists

9.5.A. Hypothyroid-like effects of amiodarone and dronedarone

The AM induced decrease in heart rate, reduction in myocardial oxygen consumption and lengthening of the QTc interval are identical to those observed in hypothyroidism (37) and can be prevented by the simultaneous administration of T_4 or T_3 (38). These findings led to the early hypothesis that AM induces hypothyroid-like effects. More hypothyroid-like effects of AM are described in Table 2, § 1. To evaluate whether the hypothyroid effects are just due to lower intracellular T_3 concentrations caused by inhibition of D1 activity by AM, a study was performed in rats treated with AM or iopanoic acid (another inhibitor of D1). This study reported that the lowering of heart rate and of the beta-adrenoceptor density in heart was observed in the AM treated but not in the iopanoic acid treated rats (39). The hypothyroid effects of AM thus can not only be due to lower T_3 concentration in tissues as the result of diminished conversion of T_4 into T_3. The additional effect of AM (but not of iopanoic acid) as an inhibitor of T_3 binding to TRs might explain many of its hypothyroid effects.

The question then arises if the concentrations of AM and DEA reached in tissues during long-term AM treatment are high enough to exert these antagonistic effects *in vivo*. In other words, are the tissue concentrations of AM and DEA in the order of the IC_{50} values found in our *in vitro* studies, so that DEA can compete with T_3 for binding to the TR *in vivo*. AM and its major metabolite DEA both accumulate in tissues during long-term AM treatment. In human tissues obtained at autopsy AM and DEA concentrations are 606 and 3815 µM/kg respectively in liver, and 62 and 274 µM/kg in the heart (Table 1, § 1). The IC_{50} value of AM is > 200µM for both TR isoforms and that of DEA 47 and 27µM for TRα_1 and TRβ_1 respectively (Table 1, § 4). These figures show that DEA concentrations in tissue can be higher than the IC_{50} values. However not only the tissue concentration of DEA is of importance, also the ratio of the DEA concentration to the T_3 concentrations is relevant (40). In this study using transfection of TRβ_1 and a reporter gene for malic enzyme (ME) in hepatocytes 1µM DEA was ineffective to reduce T_3-dependent gene expression of ME when T_3 was present in equimolar quantities (1µM T_3). However when the T_3 concentration was decreased to 1nM (1000 times lower

compared to 1µM DEA), DEA reduced the ME gene activity by 50%. AM was ineffective under these experimental conditions. These findings stress the fact that the ratio between T_3 and DEA is important for DEA to unfold its antagonistic action.

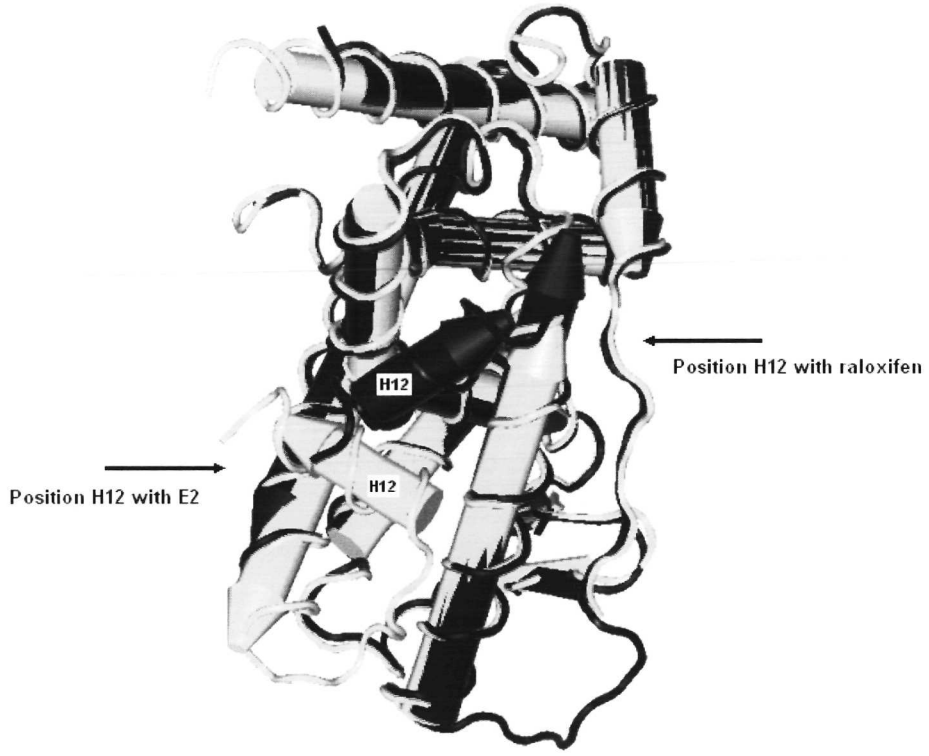


Fig. 8. Model of the crystal structure of the estrogen receptor (ER) LBD bound to estrogen (in yellow) or to raloxifen (in blue). The position of H12 in yellow (estrogen bound) creates a binding site for co-activator binding, whereas in the other position of H12 in blue (raloxifen bound) co-repressors can bind to the receptor surface. The model was generated using the VAST algorithm (NCBI website) and the program Cn3D4.1. using structures PDB # 1ERE and PDB # 1ERR.

Tissue T_3 concentrations in patients treated with AM are not available, but there are some animal data. In rats treated with 30 mg/kg AM orally for three weeks, T_3 concentrations in liver and heart are 4- to 5-fold lower than in controls (41). In rats treated with 100 mg/kg AM orally for two weeks liver and heart DEA concentrations (42) are on a molar basis 10.000 times higher compared to T_3 concentrations measured in the first study. It follows that the

DEA/ T_3 ratio is sufficiently high to allow DEA to exert its antagonist effect *in vivo* (Table 2).

Table 2. AM, DEA and T_3 concentrations in human and rat tissues after AM treatment

AM and DEA concentrations in human tissues (65)

Tissue	AM (µmol/kg)	DEA (µmol/kg)
Liver	606	3815
Heart	62	274

AM and DEA concentrations in rat tissues after 100 mg/kg AM orally for 2 weeks (43)

Tissue	AM (µmol/kg)	DEA (µmol/kg)
Liver	49	18
Heart	24	14

T_3 concentrations in rat tissues after 100 mg/kg AM orally for 2 weeks (42)

Tissue	T_3 (nmol/kg) control	T_3 (nmol/kg) AM
Liver	10	2
Heart	4	1

A minimal tissue concentration of DEA has to be reached, before DEA can inhibit the binding of T_3 to TR. In human tissues it will take time before DEA accumulates and reaches its IC_{50} concentrations. Side effects, due to tissue DEA accumulation, in patients on AM treatment are usually observed after several months. For example the increase in plasma cholesterol is observed only 12 months after the start of treatment (28), which is in line with the above calculations.

Tissue concentrations of Dron and DBDron are only known from an animal study in dogs treated with 20 mg/kg orally for 3 weeks. Myocardial content of Dron is 23 µmol/kg, and that of DBDron is 6 µmol/kg (23). The heart concentrations of Dron and DBDron in dogs are in the same order of magnitude as the AM and DEA concentrations in rat hearts. Tissue T_3 concentrations in animals treated with Dron are not available, but plasma T_3 concentrations are known from rats treated with AM or Dron (chapter 8). In both AM and Dron treated rats plasma T_3 is decreased with 25 and 21%

respectively compared to controls (Table 3). We can only speculate that the DBDron/T_3 ratio is of the same importance as the DEA/T_3 ratio (40). Lower plasma T_3 in the Dron treated rats will probably result in lower tissue T_3 concentrations, like in the AM treated rats. DBDron concentrations in the hearts of Dron treated dogs are 3 times lower compared to the DEA concentrations in the hearts of AM treated rats; however the cumulative dose of Dron was at least 2 times less compared to AM. These calculations indicate that DBDron concentrations can be high enough to exert the DBDron selective antagonistic action to the TRα_1.

Table 3. Dron, DBDron concentrations in dog tissue after Dron treatment and T_3 concentrations in rat plasma after AM or Dron treatment.

Dron and DBDron concentrations in dog tissue after 20 mg/kg Dron orally for three weeks (24)

Tissue	Dron (µmol/kg)	DBDron(µmol/kg)
Heart	23	6

T_3 concentrations in rat plasma after 100 mg/kg AM or Dron orally for 2 weeks (66)

T_3 (nmol/kg) control	T_3 (nmol/kg) AM	T_3 (nmol/kg) Dron
1.38	1.03	1.09

DEA is also reported to possess agonistic effects on TRs, at least in vitro. In HepG2 cells AM and DEA reduces the LDL receptor promoter activity, but interestingly in combination with T_3, AM and DEA have a synergistic effect on promoter activity (43). A study in pituitary NIH3T3 cells showed that high concentrations of DEA, but not AM, had an agonistic effect on the ME (malic enzyme) gene expression (40).These agonistic and antagonistic actions are probably caused by different ratios between DEA, T_3 and TR. When there is a vast excess of TR proteins, probably not all TRs are occupied with T_3, and TRs are then available for DEA binding. However T_3 has to be bound to the TR before DEA can act as an antagonist as is shown in the GRIP/TRβ_1 binding studies described in chapter 7.

The hypothyroid like effects by AM may also be mediated by non-genomic pathways, especially affecting the plasma membrane. An example of non-genomic action by AM is its effect on cardiac β-adrenoceptors. The number of β-adrenoceptor is decreased in cardiomyocytes upon AM administration,

mainly by increasing the rate of receptor disappearance from the plasma membrane. In the same study β-adrenoceptor mRNA in rat heart was not affected by AM (44).

9.5.B. Mechanistic model for the action of nuclear receptor antagonists

Thyroid hormone receptors (TRs) and other nuclear receptors (NR), like the androgen receptor (AR) and the estrogen receptor (ER), are all members of the large nuclear receptor super family. These receptors are similar in their three domain structure (Fig. 4, § 1) consisting of an N-terminal domain (NTD), a DNA binding domain (DBD) and a ligand binding domain (LBD), which is composed of 12 helices (H1-H12). Antagonists of the androgen, estrogen and progesterone receptor are frequently used in medicine. Antiandrogens are applied in the treatment of prostate cancer (45), whereas selective antagonists of the estrogen receptors are successfully used for fighting breast cancer, osteoporosis and cardiovascular diseases (20). Selective progesterone receptor modulators (SPRMs), have both agonistic and antagonistic activities depending upon the site of action. They are applied in the treatment of gynaecological disorders such as uterine fibroids and endometriosis (46). SPRMs are used for termination of pregnancy, as well for postmenopausal hormone replacement therapy (47). When the antagonist is bound to the receptor, the receptor fails to activate the transcriptional regulatory machinery, which otherwise occurs when ligand (agonist) is bound. Structural studies on the LBDs of several nuclear receptors, like the RXR, the ER, the AR and the TR showed that when ligand is tightly packed into the nuclear receptor pocket, helix 12 (H12) of the nuclear receptors shifts in position, closing the receptor pocket like a lid on a box (Fig. 5, § 1), thereby creating a surface for co-activator binding (22). The crystal structures of liganded nuclear receptors suggest a mechanism of how antagonists may work. The antagonist has to be similar enough to the ligand to bind in the receptor pocket, in this way preventing the binding of the natural ligand or an agonist. The antagonist can have extensions that can stick out of the binding pocket and in this way hinder H12 to close the receptor pocket, block proper receptor folding and block the creation of the surface for co-activator binding. In this "extension model" (9) antagonists can block gene expression by preventing co-activator binding to the nuclear receptor.

Structural studies of the estrogen receptor (ER) with its natural ligand estradiol and the antagonist raloxifen support this model (48). When estradiol is bound to the ER, H12 is packed into the body of the ER, similar to the packing of the analogous helix in the TR. In contrast, when raloxifen (48) or tamoxifen (49) is bound to the ER, H12 occupies a different position and blocks the co-activator binding (Fig. 8). The behaviour of raloxifen and tamoxifen is even more complex as these compounds have "mixed agonist/antagonist" activity. Raloxifen and tamoxifen have antagonist activity when the ER is bound to its specific DNA responsive element (ERE) and agonist activity when the ER is bound to the AP-1 complex of transcription factors (50).

From the TR crystal structures, and that of other nuclear receptors, it has become clear that after ligand binding H12 moves to close the receptor pocket. This finding was essential in the design of TR antagonists that nowadays is based on TR crystal structures.

9.6. Future perspectives

The design of TRα_1 or TRβ_1 selective agonists and antagonists might have clinical relevance.

For instance a TRβ_1 selective agonist could be very effective in the treatment of hypercholesterolemia, without inducing (TRα_1 mediated) tachycardia. A TRβ_1 selective agonist may also be useful in the treatment of resistance to thyroid hormone (RTH), caused by mutations in the TRβ_1 gene. A TRα_1 selective antagonist may be useful in lowering heart rate, without inducing hypercholesterolemia.

Amiodarone (AM) was early on described as a possible TR antagonist, and acts as described in this thesis as a TRα_1 and TRβ_1 antagonist in experimental animals as well as in humans. One of its analogues without iodine was tested in the nineties, known as SR 33589 and now named dronedarone (Dron). Dron, like AM is used as an antiarrhythmic drug. In contrast to AM however, as described in this thesis, Dron acts as a TRα_1-selective antagonist, which might have clinical advantage.

Before elucidation of the TR structure, many compounds were designed based on the chemical structure of T_3 some of them did indeed function as thyromimetics (51;52). The most useful compound from these series is triiodothyroacetic acid (Triac). Triac has a 2.7 times higher affinity for TRβ_1 than T_3, whereas the affinities of Triac and T_3 for the TRα_1 are the same (53). Triac can be used in the treatment of RTH to decrease plasma TSH and T_4 levels. Triac binds better than T_3 to the TRβ_1 and TRβ_2 in the hypothalamus and the pituitary, because of its higher affinity for TRβ_1, thereby repressing TRH and TSH gene expression (54). Another thyromimetic, 3,5-diiodothyropropionic acid (DITPA), increases dP/dt_{max} (maximum rate of LV pressure development) in hypothyroid rats (55). It has been applied in patients with heart failure, in whom it increases dP/dt and lowers plasma cholesterol without a change in heart rate (56). These findings are comparable to the increased dP/dt_{max} found in hypothyroid mice treated with GC-1 and the plasma cholesterol-lowering by GC-1 found in primates (34).

When the crystal structure of the TR was solved, it became clear that T_3 was completely buried in the receptor pocket, surrounded by amino acids and tightly packed without room for chemical groups larger than iodine at the 3'

position. Therefore the following model ("extension hypothesis" (9)) was hypothesized from the crystal structure: An agonist has to resemble T_3 so it can fit in the receptor pocket, and after agonist binding helix 12 has to close the receptor pocket and create the binding surface for the coactivator binding needed for gene transcription. Antagonists were hypothesized to have their effect by inhibiting the binding of T_3 to TRs, and disturbing TR-coactivator binding. A number of TR antagonists were designed based on this "extension hypothesis". The principle idea of the model was to design a compound that fits in the receptor pocket like a TR agonist, but to attach an extension creating a compound that sticks out of the receptor pocket, making it impossible for helix 12 to close the pocket, thereby interfering with the formation of the TR coactivator-binding surface (Fig. 5, § 1). To achieve isoform selectivity it was hypothesized that the compound has to differentiate between the single amino acid that makes contact to the ligand inside the hormone binding pocket and differs between TRα_1 and TRβ_1, namely Ser277 or Asn331 respectively (57).

As a starting point for the design of new TR agonists and antagonists the chemical structure of T_3 and the crystal structure of the TR were used. All compounds synthesized were without iodine. Compound GC-1 was the first high-affinity TRβ_1 selective agonist described in 1998 (11). It has a 10-times higher binding affinity to TRβ_1 compared to TRα_1. GC-1 contains several structural changes compared to the natural ligand T_3. These changes include the replacement of the three iodine atoms with methyl and isopropyl groups, replacement of the diphenyl ether linkage with a methylene linkage, and replacement of the amino-acid side chain with an oxyacetic-acid (Fig. 8). It is this oxyacetic-acid side chain which is critical in conferring TRβ_1 selectivity (58). GC-1 lowered plasma cholesterol in hypercholesterolemic rats by 75% and plasma TSH by 50% in a dose-dependent manner, without having an effect on heart rate (33). This full TRβ_1 antagonistic effect was confirmed in primates. In cynomolgus monkeys treated for 7 days, plasma cholesterol was reduced 33% and 37% by T_3 and GC-1 respectively, whereas T_3 but not GC-1 induced tachycardia. Plasma TSH was reduced in parallel with the fall in cholesterol by both T_3 and GC-1. In addition to cholesterol reduction, lipoprotein (a) levels were reduced by 34 and 40 % by T_3 and GC-1 treatment respectively. GC-1 is as potent as statins (HMG-CoA reductase inhibitors) in

decreasing plasma cholesterol concentrations in primates. However statins, which are widely used for cholesterol-lowering therapy in humans, have no additional benefit of reducing the atherogenic risk factor lipoprotein (a). Therefore in this respect GC-1 may add a superior therapeutic property compared to statins (34;59). However no studies in humans are described yet. A large number of derivatives of the TRβ_1 selective compound GC-1 has been synthesized and some of them show TR agonistic of antagonistic action. The most potent compounds are listed in Table 4.

Table 4. TR agonists, antagonists and their isoform selectivity

TR agonists	In vivo	TR antagonists	In vivo
Unselective TR agonists		Unselective TR antagonists	
T_3	+	amiodarone	+
		NH3	+
TRβ_1 selective agonists		DIBRT	
TRIAC	+	HY-4	
GC-1	+	KB 130015	+
GC-24			
KB-141	+	TRβ_1 selective antagonists	
		none	
TRα_1 selective agonists			
None		TRα_1 selective antagonists	
		dronedarone	+

In vivo + means: the compound is tested *in vivo*

GC-24 is, until now, the last TR agonist reported (60). GC-24 is a highly selective agonist for TRβ_1. The 3'-isopropyl group in GC-1 is substituted for a benzyl group. Although the benzyl group is too large to fit into the enclosed pocket of the receptor, the crystal structure of hTRβ_1 with GC-24 bound explains its agonist activity and unique isoform specificity. The "too large" benzyl is accommodated through shifts of 3-4 Å in two helices (helix 3 and helix 11) and forms a stable hydrophobic cluster with the hydrophobic amino

acid residues in the receptor pocket. Helix 3 and 11 are required for ligand binding and positioning of the critical helix 12. Despite these changes, the complex associates with coactivator as tightly as hTR bound to T_3, and is fully active in cellular assays. GC-24 binds to TR$ß_1$ with a K_d that is slightly weaker than that of T_3 but showed an average preference for TR$ß_1$ of ≈ 40-fold over TR$α_1$. GC-1 showed an average preference for TR$ß_1$ of only ≈ 3- to 5-fold greater than for TR$α_1$ (11). It is speculated that helix 11 is better packed in TR$α_1$, and that changes in the spatial volume near the ligand substitution are probably less tolerated in the TR$α_1$ subtype. Thus, addition of a bulky phenyl extension to the 3' position of the first aryl ring of GC-1 improves the specificity of binding to TR$ß_1$ with no reduction in binding affinity. No *in vivo* studies with CG -24 have yet been published.

Another compound that binds to TRs with high affinity in the nanomolar range is NH-3. The TR antagonist NH-3 is a derivative of GC-1 and contains a nitrophenyl acetylene moiety attached to the 5'-position of the thyronine core. NH-3 has similar binding affinities for in vitro translated Xenopus leavis TR$α_1$ and TR$ß_1$. This is consistent with findings using in vitro translated human TRs, implying that this modification of GC-1 decreases isoform selectivity. X.laevis tadpole metamorphosis, which is a T_3-dependent developmental process, is inhibited by NH-3 in a dose-dependent manner. Spontaneous metamorphosis is arrested by NH-3 with the same effectiveness as the thyroid hormone synthesis inhibitor methimazole. NH-3 demonstrates potent inhibition of T_3 action on TR$α_1$ and TR$ß_1$ *in vitro* and *in vivo*.

DIBRT (with two bromide atoms), HY-4 and GC-14 are also derivatives of GC-1. Although they bind directly to TRs and inhibit T_3 induced transcription in cultured cells, these compounds have weak TR binding affinity. GC-14 is TR$β_1$ selective, but DIBRT and HY-4 do not show isoform selectivity. Their weak binding affinity and low potency are the reason that these compounds have not been tested in an animal model yet.

Compound KG-141 resembles GC-1. It contains two chloride atoms at the 3- and 5-position instead of methyl groups, an acetic acid side chain instead of the oxy-acetic acid side chain, and a diphenyl ether linkage between the two phenyl groups (like in T_3) instead of the methylene linkage in GC-1. *In vitro* and *in* vivo KB-141 acts as a TR$ß_1$ selective agonist. In primates KB-141 caused significant cholesterol, lipoprotein (a), and body weight reduction with

no effect on heart rate. Binding affinity of KB-141, compared to CG-1 or T_3, to the TR$ß_1$ is not described; however, the IC_{50} value of KB-141 for binding to the TR$ß_1$ is 4 times higher compared to the IC_{50} value or T_3.
The last compound described here is KB130015. KB130015 is not developed as a TR agonist or antagonist, but as an anti-arrhythmic. It is one out of a series of newly developed AM derivatives, which all have two iodine atoms, like AM. KB130015 inhibits the binding of T_3 to the human TR$α_1$ and TR$ß_1$ *in vitro* and T_3 antagonism was confirmed in transfection studies. KB130015 and AM induced similar changes in thyroid hormone parameters in rats. The hypercholesterolemic effects of KB130015, however, are less pronounced when compared to AM. Liver damage, measured as serum transaminases (ASLAT and ALAT), occurs to a lesser extent in the KB130015 treated group compared to the AM treated animals. In Guinea pig papillary muscle KB130015 induced a significant increase of the action potential duration (APD) compared to controls. These findings led the authors to the following conclusion: "KB130015 appears to be less toxic than amiodarone while maintaining its electrophysiologic properties consistent with antiarrhythmic activity" (61). The rats in this study were treated with AM for only two weeks it remains to be seen if KB130015 is tolerated better than AM, especially because nothing is mentioned about the side effects due to iodine excess. Since it is known that the side effects of AM and DEA occur only after long-term treatment because it takes time before both compounds accumulate in tissues, the same may be true for KB130015. No studies in primates or humans have yet been reported. Based on these preliminary results, it seems to be premature to prefer KB130015 over AM in treating arrhythmias.

Summarizing, there are three TR$ß_1$ selective agonists, namely GC-1, GC-24 and KB-141. Only two of them GC-1 and KB-141 have been tested *in vivo.* Their TR$ß_1$ selective agonistic effect in primates comprises significant lowering of plasma cholesterol, lipoprotein (a) and plasma TSH with no effect on heart rate. These compounds may be potentially useful in the treatment of obesity, reduction of LDL cholesterol and reduction of the atherogenic risk factor lipoprotein(a), but also in decreasing plasma TSH and T_4 levels in patients with resistance to thyroid hormone (RTH). NH-3 is the only TR antagonist with

binding affinity similar to T_3, but unfortunately it has no isoform selective properties.

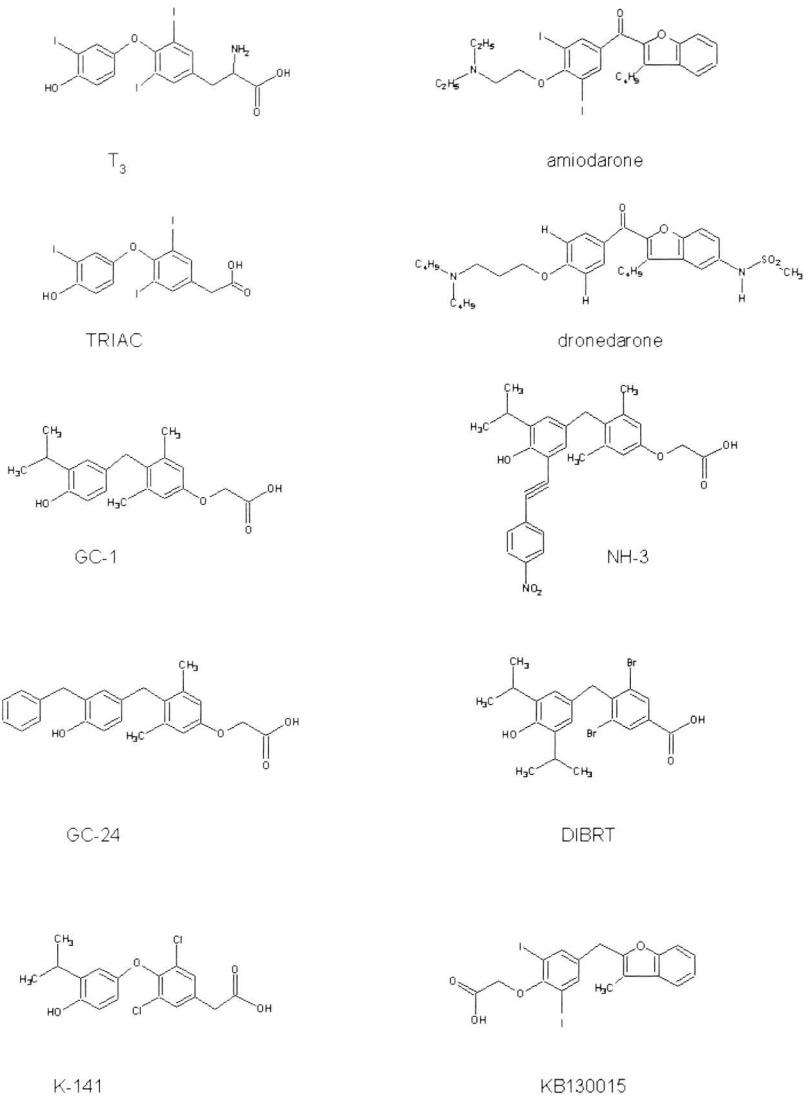


Fig. 9: Chemical structures of thyroid hormone agonists and antagonists.

The only TRα_1 selective antagonist known until now is dronedarone; its *in vitro* and *in vivo* antagonistic actions are described in this thesis. Dron does have

important advantages compared to AM. The thyroidal side effects caused by AM induced iodine excess were not observed in humans on Dron treatment (35), and AM induced hypercholesterolemia (TRβ_1 mediated) was not observed in rats and humans receiving Dron.

No TRα_1 selective agonist and TRβ_1 selective antagonists are reported until now and their clinical relevance is disputable. A TRα_1 selective agonist may be useful in the treatment of bradycardia, when thyroid hormone parameters are normal, without having an effect on lipids. However TRα_1 mediated processes in other organs will be up regulated as well, and the consequences of this activation are difficult to predict. The clinical relevance for a TRβ_1 selective antagonist is hard to defend. A TRβ_1 selective antagonist causes hypercholesterolemia, a situation that has to be prevented and not induced by drugs. However, selective TRα_1 agonists and TRβ_1 antagonists can be helpful in research in further elucidating TR isoform-mediated actions.

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Summary

Amiodarone (AM) is a highly effective drug against life-threatening cardiac arrhythmias and angina pectoris. In a minority of patients treated with amiodarone amiodarone-induced hypothyroidism (AIT) and amiodarone-induced thyrotoxicosis (AIT) occur. These thyroidal side effects are due to the excess of iodine that is generated during biotransformation of the drug, and/or cytotoxic effects of the drug on thyroid follicular cells. In contrast, in all patients treated with amiodarone, the drug prolongs the duration of the action potential in the heart, and it decreases heart rate and reduces myocardial oxygen consumption. Because these cardiac changes resemble those observed in hypothyroid patients, it was hypothesized that inducing a local "hypothyroid-like" condition in the heart is one of the mechanisms of action of AM.

The studies described in this thesis are aimed to evaluate whether such a hypothyroid-like condition could be explained by an inhibitory effect of amiodarone (AM) on the binding of T_3 to its specific nuclear thyroid hormone receptors TRα_1 and TRβ_1.

In chapter 2 and 3 it is described that not AM itself, but its major metabolite desethylamiodarone (DEA), is an inhibitor of the binding of T_3 to TRs. DEA is a noncompetitive inhibitor of the binding of T_3 to TRβ_1. In contrast, DEA is a competitive inhibitor of T_3 binding to the TRα_1. From these studies it became evident that not the drug itself, but its major metabolite DEA can probably act as a TR antagonist.

To further elucidate structure-function relationship of the interaction between AM and TRs several studies were performed. In a first approach we looked at the effect of changes in the amiodarone molecule itself (*chapter 4*). A number of amiodarone metabolites and analogues were evaluated to gain insight in which atoms and/or structures in the drug molecule are important for the inhibitory properties. The compounds were tested in an *in vitro* assay for binding of T_3 to TRα_1 and TRβ_1 proteins. From these studies it became apparent that the size of the diethyl-side group and the two bulky iodine atoms in the parent drug hamper the binding of AM at the T_3-binding site.

Some of the tested compounds exert the same type of inhibition (competitive) for both TRα_1 and TRβ_1, whereas the mode of inhibition of other compounds is different (competitive for TRα_1 and noncompetitive for TRβ_1).

The structure-function relationship between DEA and TR was further delineated by studying the inhibitory effect of DEA on the binding of T_3 to

mutated TRβ_1 proteins (*chapter 5*). From this study it became clear that TRβ_1 mutant E457A, situated in helix12 (H12), has a greater affinity for DEA than for the wild type receptor, indicating a possible binding site for DEA on the receptor close to helix 12.

Interactions of the hydrophobic region (which includes H12) of the TRβ_1 with the NR box of co-activators have been described. Consequently, the question arose whether DEA could interfere with the binding of co-activator GRIP1 to the TRβ_1. To study the protein-protein interaction between receptor and co-activator a nonradioactive "Pull-Down" assay was developed (*chapter 6*). In the presence of DEA, the T_3-dependent binding of co-activator GRIP1 to TRβ_1 was dose-dependently decreased (*chapter 7*). This finding indicates that DEA also interferes with receptor/co-activator interaction, thereby probably having an additional effect on T_3-dependent gene regulation.

In *chapter 8,* the inhibitory properties of a related new anti-arrhythmic drug dronedarone (Dron), devoid of iodine, on the binding of T_3 to the TRα_1 and TRβ_1 is studied. *In vitro* debutyldronedarone (DBDron), the major metabolite of Dron, and not Dron itself has a strong inhibitory effect on the binding of T_3 to TRα_1 (predominantly expressed in heart), but a less potent inhibitory effect on the binding of T_3 to TRβ_1 (predominantly expressed in liver). From the results of these binding studies it was hypothesized, that Dron could act as a TRα_1-selective antagonist. To test this hypothesis an *in vivo* study in rats, treated with AM or Dron, was performed. Plasma cholesterol increased and liver LDL receptor protein and type 1 deiodinase (D1) activities decreased in AM-treated, but not in Dron-treated animals; these are all TRβ_1-dependent effects. In the heart the TRα_1-mediated lengthening of the QTc interval was present in both AM- and Dron-treated animals. These findings suggest that Dron, via its major metabolite DBDron indeed acts as a TRα_1-selective antagonist *in vivo*. Dronedarone is the only TRα_1-selective antagonist known until now and has important advantages compared to AM. Dron has a beneficial effect on the heart like AM; however the thyroidal side effects caused by AM, are not observed in humans on Dron treatment. In addition, the AM-induced hypercholesterolemia is not observed in rats or humans receiving Dron.

From the studies described in this thesis it can be concluded, that desethylamiodarone (DEA) is a TRα_1 and TRβ_1 antagonist, whereas debutyldronedarone (DBDron) is a TRα_1 selective antagonist.

Samenvatting

Amiodaron (AM) is een zeer doeltreffend medicijn tegen levensbedreigende hartritmestoornissen en angina pectoris. Bij een minderheid van de patiënten die met amiodaron worden behandeld, leidt het gebruik van amiodaron tot hypothyreoidie en thyrotoxicose. Deze bijwerkingen op de schildklier worden veroorzaakt doordat er een teveel aan jodium vrijkomt bij de afbraak van het medicijn, en/of door cytotoxische effecten van het medicijn op de schildklierepitheelcellen. Daarentegen veroorzaakt het medicijn bij alle patiënten verlenging van de actiepotentiaal van het hart, verlaging van de hartslag en vermindering van het zuurstofgebruik door het hart. Omdat deze cardiale effecten eveneens voorkomen bij patiënten met hypothyreoidie, werd de hypothese gesteld dat een van de mechanismen van AM bestaat uit het opwekken van een plaatselijke "hypothyreoidie-achtige" conditie in het hart.
De onderzoeken die in dit proefschrift worden beschreven hebben tot doel om vast te stellen of die "hypothyreoidie-achtige" conditie kan worden verklaard door een remmende werking van AM op de binding van T_3 aan de specifieke kernreceptoren voor schildklierhormoon, TRα_1 en TRβ_1.
In hoofdstuk 2 en 3 wordt beschreven dat niet AM, maar zijn voornaamste metaboliet desethylamiodaron (DEA), een remmer is van de binding van T_3 aan TR's. DEA is een non-competitieve remmer van de binding van T_3 aan TRβ_1, maar is daarentegen een competitieve remmer van de binding van T_3 aan TRα_1. Uit deze onderzoeken werd duidelijk dat niet het medicijn als zodanig, maar zijn voornaamste metaboliet DEA waarschijnlijk werkt als een TR antagonist.
Om de structuur/functie relatie van de interactie tussen AM en TR's nader te belichten zijn verschillende onderzoeken gedaan. In een eerste benadering keken we naar het effect van veranderingen in het amiodaron molecuul (*hoofdstuk 4*). We onderzochten een aantal amiodaron analogen om inzicht te krijgen in welke atomen en/of structuren in het medicijnmolecuul van belang zijn voor de remmende eigenschappen. De analogen werden getest in een *in vitro* analyse op binding van T3 aan TRα_1 en TRβ_1 eiwitten. Uit deze onderzoeken werd duidelijk dat de grootte van de diethyl zijketen en de twee omvangrijke jodiumatomen de binding van AM aan de T_3-bindingsplaats verhinderen.

Enkele van de onderzochte analogen bewerkstelligen hetzelfde type remming (competitief) op zowel TRα_1 als TRβ_1, terwijl het type remming van andere analogen verschillend is (competitief voor TRα_1 en non-competitief voor TRβ_1). Om de structuur/functie relatie tussen DEA en TR verder te kunnen ophelderen, onderzochten we de remmende werking van DEA op de binding van T3 aan gemuteerde TRβ_1 eiwitten (*hoofdstuk 5*). Uit dit onderzoek werd duidelijk dat de TRβ_1 mutant E457A, gelegen in helix12 (H12), een grotere affiniteit heeft voor DEA dan voor de wild type receptor, waarmee een mogelijke bindingsplaats voor DEA op de receptor dichtbij helix 12 werd gevonden.

Daar er interacties van het hydrofobe gebied (waarin zich H12 bevindt) van de TRβ_1 met het kernreceptor bindingsdomein van co-activatoren beschreven waren, ontstond de vraag of DEA invloed zou uitoefenen op de binding van de co-activator GRIP-1 aan TRβ_1. Om de eiwit-eiwit interactie tussen receptor en co-activator te onderzoeken, hebben we een niet-radioactieve "Pull-Down" bepaling opgezet (*hoofdstuk 6*). In aanwezigheid van DEA werd de T_3-afhankelijke binding van co-activator GRIP-1 aan TRβ_1 dosis-afhankelijk verlaagd (*hoofdstuk 7*). Deze bevinding geeft aan dat DEA eveneens invloed heeft op de receptor/co-activator interactie, en daarbij waarschijnlijk ook een bijkomend effect heeft op T_3-afhankelijke gen regulering.

In *hoofdstuk 8* worden de remmende eigenschappen op de binding van T_3 aan TRα_1 en TRβ_1 onderzocht van Dronedaron (Dron), een nieuw medicijn tegen hartritmestoornissen gelijkend op AM maar zonder jodium. *In vitro* heeft debutyldronedaron (DBDron), de voornaamste metaboliet van Dron, en niet Dron zelf, een sterk remmende werking op de binding van T_3 aan TRα_1 (de voornaamste isovorm van de schildklierhormoonreceptor in het hart), maar een minder krachtig remmende werking op de binding van T_3 aan TRβ_1 (die in de lever de meest abundante receptor is). Op basis van deze resultaten veronderstelden we dat Dron zou kunnen werken als een TRα_1-selectieve antagonist. Om deze hypothese te testen hebben we een *in vivo* studie gedaan met ratten die waren behandeld met AM of Dron. In ratten die met AM waren behandeld, werd een toename van plasma cholesterol, een afname van het LDL receptor eiwit en verminderde type 1 deiodinase (D1) activiteit in de lever gemeten. Deze TRβ_1-afhankelijke effecten traden niet op in dieren die met Dron waren behandeld. De TRα_1-afhankelijke verlenging van het QTc

interval was aanwezig in zowel met AM als met Dron behandelde dieren. Deze bevindingen suggereren dat Dron, via zijn voornaamste metaboliet DBDron, inderdaad werkt als een TRα_1-selectieve antagonist in vivo. Dronedaron is de enige tot nu toe bekende TRα_1-selective antagonist en heeft belangrijke voordelen in vergelijking met AM. Dron heeft net als AM een gunstige werking op het hart; echter, de door AM veroorzaakte bijwerkingen op de schildklier worden niet waargenomen bij mensen die met Dron worden behandeld. Ook de door AM veroorzaakte hypercholesterolemie wordt niet waargenomen in ratten of mensen met Dron medicatie.

Uit de onderzoeken die in dit proefschrift worden beschreven kan worden geconcludeerd, dat desethylamiodaron (DEA) een TRα_1 en TR$ß_1$ antagonist is, terwijl debutyldronedaron (DBDron) een TRα1-selective antagonist is.

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Stellingen

1 Amiodaron is zowel een schildklierhormoon receptor-α_1 (TRα_1) als een schildklierhormoon receptor-β_1 (TRβ_1) antagonist.
Dit proefschrift

2 Dronedaron is een schildklierhormoon receptor-α_1 (TRα_1)-selectieve antagonist.
Dit proefschrift

3 GC-1, een TRβ_1 agonist, verlaagt plasma cholesterol, plasma triglyceriden en lipoproteïnen sterker dan statines.
Trends Endocrinol.Metab.2004;15:154-7

4 Mono- en diiodothyronines, tot nu toe gezien als louter inactieve afbraakproducten van het actieve schildklierhormoon T_3, kunnen wel degelijk biologische relevantie bezitten.
Mol. Cell Endocrinol.2003;213:1-11

5 Bij geavanceerde technieken, zoals de meting van mRNA middels Real time PCR, is de eenvoud van de praktische handelingen in onverwacht contrast met de kennis en ervaring nodig voor interpretatie en kwaliteitscontrole.

6 Vaak is in vivo onderzoek bij intacte proefdieren noodzakelijk om hypotheses gebaseerd op in vitro waarnemingen te valideren.

7 Te veel regels verstikken het persoonlijk initiatief.

8 Jong moeder worden is goed voor moeder en kind.

9 Een goed kind regeert zichzelf.
Mijn oma

10 Schoon glaswerk is essentieel voor de uitkomst van een analytisch experiment, evenals voor het tappen van een goed glas bier.

Stellingen behorende bij het proefschrift
"Amiodarone and thyroid hormone receptors".

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