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Induction of the adrenoleukodystrophy-related gene (*ABCD2*) by thyromimetics

Emmanuelle C. Genin[a,b], Catherine Gondcaille[a,b], Doriane Trompier[a,b], Stéphane Savary[a,b,*]

[a] *INSERM, U866, Dijon, F-21000, France*
[b] *Université de Bourgogne, Faculté des Sciences Gabriel, Centre de Recherche Lipides, Nutrition, Cancer – Laboratoire de Biochimie Métabolique et Nutritionnelle (LBMN), Dijon, F-21000, France*

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ABSTRACT

X-linked adrenoleukodystrophy (X-ALD) is a peroxisomal disorder caused by mutations in the *ABCD1* (*ALD*) gene. The *ABCD2* gene, its closest homolog, has been shown to compensate for *ABCD1* deficiency when overexpressed. We previously demonstrated that the *ABCD2* promoter contains a functional thyroid hormone response element. Thyroid hormone (T3) through its receptor TRβ can induce hepatic *Abcd2* expression in rodents and transiently normalize the VLCFA level in fibroblasts of *Abcd1* null mice. In a therapeutic perspective, the use of selective agonists of TRβ should present the advantage to be devoid of side effects, at least concerning the cardiotoxicity associated to TRα activation. In this study, we compared the effects of T3 with those of two thyromimetics (GC-1 and CGS 23425) specific of TRβ. Using a gene reporter assay, we demonstrated that the rat *Abcd2* promoter responds to the thyromimetics in a dose-dependent way similar to what is observed with T3. We then investigated the effects of 2-, 4- and 10-day treatments on the expression of *ABCD2* and its paralogs *ABCD3* and *ABCD4* in human cell lines by RT-qPCR. Both thyromimetics trigger up-regulation of *ABCD2–4* genes in HepG2 cells and X-ALD fibroblasts. Interestingly, in X-ALD fibroblasts, while T3 is associated with a transient induction of *ABCD2* and *ABCD3*, the treatments with thyromimetics allow the induction to be maintained until 10 days. Further in vivo experiments in *Abcd1* null mice with these thyromimetics should confirm the therapeutic potentialities of these molecules.

1. Introduction

X-linked adrenoleukodystrophy (X-ALD, OMIM 300100), the most frequent genetic peroxisomal disorder, presents distinct phenotypes varying both in symptoms and onset [1,2]. The childhood cerebral form (CCALD) and the adrenomyeloneuropathy (AMN) represent the main phenotypes. Whatever the clinical pattern, X-ALD patients display a characteristic accumulation of saturated and monounsaturated very-long-chain fatty acids (VLCFA, C > 22) in plasma and tissues. The exact link between this accumulation and the pathogenesis is still unclear making therapeutic progresses so difficult. In fact, hematopoietic stem cell transplantation is currently the only therapy for CCALD but its high risk of mortality, the difficulty to find compatible donors, and the need to do the transplant in early stages of the disease, lead to search for other therapeutic strategies.

X-ALD is caused by mutations in the *ABCD1* gene (formerly called *ALD*) encoding for a half ABC transporter of the peroxisomal membrane [3]. ALDP, as a homodimer or as a heterodimer with one of the three other peroxisomal ABC transporters (ALDRP, PMP70 and PMP69), is thought to import VLCFA-CoA esters into the peroxisome allowing their β-oxidation. ALDRP (adrenoleukodystrophy-related protein) [4], PMP70 (70 kDa peroxisomal membrane protein) [5] and PMP69 (69 kDa peroxisomal membrane protein) [6,7] (whose peroxisomal localization has recently been questioned [8]) are encoded respectively by the *ABCD2*, *ABCD3* and *ABCD4* genes. *ABCD1* and *ABCD2* (and *ABCD3* to a lesser extent) have been shown to present a partial functional redundancy [9]. The overexpression of the *ABCD2* gene in fibroblasts from X-ALD patients corrects VLCFA β-oxidation [10,11]. Moreover, in *Abcd1* null mice, the *Abcd2* overexpression prevents VLCFA accumulation and onset of neurodegenerative phenotype [12]. We and others have shown that the *Abcd2* gene is inducible in vivo by drugs, like fibrates or 4-phenylbutyrate (4-PBA) [13–17]. Hormones such as DHEA and thyroid hormones (thyroxine (T4) and triiodothironine (T3)) can also induce *Abcd2* expression [18,19]. Since this induction can prevent VLCFA accumulation and restore β-oxidation in *Abcd1* deficient cells and tissues [11,18], pharmacological therapy targeting the

* Corresponding author at: LBMN, INSERM U866, Université de Bourgogne, 6 Bd Gabriel, Dijon, F-21000, France. Tel.: +33 380396273; fax: +33 380396250.
E-mail address: stsavary@u-bourgogne.fr (S. Savary).

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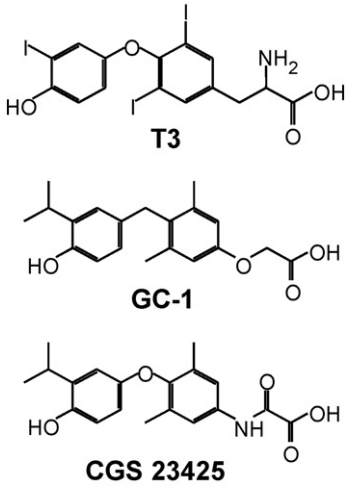

Fig. 1. Chemical structure of thyroid hormone T3 and thyromimetics (GC-1 and CGS 23425).

induction of *ABCD2* could therefore represent a potential therapeutic alternative provided that inducers are devoid of major side effects.

Thyroid hormones are known to induce peroxisomal biogenesis and β-oxidation [20,21] and were previously demonstrated to induce *Abcd2* expression in vitro and in vivo, the *Abcd2* promoter containing a functional thyroid hormone response element (TRE) [18]. These hormones exert their effects in almost all tissues and are involved in a lot of physiological processes essential for normal growth, development and metabolism (particularly lipid metabolism) [22]. They are known to act through their binding to nuclear receptors, TRα1 and TRβ1, which are widely distributed, and TRβ2 which is mainly expressed in the pituitary gland [23,24]. However, when high doses are administered, they can have deleterious effects on the heart, bones and muscles, mainly because of TRα activation. Moreover, the local and systemic availability and activity of thyroid hormones is subjected to a strict regulation through the action of deiodinases (for a review, see [25]). Therefore, several halogen-free thyromimetics have been developed to selectively activate TRβ receptors and avoid cardiotoxicity [26,27]. Here, we were interested in two of these thyromimetics (GC-1 [28] and CGS 23425 [29]) (Fig. 1). While GC-1 and T3 have comparable affinity for TRβ1, GC-1 displays an isoform selectivity with an approximately 10-fold higher affinity for TRβ1 than for TRα1 [28]. In vivo, GC-1 causes physiologic responses different from T3, in particular for lipid metabolism [30–32] and cerebral development [33]. GC-1 has recently been described as a promising molecule to treat a variety of lipid disorders, the last one being non-alcoholic fatty liver disease [34]. CGS 23425 has been shown to have a 50-fold higher preference for TRβ1 over TRα and to be devoid of cardiotoxicity in rats treated with up to 10 mg/kg drug [29].

In this study, we have compared the effects of GC-1 and CGS 23425 with those of T3 on the promoter of *ABCD2* by using gene reporter assay. We have also investigated the effects of GC-1 and CGS 23425 treatments on the expression of *ABCD2* and its paralogs *ABCD3* and *ABCD4* in human cell lines (HepG2 cells and X-ALD fibroblasts).

2. Materials and methods

2.1. Chemicals

T3 (3,3′,5-triiodo-L-thyronine sodium salt, Sigma) was dissolved in 0.01 N NaOH at 1 mM. GC-1 (3,5-dimethyl-4[(4′-hydroxy-3′-isopropylbenzyl)-phenoxy] acetic acid) and CGS 23425 (*N*-[3,5-dimethyl-4-(4′-hydroxy-3′-isopropylphenoxy)-phenyl]-oxamic acid), kindly provided by Dr. M. Issandou (GlaxoSmithKline, les Ullis, France) were dissolved in sterile water at 1 mM. All the chemicals were sterilized by passing through a 0.2 μm filter, aliquoted and stored at −20 °C. For cell treatment, stock solutions were diluted in sterile water at concentrations of 1, 10 and 100 μM and solutions were added in cell culture medium to obtain the appropriate concentration for treatment.

2.2. Cell culture

COS-7 cells (Transformed African Green Monkey cell, ATCC: CRL 1651), HepG2 (Human hepatoma cell line, HBS8065), human skin X-ALD fibroblasts (the ALD-3 cell line [10], which is a non-transformed cell line with a large deletion in the *ABCD1* gene were kindly provided by Dr. A. Pujol, Barcelona, Spain) and WT human skin fibroblasts (Coriell Institute GM03348) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C under 5% CO_2 in the absence of antibiotics. For the time-course experiment, after an attachment period of 24 h, the cells were treated with 100 nM of appropriate molecule during 2, 4 or 10 days. The medium was changed daily.

2.3. Transfection and gene reporter assay

COS-7 cells (50,000 cells/well) were co-transfected using Exgen 500 (Euromedex) with 650 ng of p2206, 100 ng of pCMV-βgal (Clontech), and 250 ng of the TRβ1 expressing vector (pTRβ1-pSG5) or 250 ng of the empty vector pSG5 (Stratagene) as previously described [15]. The transfected cells were incubated with or without T3, GC-1, CGS 23425 at 1, 10, 100 or 1000 nM for 48 h, and assayed for luciferase activity as previously described [15,18]. Luciferase activity was corrected according to the protein content (Bradford assay). The correction of the luciferase activity for transfection efficiency with β-galactosidase activity was not possible because of variations observed in the presence of thyromimetics (but not with T3).

2.4. Quantitative RT-PCR

Cells were harvested with 0.25% trypsin/EDTA (Sigma) and washed twice with PBS. Total RNA of HepG2 cells was isolated using Tri Reagent (Euromedex) and then purified using RNeasy Mini kit (Qiagen) following manufacturer's instruction. Total RNA from X-ALD fibroblasts were extracted using RNeasy Mini kit (Qiagen) following manufacturer's instructions. cDNA was generated by reverse transcription and analyzed by quantitative PCR using the SYBR Green real time PCR technology and an iCycler iQ Real Time Detection System (Bio-Rad). The primers described in Table 1 were chosen using the Beacon Designer Software (Bio-Rad). Briefly, to discard genomic DNA, 4 μg RNA was treated for 30 min with 1 μl 10× RQ1 DNase (1 U/μl) (Promega) in a final volume of 10 μl. 1 μg of random hexamers was then added and the volume was adjusted to 17 μl before denaturation (70 °C, 5 min). First strand cDNA was synthesized using 1 μl of M-MLVRT reverse transcriptase enzyme (Moloney-Murine Leukemia Virus-Reverse Transcriptase) (200 U/μl) (Promega), 5 μl of 5× M-MLVRT buffer, 1.25 μl of dNTPs (10 mM) (Promega). The reaction was performed for 1 h at 37 °C. PCR reactions were carried out in duplicate in a final volume of 25 μl containing 12.5 μl of SYBR Green qPCR Mastermix (Eurogentec), 1 mM EDTA, 300 nM of forward and reverse primers and 5 μl of cDNA. The PCR enzyme (*Taq* DNA polymerase) was heat-activated at 95 °C for 10 min, and the DNA was amplified for 40 cycles at 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s, followed by a melting curve analysis to control the absence of non-specific products. For

Table 1
Primer sequences for PCR.

Gene	Forward primer	Reverse primer
ABCD2	5′-GAACTGCTGTCATTCAAGAATCTG-3′	5′-TGCCAATGTGTCACTGAGAGG-3′
ABCD3	5′-GTGGTATCATTGGTCGTAGCAG-3′	5′-AGCCTTACTCGGAAGCACAG-3′
ABCD4	5′-TGGAGCACATGAGGACAGAC-3′	5′-AAGGTGTTGATGCCGATGTAC-3′
Dio3	5′-GCCTCAAAGCAGCACCTAAACC-3′	5′-ACCTCGCCAATGCCTCTCAAG-3′
TRβ	5′-GTCCTGTGATGTGAATGCTCTGAAG-3′	5′-GGTGCTGGTGAGTTAATGATTGTC-3′
36B4	5′-CTCCTTTGGGCTGGTCATCC-3′	5′-CAGACAGACACTGGCAACATTG-3′

each transcript, the amplification efficiency was determined by the slope of the standard curve generated from 2-fold serial dilutions of cDNA. Quantification of gene expression was performed using cycle to threshold (Ct) values and normalized by the *36B4* reference gene encoding for the acidic ribosomal phosphoprotein P0.

2.5. Statistical analyses

Comparisons of treated samples with controls were performed by Mann–Whitney test using the StatView statistical software. The differences between the treated and control groups are indicated as significant ($p < 0.05$) or highly significant ($p < 0.01$).

3. Results

3.1. Both GC-1 and CGS 23425 thyromimetics stimulate the Abcd2 promoter-driven luciferase expression

The rat *Abcd2* promoter was previously demonstrated to mediate induction of luciferase reporter gene in T3-treated COS-7 cells co-transfected with TRβ1 [18]. Using the same construct, i.e., the p2206 plasmid, which contains approximately 2 kb of the rat *Abcd2* promoter cloned into pGL3 (Promega), we compared the effects of different doses (1, 10, 100 or 1000 nM) of GC-1 and CGS 23425 to those of T3. As shown in Fig. 2, in cells co-transfected with the TRβ1 expressing vector (TRβ1-pSG5), each treatment resulted in a dose-dependent increased luciferase activity, the maximal effect (2.6–2.7-fold induction) being obtained at the dose of 1 μM. As expected for GC-1 since the affinity for TRβ1 of GC-1 and T3 is similar, we observed no significant difference between T3 and GC-1 treatments. Surprisingly since this compound has been shown to compete for hepatic nuclear T3-binding site is about 6-fold better than T3 [29], the effect of CGS 23425 was undistinguishable from that of T3. This apparent contradiction could be linked to the experimental process which depends on the high expression level of TRβ1 (co-transfection of TRβ1-pSG5 plasmid which contains a SV40 early promoter and polyadenylation signal to promote expression).

3.2. Compared effect of T3, GC-1 and CGS 23425 treatments on the expression of ABCD genes in human HepG2 cells

The proximal part of the *ABCD2* promoter, which contains the thyroid hormone response element, is strikingly well conserved in rodent and human [15]. Moreover, using gene reporter assay with constructs containing the isolated TRE upstream of a globin promoter, we demonstrated similar ability between human and rat

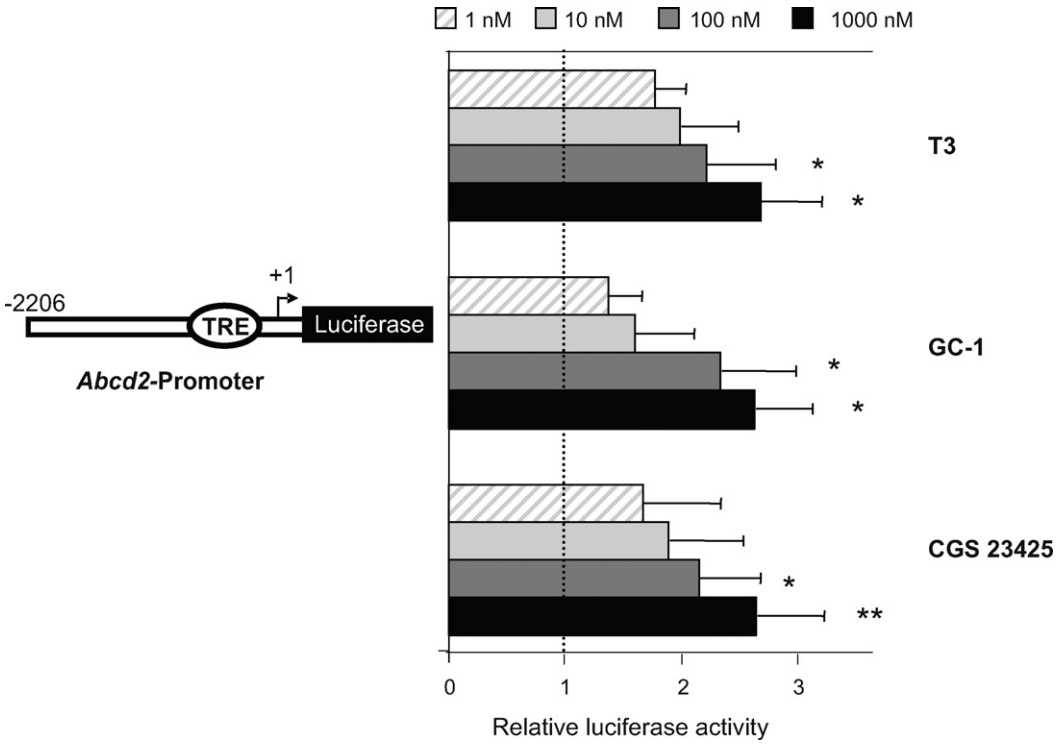

Fig. 2. Dose-dependent induction of *Abcd2* promoter-driven luciferase expression by T3 and thyromimetics. COS-7 cells were co-transfected with p2206, which contains the rat *Abcd2* promoter and its functional thyroid hormone response element (TRE) and with TRβ1-pSG5. Cells were treated for 48 h with 1, 10, 100 or 1000 nM of T3, GC-1 or CGS 23425. Luciferase activity was expressed relative to the activity of control cells (untreated cells co-transfected with p2206 and pSG5), activity arbitrarily taken equal to 1. Treatment without co-transfection of pTRβ1-pSG5 was not significantly different from the control (data not shown). Data represent the mean ± SD of five to six independent experiments performed in triplicate wells. Statistically significant differences from controls by Mann–Whitney test are indicated by asterisks (*$p < 0.05$, **$p < 0.01$).

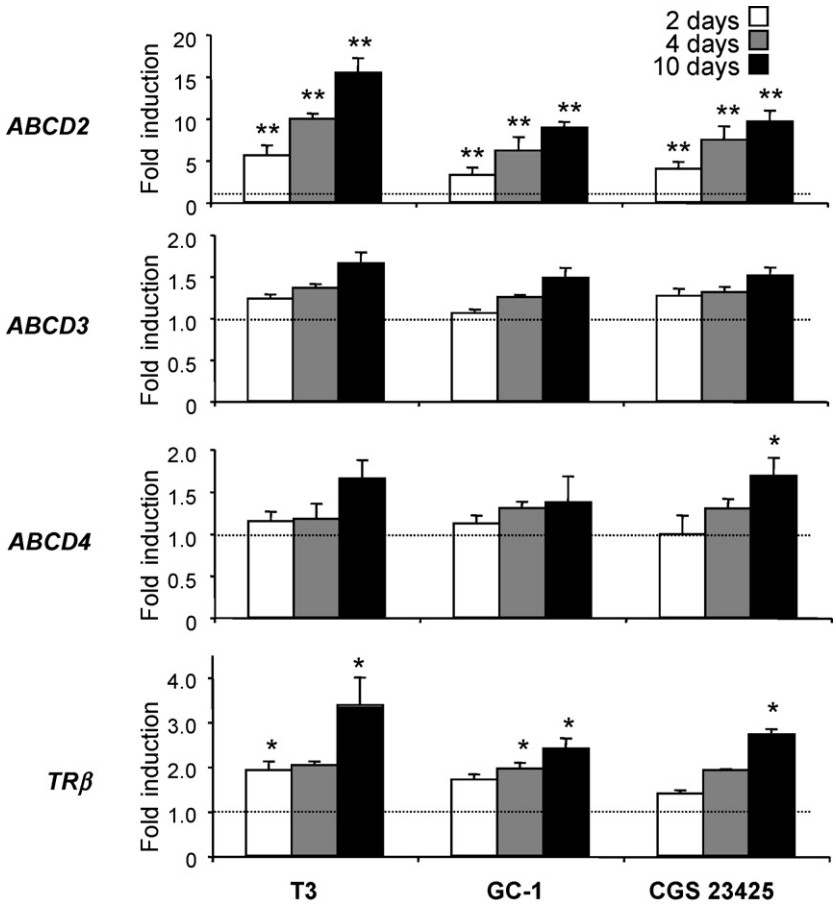

Fig. 3. Compared effects of 2-, 4- or 10-day treatments with T3 or thyromimetics on gene expression in human HepG2 cells. The mRNA levels were measured using real time RT-qPCR and normalized to 36B4. Data represent the mean ± SD of three to nine experiments and are expressed as fold induction in comparison with the gene expression level in untreated cells taken arbitrarily equal to 1. Statistically significant differences from control by Mann–Whitney test are indicated by asterisks (*$p < 0.05$, **$p < 0.01$).

motifs to induce luciferase activity [18]. The expression of *ABCD2* in human cells might therefore be inducible by T3 and thyromimetics. From our previous in vivo studies in rodent, liver was the main tissue where we could observe induction of the *Abcd2* and *Abcd3* genes. We then decided to explore the capability of T3 and thyromimetics to induce *ABCD2* expression in the human hepatoma HepG2 cell line. Gene expression was measured by RT-qPCR in cells treated during 2, 4 or 10 days with 100 nM of T3, GC-1 or CGS 23425. As shown in Fig. 3, *ABCD2* expression was induced whatever the treatment. The increase in duration of treatment was directly correlated with the induction level. The induction levels observed with GC-1 and CGS 23425 were not significantly different ranging from 3- to 9-fold but were inferior to those observed with T3 (×5.6, ×9.9, ×15.4 after 2, 4 and 10 days, respectively). The expression of *TRβ* was also induced in a time-dependent manner by T3 and both thyromimetics. Although nearly under the limit of statistical significance, *ABCD3* and *ABCD4* expression were found weakly increased in a time-dependent manner.

3.3. Compared effect of T3, GC-1 and CGS 23425 treatments on the expression of ABCD genes in human X-ALD fibroblasts

We previously showed that T3-treatment in murine *Abcd1*-deficient fibroblasts resulted in a transitory correction of the VLCFA accumulation correlated with a transitory induction of *Abcd2* expression [18]. To assess the « therapeutic » potential of thyromimetics for X-ALD, we treated human X-ALD fibroblasts with T3 and thyromimetics during 2, 4 and 10 days and quantified the expression level of the genes of interest (Fig. 4). We observed a weak induction of *ABCD2* after 2 days of treatment with T3 (×1.5). After 4 days, the induction level peaked at ×1.7 and then decreased to ×1.3 after 10 days, a result compatible with the results observed in mouse *Abcd1*-deficient fibroblasts [18]. Induction levels in the same range were obtained in WT fibroblasts for *ABCD2*, *ABCD3* and *TRβ* (data not shown). Interestingly, this transitory induction of *ABCD2* was not found in GC-1- and CGS 23425-treated cells. The 2-day treatments resulted in a weak induction (x1.3 for GC-1 and ×1.6 for CGS 23425) that was maintained or amplified after 4 and 10 days. Similar results were obtained for *ABCD3* and *ABCD4*. While far less pronounced than in HepG2 cells, the *TRβ* expression appeared to be correlated to those results: a weak induction is maintained after 10 days of treatment with thyromimetics while the induction peaks at 4 days and comes back to the initial level after 10 days of treatment with T3.

4. Discussion

Our previous study on the T3-response of the *ABCD2* gene [18] and the recent work of Weinhofer et al. [35] suggest that the ligand binding on TRβ1 results in a derepression of the *Abcd2* promoter. In the current study, we addressed whether GC-1 and CGS 23425, two thyroid hormone homologs showing selectivity for the TRβ1 isoform, could induce the expression of the *ABCD2* gene. We first demonstrated by gene reporter assay that the rat *Abcd2* promoter shows equivalent dose–response to T3 and thyromimetics. Since both thyromimetics appear as efficient as T3 to stimulate the *Abcd2* promoter-driven luciferase expression, we then explored their effects on gene expression in human cells, the hepatoma

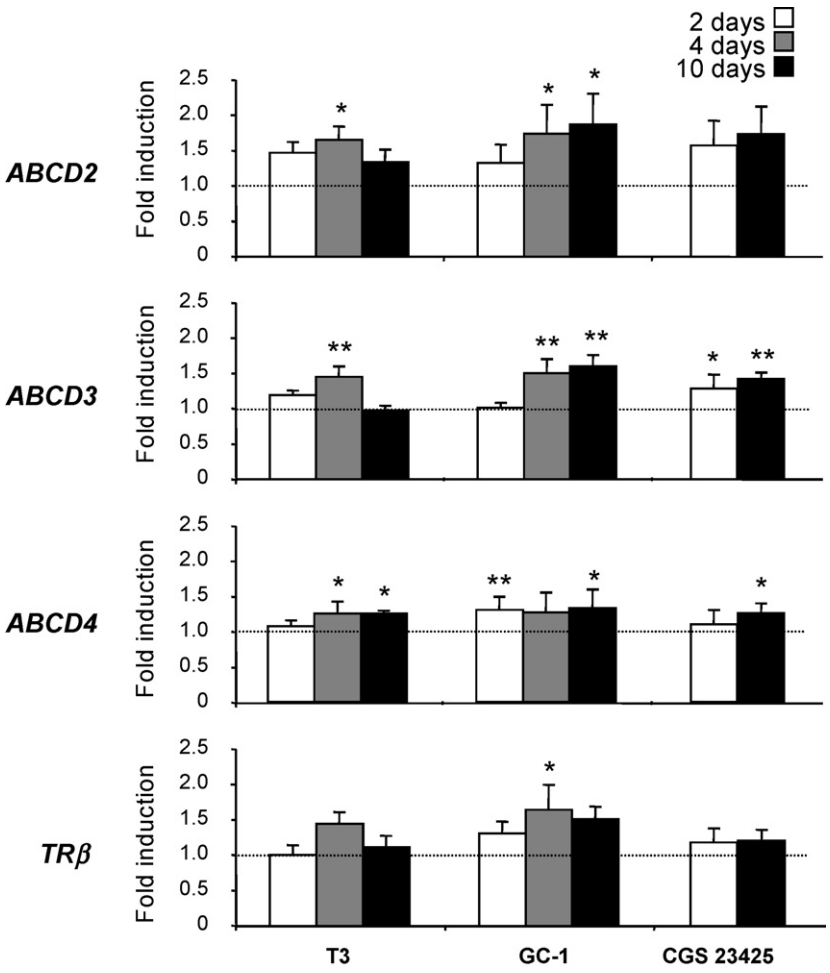

Fig. 4. Compared effects of 2-, 4- or 10-day treatments with T3 and thyromimetics on gene expression in human X-ALD fibroblasts. The mRNA levels were measured using real time RT-qPCR and normalized to 36B4. Data represent the mean ± SD of three to nine experiments and are expressed as fold induction in comparison with the gene expression level in untreated cells taken arbitrarily equal to 1. Statistically significant differences from control by Mann–Whitney test are indicated by asterisks ($^{*}p < 0.05$, $^{**}p < 0.01$).

HepG2 cell line and X-ALD fibroblasts. All in all, both thyromimetics revealed to be inducers of *ABCD2* expression. However, we noticed some differences with the effects of a T3-treatment according to the level of induction, the cell type and the capability of the induction to be "long-term" maintained.

In HepG2 cells, both thyromimetics were found to increase the expression level of *ABCD2* but to a lower extent than T3. This is probably linked to the presence of TRα1 in these cells [36] and to the capability of *ABCD2* to be induced by T3 through TRα1 activation [35]. The induction level observed after a treatment of 2 days was amplified with time reaching a maximum after 10 days. The expression of *TRβ*, which is known to be tightly regulated by T3 [37] was found to be up-regulated in a similar manner than *ABCD2*. In contrast, in X-ALD fibroblasts, we observed a transitory induction of both *ABCD2* and *TRβ*. The induction level was weaker than in the HepG2 cells with a peak after 4 days (×1.5). The differences observed between the two cell types could be due to differences in the basal level of expression of TRβ1 (TRβ1 is more expressed in HepG2 cells than in fibroblasts (three to four Ct differences)). The existence of cell specific mechanisms of inactivation of T3, likely depending on deiodinase expression could also contribute to the observed differences. The deiodinases are known to control the thyroid hormone homeostasis since they can activate or inactivate thyroid hormones [25]. In general, a given cell type expresses only one type of deiodinase. Deiodinase 3 (Dio3) activity, which inactivates T3, was found to be absent in HepG2 cells [38]. By RT-qPCR, we confirmed the absence of Dio3 in HepG2 cells and found Dio3 expressed in X-ALD fibroblasts (data not shown) in agreement with the fact that skin (the tissue origin of these cells) expresses high levels of Dio3 [39]. Expression of Dio3 in X-ALD fibroblasts was not modified by T3-treatment (data not shown).

While in HepG2 cells, induction of *ABCD2* and *TRβ* is maintained after 10 days whatever the treatment, in X-ALD fibroblasts, *ABCD2* is transiently induced by T3 but remains induced after 10 days of treatment with GC-1 or CGS 23425. If the cell type differences in the T3-dependent induction level of *ABCD2* may be linked in part to the TRβ1 and Dio3 basal level of expression, the transient effect apparently associated to the transient induction of *TRβ* remains to be explained. Altogether, this capability of thyromimetics to bypass the negative feedback regulation observed with T3 on the expression of *TRβ* may represent a major point for a further therapeutic use.

Beyond the demonstration that *ABCD2* responds to the thyromimetics, our study confirmed for *ABCD3*, and showed for the first time for *ABCD4*, that these two genes are regulated by T3 and can be positively modulated by thyromimetics although to a lesser extent than *ABCD2*. In X-ALD fibroblasts, the expression levels of *ABCD3* and *ABCD4* follow the same pattern as *ABCD2* with a transitory induction in the presence of T3 (peak at day 4 mainly visible for *ABCD3*) and an up-regulation maintained after 10 days with thyromimetics. We failed to identify a thyroid hormone response element in the promoter of both genes by an in silico approach; we could only identify half "AGGTCA" sites which could be part of

degenerated response elements that could explain why a weaker response is observed in comparison with *ABCD2*. The partial functional redundancy of *ABCD3* with *ABCD1* has already been shown. *ABCD4* has never been demonstrated to compensate for *ABCD1* deficiency when overexpressed but its level of expression appears to be correlated with the severity of the X-ALD phenotypes [40]. Altogether, the capability to modulate by the thyroid hormone pathway, not only the expression of *ABCD2* but also the expression of *ABCD3* and *ABCD4* genes, opens a new field of investigation in a therapeutic perspective for X-ALD.

From our results in rodent liver with T3 [18] and from the results in HepG2 cells, we can reasonably think that thyromimetics would be able to induce the hepatic expression of *Abcd2*, *Abcd3* and *Abcd4* in vivo. Moreover, both GC-1 [30–32] and CGS 23425 [41] were demonstrated to mediate hypocholesterolemia in vivo. That should result in a subsequent activation of SREBP (sterol regulatory element-binding protein) (for a review, see [42]). Cholesterol depletion and SREBP1c have been shown to induce the *Abcd2* expression [43,44]. Thus, the use of thyromimetics could lead to a direct stimulation of the hepatic *ABCD2* expression through TRβ1 activation and to a further stimulation associated with the activation of the SREBP pathway. As already demonstrated in another model of compensation of peroxisomal deficiency [45], it cannot be excluded that a compensatory effect in the liver would result in an improvement in brain and neural tissues. However, a direct modulation of gene expression in brain would further reinforce the therapeutic hope on these molecules. In spite of a probable positive effect in liver, it is not evident whether such a treatment will lead to increased expression level of the *ABCD2–4* genes or at least of *ABCD2* in brain. All TR isoforms are expressed in brain even though TRα1 predominates in most of the brain cells. However, although less efficient than T3, GC-1 was shown to be able to enter the brain and be effective to induce gene expression in brain tissues containing TRβ1 [33]. Moreover, TRβ1 is known to be expressed in mature myelin producing cells, to be up-regulated (post-transcriptionally) by T3 in oligodendrocytes, to participate actively to myelination, and apparently to play a predominant role in oligodendrocyte precursor cells [46–49].

Further in vivo studies in *Abcd1*-deficient mice or other animal models will be needed to confirm whether GC-1, CGS 23425 or other thyromimetics specific for TRβ1 are able to induce *ABCD* genes in target tissues of X-ALD (brain, adrenals and testis) and confirm the therapeutic potentialities of these molecules.

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