3,5-di-iodothyronine stimulates tilapia growth through an alternate isoform of thyroid hormone receptor β 1

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Abstract

Recent studies in our laboratory have shown that in some teleosts, 3,5-di-iodothyronine (T₂ or 3,5-T₂) is as bioactive as 3,5,3'-tri-iodothyronine (T₃) and that its effects are in part mediated by a TR β 1 (THRB) isoform that contains a 9-amino acid insert in its ligand-binding domain (long TR β 1 (L-TR β 1)), whereas T₃ binds preferentially to a short TR β 1 (S-TR β 1) isoform that lacks this insert. To further understand the functional relevance of T₂ bioactivity and its mechanism of action, we used *in vivo* and *ex vivo* (organotypic liver cultures) approaches and analyzed whether T₃ and T₂ differentially regulate the S-TR β 1 and L-TR β 1s during a physiological demand such as growth. *In vivo*, T₃ and T₂ treatment induced body weight gain in tilapia. The expression of L-TR β 1 and S-TR β 1 was specifically regulated by T₂ and T₃ respectively both *in vivo* and *ex vivo*. The TR antagonist 1–850 effectively blocked thyroid hormone-dependent gene expression; however, T₃ or T₂ reversed 1–850 effects only on S-TR β 1 or L-TR β 1 expression, respectively. Together, our results support the notion that both T₃ and T₂ participate in the growth process; however, their effects are mediated by different, specific TR β 1 isoforms.

Key Words

- ▶ 3,5-T₂
- ▶ growth
- ▶ thyroid hormone receptor
- ▶ 1–850

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Introduction

The pleiotropic effects exerted by thyroid hormones (THs) have led to growing interest to identify, besides the amply studied 3,5,3'-tri-iodothyronine (T₃), other possible bioactive iodothyronines that could explain this diversity of functions. Accordingly, studies from several laboratories have suggested that 3,5-di-iodothyronine (3,5-T₂ or T₂), a putative product of the outer ring deiodination pathway involved in T₃ metabolism, also possesses bioactivity (Goglia 2005). In fact, studies from our laboratory have shown that both T₃ and T₂ act directly on TH-dependent genes in teleost liver (García-G *et al.* 2007) and that these genomic actions are mediated by different isoforms of

TR β 1 (Mendoza *et al.* 2013). Indeed, results from binding and transactivating assays revealed that the effects of T₂ on genomic regulation appear to be mediated by a different isoform of TR β 1 that is found only in fish and contains a 9-amino acid insert in its ligand-binding domain, and which we have denominated long TR β 1 (L-TR β 1). By contrast, T₃ preferentially binds to and activates the short TR β 1 (S-TR β 1; Mendoza *et al.* 2013). Furthermore, hepatic *in vivo* expression of L-TR β 1 is 10⁶-fold higher than that of S-TR β 1, suggesting that, at least in teleosts, the effects of THs are mainly mediated through this isoform. These observations, together with our findings that *in vivo*,

T_2 and T_3 modulate the expression of L-TR β 1 and S-TR β 1 respectively, suggest a distinct signaling pathway for each TH in teleosts and prompted us to propose an extra level in the cascade of TH signaling for which T_2 is specifically made and regulated (Mendoza *et al.* 2013).

In this study and with the aim of further understanding the functional relevance of T_2 bioactivity and its mechanism of action, two approaches were employed. *In vivo*, we analyzed whether T_3 and T_2 differentially regulate the S-TR β 1 and L-TR β 1 isoforms during a physiological demand such as body growth. *Ex vivo*, we closely analyzed the action mechanisms of the observed T_3 and T_2 effects and explored whether these mechanisms were indeed mediated by a TR β 1 isoform. Our results support the idea that at least in teleosts, T_2 is a relevant, physiologically bioactive TH whose genomic effects are mediated by a distinct TR β 1 isoform.

Materials and methods

Animals

Tilapia juveniles ($\sim 0.8 \pm 0.1$ g) were kindly provided by SAGARPA (Querétaro, Mexico). Fish were kept in 10 l tanks containing constantly aerated fresh water at a temperature of 25 °C and maintained on a 12 h light:12 h darkness cycle. The fish were fed twice a day (~ 40 mg/fish per day) with a commercial diet (Sera Marin, Sera, Germany). All animal experimentation (handling and killing) was conducted in accordance with accepted procedures regarding handling and killing of the animals, as reviewed and approved by the Animal Welfare Committee of our Institute.

In vivo experimental design

In all cases, initial fish body weight was recorded before treatments. Experimental groups consisted of ten individuals per group, and all experiments were performed in duplicate tanks (total of 20 fish per experimental group). Fish were treated by immersion in a 1 nM solution of one of the following TH (Sigma): T_3 , T_2 , or the inactive isomer rT_3 . A negative control group was treated with 4.5 mM methimazole (MMI; Sigma). The administration protocol was previously described (Navarrete-Ramírez *et al.* 2011) and consisted of adding a fresh batch of MMI or TH previously solubilized in 0.05 M NaOH to the corresponding tank in the morning (0900 h). The treatment was terminated after 8 h by changing the culture water, and this drug administration protocol was

repeated three times per week. Treatments were administered for 1 month, after which the fish were weighed and decapitated, and their livers were rapidly removed. In all cases, livers from each experimental group were divided into two and used for RNA and TH extraction (see below).

Ex vivo experimental design

Organotypic cultures of tilapia liver For each experiment, individual juvenile tilapia livers were dissected and placed in ice-cold 50% Hank's Balanced Salt Solution (Gibco), which contained 50% Minimum Eagle's Medium (MEM, Gibco), 10 mM HEPES, and 8 mM glucose (pH 7.2). Livers were sectioned at 400 μ m thickness on a McIlwain tissue chopper (about 18 slices per liver). For each treatment, at least five tilapia livers were sliced and randomly placed onto inserts of semiporous membranes made of Biopore CM hydrophilized PTFE, pore size 0.4 μ m (six slices per membrane (Millipore, Herts, UK)), and maintained in an incubator at 18 °C in 5% CO_2 . The culture medium consisted of 50% MEM, 10 mM HEPES, and 35% Hank's Balanced Salt Solution supplemented with 10% horse serum (Gibco), 8 mM glucose, and 5% penicillin–streptomycin (Gibco).

Treatments After 48 h of stabilization, the medium of each well (1.1 ml/well) was removed and replaced by the appropriate amount of fresh medium, which contained the different drug treatments: T_3 or T_2 at concentrations of 0.1, 1, 10, and 100 nM previously solubilized in NaOH or the TR antagonist 1–850 (Schapira *et al.* 2003; Calbiochem, Darmstadt, Germany) previously solubilized in DMSO at concentrations of 0.1, 1, 10, and 20 μ M alone or combined with 100 nM T_3 or T_2 . In all cases, after a 24-h incubation, slices from each plate were removed and pooled for mRNA extraction and quantification. All experiments were performed independently at least twice in duplicate.

Quantification of intrahepatic T_3

TH extraction Liver segments from five individual fish per experimental groups were pooled (~ 50 mg tissue/pool) and homogenized in ten volumes of methanol: ammonium hydroxide (99:1) solution, as described previously (García-G *et al.* 2004). The homogenates were centrifuged for 10 min at 700 g. The supernatants were collected, evaporated in a speed vacuum, and suspended 1:5 (initial w/v) in assay buffer Tris–HCl (0.05 M; pH 8.6).

T₃ RIA Intrahepatic T₃ content was measured by RIA as described previously (Orozco *et al.* 1992). The inter- and intra-assay coefficients of variation were 9.5 and 6.6% respectively. The incubation mixture contained assay buffer and a working dilution (1:8000) of anti-T₃ serum (Sigma), standard (standard curve, 7.8–1000 pg/dl), the radioactive solution (10 pg/100 µl of the labeled T₃ plus 10 mg/10 ml 8-anilino-1-naphthalene sulfonic acid (Sigma)), and 50 µl of the experimental sample. Free and antibody-bound radioactive T₃ were separated using 0.5% activated charcoal/dextran solution (Sigma).

Measurement of mRNA expression

In all cases, mRNA was quantified by real-time PCR as described previously (Mendoza *et al.* 2013). Briefly, total RNA was extracted from juvenile tilapia liver (TRIzol, Invitrogen), and RT (M-MLV, Promega) from 2 µg of total hepatic RNA was performed using oligo(dT) primer (final volume of 25 µl). β-actin was used as an internal standard in reactions that contained 1 µl of the RT reaction, 6 µl Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, Waltham, MA, USA), and 500 nM forward and reverse primers in a final volume of 12 µl. Oligonucleotides and PCR protocols used for all gene amplification are specified in Table 1. In all cases, the standard curve ranged from 10¹ to 10⁹ molecules/µl, and a Light Cycler instrument was used for detection and data analyses according to the manufacturer's instructions (Roche Molecular Biochemicals). The mRNA concentration was expressed as molecules per microgram of total mRNA used in the RT reaction (2 µg) and obtained by comparison with the standard curve, normalized to the concentration of β-actin in each experimental sample. Identical PCRs from the RNA samples before the RT reaction yielded no detectable products.

Statistical analysis

Results obtained in all experiments were analyzed using a one-way ANOVA coupled with the Tukey's multiple-comparison test (control vs treatments). Differences were considered statistically significant at *P* values of ≤0.05.

Results

In vivo experiments

Validation of iodothyronine treatment An initial aim of this study was to test T₂ bioactivity. To this end, effects of T₂ on body growth were measured as weight gain. As this is a long-term treatment, we designed an administration protocol that consisted in exposing the fish to very low doses of TH (1 nM in water culture) and limited the exposure to 8 h/day, 3 days/week for 1 month. This hormone concentration is at least ten times lower than that employed in other studies (Lam 1980, Nacario 1983, Lam & Sharma 1985, Reddy & Lam 1992, De Jesus *et al.* 1998, Lam *et al.* 2005). Furthermore, the administration protocol had a positive effect on fish growth and no negative effects on fish survival, while it induced a burst in growth of ~30% in both T₃- and T₂-treated fish (Table 2). Most importantly, the low TH concentrations used did not alter the thyroidal homeostasis of the fish, as judged by the intrahepatic T₃ levels, which were similar to those recently reported for intramuscular zebrafish T₃ and T₂ (1.8±0.2 and 2.8±0.4 ng/g respectively; Little *et al.* 2013). Indeed, despite the fact that intrahepatic T₃ concentrations remained within the euthyroid range in all tilapia treated with the different THs, only fish treated with 1 nM T₃ or T₂ presented a four fold body weight gain compared with the control group (*P*<0.001; Fig. 1).

Table 1 Oligonucleotide sequences and real-time PCR protocols used to amplify the different genes. In all cases, an initial 10-min denaturing step was included

Gene	Amplicon (bp)	Forward primer	Reverse primer	Real-time PCR protocol	R
β-actin	170	ACT TCG AGC AGG AGA TGG	GGT GGT TTC GTG GAT TCC	3 s at 95 °C; 7 s at 52 °C; and 8 s at 72 °C for 45 cycles	1.96
IGF1	217	AAC CTT GGG TGC TCT TGG CAT G	GTC TGT GGA GAG CGA GGC TTT	3 s at 95 °C; 8 s at 56 °C; and 9 s at 72 °C for 45 cycles	1.80
L-TRβ1	232	GTG AAG GAA GCT AAG CCT GA	CAC AAG GCA GCT CAC AGA AC	3 s at 95 °C; 10 s at 52 °C; and 10 s at 72 °C for 55 cycles	1.96
S-TRβ1	135	GCG GAA ATT CCT GCC TGA G	GCA GCT CAC AGA ACA TGG GC	2 s at 95 °C; 8 s at 52 °C; and 7 s at 72 °C for 45 cycles	2.01
D2	249	GAA ACT TGG CTG TGA GGC	CTC GTC GAT GTA GAC CAG	2 s at 95 °C; 7 s at 58 °C; and 10 s at 72 °C for 55 cycles	2.08

R, amplification efficiency for the standard curve.

Table 2 Juvenile tilapia weights before and after a 30-day treatment with the different thyroid hormones (1 nM) or methimazole (4.5 mM). Values are means $\pm$ s.e.m. ($n=20$)

Treatment	Initial weight (g)	Final weight (g)
Control	0.885 $\pm$ 0.015	1.019 $\pm$ 0.625
rT ₃	0.916 $\pm$ 0.006	1.041 $\pm$ 0.710
T ₃	0.853 $\pm$ 0.0375	1.690 $\pm$ 0.880
T ₂	0.810 $\pm$ 0.010	1.628 $\pm$ 0.865
MMI	0.788 $\pm$ 0.0025	0.865 $\pm$ 0.350

By contrast, weight gain of rT₃-treated fish was similar to that observed in euthyroid tilapia, while those exposed to MMI exhibited the lowest intrahepatic concentration of T₃ ($P<0.001$) and the lowest rate of weight gain (Fig. 1).

Regulation of TR β 1 isoforms by T₃ and T₂

Given that T₃ and T₂ treatment induced growth to a similar extent, we then analyzed whether their effects were differentially mediated by the S-TR β 1 and L-TR β 1 isoforms. To this end, hepatic expression of the mRNA for these two TR β 1s was quantified. As shown in Fig. 2, hepatic expression of the short isoform of TR β 1 was higher only in fish exposed to T₃. By contrast, expression of the L-TR β 1 isoform was upregulated only in the liver of tilapia treated with 1 nM T₂; no upregulation was observed in the group of rT₃-treated fish (Fig. 2). mRNA levels of both TR β 1 isoforms in MMI-treated fish were significantly decreased compared with the control group. The fact that T₂ and T₃ exclusively upregulate the expression of the long and short isoforms of TR β 1 respectively suggests that the effects of these two THs are mediated by two distinct isoforms of TR β 1.

Effect of T₃ and T₂ on hepatic IGF1 and D2 expression

In order to analyze whether the differential observed effects of T₃ and T₂ on the expression of L-TR β 1 and S-TR β 1s were specific, hepatic expression of two other TH-regulated genes was quantified. IGF1 (*igf1*) and D2 (*d2*) genes were chosen as they are positively and negatively regulated by THs respectively (Schmid *et al.* 2003). As depicted in Fig. 2, treatment with T₃ and T₂ equivalently enhanced hepatic expression of IGF1 and blocked that of D2; thus, T₃ and T₂ do not differ in how they regulate the expression of these two genes. rT₃-treated fish showed euthyroid levels of hepatic expression of IGF1 and D2, while those in MMI-treated fish were significantly decreased (IGF1) and increased (D2).

Ex vivo experiments

Regulation of gene expression by T₃ and T₂

Organotypic liver cultures responded to TH treatment. Expression of mRNA for IGF1 and D2 was up- and downregulated by T₃ and T₂ treatments respectively (Fig. 3). This pattern of regulation was similar to that observed in the *in vivo* experiments, validating the use of organotypic liver cultures to study the regulation of TH-regulated genes. We then analyzed whether cultures treated with T₃ or T₂ showed differential regulation of the two TR β 1 isoforms. Interestingly, the pattern of TR β 1 regulation by T₃ and T₂ was inverted when compared with the *in vivo* studies. Indeed, the expression of the short isoform of TR β 1 was lower only in fish exposed to T₃, whereas the expression of the L-TR β 1 isoform was downregulated only in the liver cultures treated with T₂ (Fig. 4). Furthermore, in agreement with the reported affinity of the tilapia TR β 1 isoforms for their corresponding ligands (Mendoza *et al.* 2013), T₂ treatment did not influence

Figure 1

(Top panel) Intrahepatic T₃ concentrations. (Bottom panel) Body weight gain. Tilapia were treated for 30 days with 1 nM T₃, T₂, or rT₃. A negative control group was treated with 4.5 mM MMI. Values are means $\pm$ s.e.m. ($n=20$). Significance is indicated (* $P<0.05$ and ** $P<0.001$).

Figure 2

Hepatic mRNA expression of short TRβ1, long TRβ1, IGF1, and D2. Tilapia were exposed to 1 nM T₃, T₂, or rT₃ for 30 days. The negative control was exposed to 4.5 mM MMI. Values are means ± S.E.M. Significance is indicated (*P < 0.05 and **P < 0.001).

S-TRβ1 expression even at the highest concentrations tested (100 nM), although this concentration of T₃ did downregulate L-TRβ1. The fact that T₂ and T₃ specifically downregulate the expression of the long and short isoforms of TRβ1 respectively further supports the notion that the effects of these two THs are mediated by two distinct isoforms of TRβ1 (Fig. 4).

Effects of the TR antagonist 1-850 upon gene expression

As shown in Fig. 3, and consistent with their regulation by THs, 1-850 effectively blocked IGF1 expression while it increased that of D2, further supporting the idea that both hormones act through a TR pathway. Furthermore, the co-treatment with 100 nM T₃ or T₂ reversed the effects of the TR antagonist 1-850, an effect that was blunted at 10 and 20 μM antagonist, probably because 1-850 has a 1000-fold lower affinity than T₃ for the receptor (Schapira *et al.* 2003). The expression of the long and short isoforms of TRβ1 was not affected by 1-850 treatment; however, T₃ and T₂ co-administration downregulated S-TRβ1 and L-TRβ1 respectively only at low (0.1 and 1 μM) concentrations of 1-850 (Fig. 4).

Discussion

Our previous studies support the notion that T₂ bioactivity in fish is mediated by the L-TRβ1 isoform, whereas T₃ acts by binding to the S-TRβ1 isoform (Mendoza *et al.* 2013). As we used either mammalian GH3 cells, or fish acutely treated with T₃ and T₂ in those experiments, the observed TRβ1 isoform specificity for a given ligand could not be functionally corroborated. Thus, one of the goals of this study was to analyze whether T₂, like T₃, could regulate a physiological demand such as growth and whether this regulation was mediated by the L-TRβ1 isoform. We here show for the first time that treatment with T₂ effectively stimulates growth in tilapia fingerlings in the same manner as observed for T₃. Therefore, and although the effects of THs on growth have been solely attributed to T₃ (Power *et al.* 2001), our present results clearly show that T₂ is also a physiologically active player in this process, at least in tilapia, validating fish growth as a useful marker to analyze the possible differential regulation of the TRβ1 isoforms that mediate T₃ and T₂ effects.

Results from both *in vivo* and *ex vivo* T₃ or T₂ treatments revealed an equivalent enhancement of IGF1 and blockade of D2 liver expression, showing that T₃ and T₂ do not differ in how they regulate the transcription

Figure 3

mRNA expression from organotypic liver cultures of IGF1 and D2. Tilapia organotypic liver cultures were treated for 24 h with 0.1–100 nM T₃ (top panel) or T₂ (bottom panel), or with 0.1–20 μM of 1–850 without or with 100 nM T₃ (top panel) or T₂ (bottom panel, $n=6$ slices per experimental group). Values are means $\pm$ s.e.m. of three independent experiments. Significance is indicated (* $P<0.01$ and ** $P<0.001$).

of these two genes. By contrast, results regarding the regulation of the TR β 1 gene by T₃ and T₂ under the two experimental approaches were different. In previous studies, in which tilapia were acutely treated with either T₃ or T₂, the classical downregulatory response of the liver S-TR β 1 and L-TR β 1 mRNAs respectively was observed (Mendoza *et al.* 2013); this effect was also seen here in the organotypic liver cultures (Fig. 4) and was previously reported for the TR β 1 gene in other vertebrates treated with T₃ (Sadow *et al.* 2003). Interestingly, however, in the present long-term (1 month) *in vivo* studies, the expression of mRNAs for both the L-TR β 1 and S-TR β 1 was upregulated by T₂ and T₃ treatment respectively (Fig. 2). This regulatory pattern resembles that of tadpoles (Sachs *et al.* 2000, Tata 2000) and flatfish (Yamano & Miwa 1998, Marchand *et al.* 2004, Galay-Burgos *et al.* 2008), in which a pre-metamorphic rise in circulating THs is followed by an increase in the expression of TR β 1. The auto-induction of TR and other nuclear receptors is linked to developmental actions and seems to be a prerequisite for a sequential activation of target genes involved in a specific biological action (Tata 2002). As there were no age or environmental distinctions between tilapia in previous study (Mendoza *et al.* 2013) and this study, it is reasonable to suggest that the observed differences in TR β 1 regulation are mainly due to the administration protocols. Thus, we hypothesize that the intermittent administration protocol used in the present set of experiments partially mimics the pulsatile physiological stimulus of THs and increases the number of receptors, which in turn activate growth-related target genes to stimulate body growth, an event that, like metamorphosis, involves proliferative effects.

We had previously shown that MMI treatment induced the classic upregulation of both hepatic TR β 1 isoforms in juvenile tilapia (Mendoza *et al.* 2013). Surprisingly, the partial blockade of TH synthesis which results from the intermittent MMI administration protocol elicited the opposite regulatory effect, despite the fact that intrahepatic T₃ concentrations were below euthyroid levels in both experiments. Interestingly, others have reported a TR β , but not TR α , downregulation after up to 15 days of exposure to the goitrogen thiourea (400 μM) in metamorphosing *Solea senegalensis* larvae (Manchado *et al.* 2009). The fact that both THs and MMI intermittent treatments regulated the TR β gene in a manner similar to that observed in metamorphosing fish or amphibian is intriguing, especially as none of the other studied genes elicited such a regulatory response. The underlying mechanisms for this opposite regulation

Figure 4

mRNA expression from organotypic liver cultures of short TRβ1 and long TRβ1. Organotypic tilapia liver cultures were treated for 24 h with 0.1–100 nM T_3 (top panel) or T_2 (bottom panel) or with 0.1–20 μM, 1–850 without or with 100 nM T_3 (top panel) or T_2 (bottom panels; $n=6$ slices per experimental group). Values are means $\pm$ s.e.m. of two independent experiments. Significance is indicated (* $P<0.05$ and ** $P<0.001$).

are not known, and further studies would be needed to clarify this issue.

To further support the notion that the actions of T_2 upon body weight gain were indeed mediated by the L-TRβ1 isoform, we treated organotypic liver cultures with the TR antagonist 1–850. This antagonist has been reported to bind with high affinity to both TRα and TRβ in mammalian systems (Schapira *et al.* 2003). In these studies, 1–850 treatment effectively blocked transcription of a positively regulated gene (IGF1); however, it either enhanced (D2) or had no effect (TRβ1s) on transcription of negatively regulated genes. A similar lack of effect on TRβ1 expression by the TH antagonist NH3 had previously been observed in *Xenopus* TRβA (Opitz *et al.* 2006). However, the different response patterns of the two negatively regulated genes are intriguing. Several factors may contribute to this response, i.e. unliganded TRs have ligand-independent actions; thus, unliganded- or 1–850-liganded TRs may adopt altered conformations, which could change coregulator recruitment and specific gene expression independently of the effects on TH binding (Shah *et al.* 2008). Nonetheless, in all cases, the effects of 1–850 were blunted after treatment with T_3 or T_2 . Importantly, T_3 reversed the effects of 1–850 only upon the expression of the S-TRβ1 isoform and T_2 had similar effects only upon that of the L-TRβ1 isoform, further demonstrating that this L-TRβ1 isoform selectively mediates T_2 actions.

Aside from its well-described extranuclear effects upon metabolism in mammals (Goglia 2005), the physiological relevance of T_2 has been unnoticed until recently, and T_3 has been accepted as the foremost TH capable of genomic action. A major finding in this study consists in the demonstration that, through interacting with a specific and distinct TRβ1 isoform, T_2 effectively triggers an outburst of body growth in tilapia fingerlings, with an efficiency comparable to that of T_3 . These results confirm and extend previous reports from our laboratory and others suggesting that T_2 is also an important transcriptional regulator, at least in teleosts (García-G *et al.* 2004, 2007, Little *et al.* 2013, Mendoza *et al.* 2013). The lack of available data on circulating or intratissular T_2 concentrations has masked the importance of this TH. However, very recent studies have shown that zebrafish intramuscular T_2 concentrations closely resemble those of T_3 (Little *et al.* 2013). These data together with our previous findings that T_2 preferentially binds to and activates the L-TRβ1, an isoform that is expressed *in vivo* at 10⁶-fold higher levels than S-TRβ1, support the notion that, at least in teleosts, T_2 is physiologically relevant

(Mendoza *et al.* 2013). As previously described for T₃ and the *THRA* gene in HepG2 cells (Timmer *et al.* 2003), the present results are also consistent with the notion that T₃ and T₂ can differentially regulate the alternative splicing of the TRβ1 pre-mRNA in tilapia (Mendoza *et al.* 2013). The other genes analyzed here are regulated in the same manner by T₃ and T₂ in this species; however, the fact that the two TRβ1 isoforms are differentially regulated by these two ligands raises the possibility that additional genes may also be differentially regulated, a suggestion that warrants further attention.

Declaration of interest

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