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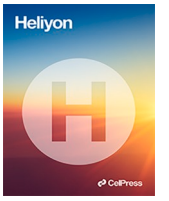

Research article

L-Thyroxine and L-thyroxine-based antimicrobials against Streptococcus pneumoniae and other Gram-positive bacteria

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ABSTRACT

Objectives: The rise of antibiotic-resistant *Streptococcus pneumoniae* (*Sp*) poses a significant global health threat, urging the quest for novel antimicrobial solutions. We have discovered that the human hormone L-thyroxine has antibacterial properties. In order to explore its drugability we perform here the characterization of a series of L-thyroxine analogues and describe the structural determinants influencing their antibacterial efficacy.

Method: We performed a high-throughput screening of a library of compounds approved for use in humans, complemented with ITC assays on purified *Sp*-flavodoxin, to pinpoint molecules binding to this protein. Antimicrobial *in vitro* susceptibility assays of the hit compound (L-thyroxine) as well as of 13 L-thyroxine analogues were done against a panel of Gram-positive and Gram-negative bacteria. Toxicity of compounds on HepG2 cells was also assessed. A combined structure-activity and computational docking analysis was carried out to uncover functional groups crucial for the antimicrobial potency of these compounds.

Results: Human L-thyroxine binds to *Sp*-flavodoxin, forming a 1:1 complex of low micromolar K_d. While L-thyroxine specifically inhibited *Sp* growth, some derivatives displayed activity against other Gram-positive bacteria like *Staphylococcus aureus* and *Enterococcus faecalis*, while remaining inactive against Gram-negative pathogens. Neither L-thyroxine nor some selected derivatives exhibited toxicity to HepG2 cells.

Conclusions: L-thyroxine derivatives targeting bacterial flavodoxins represent a new and promising class of antimicrobials.

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1. Introduction

Streptococcus pneumoniae (*Sp*) is a Gram-positive pathogenic bacterium. While part of the normal upper respiratory tract microbiota, *Sp* is also the major cause of community acquired pneumonia and meningitis in children and the elderly [1] and several other diseases such as otitis media, sinusitis or conjunctivitis. In some cases, *Sp* infections can lead to bacteraemia and sepsis [2,3]. Widespread vaccination and antimicrobial treatments are effective ways of controlling *Sp* infections [4]. However, it is estimated that 30% of *Sp* infections in the US are caused by drug resistant pneumococci, and, according to the European Centre for Disease Prevention and Control (ECDC), "antibiotic resistance by *Sp* is an increasing problem across Europe and susceptibility to macrolide antimicrobials, penicillins and cephalosporins can no longer be assumed in many countries" [5]. Antimicrobial resistance, together with the need to reduce the use of broad-spectrum antibiotics (hence less noxious to microbiota) [6,7], calls for the development of new, narrower-spectrum antimicrobials to be added to the therapeutic arsenal of drugs available to combat *Sp* infections.

One way towards discovering novel active molecules is target-oriented high-throughput screening (HTS) of chemical libraries, which some of us have used as the starting point to discover [8–10] narrow-spectrum antimicrobials against, for example, the Gram-negative *Helicobacter pylori* (*Hp*). For that, an essential *Hp* protein, flavodoxin (Fld), was purified and used to identify binding inhibitory hit compounds that were further developed into lead compounds. *Sp* contains a gene encoding a flavodoxin that has been described as an essential protein for *Sp* replication during experimental meningitis in mice [11]. In a first step to identify novel anti-*Sp* antimicrobials, the gene for *Sp*-Fld from strain TIGR4 was cloned and expressed in *E. coli*. Then, the X-ray structure of the active holoprotein was solved, and its folding and FMN binding equilibria were characterized [12]. *Sp*-Fld is a short-chain 146-residue flavodoxin that carries a non-covalently bound FMN cofactor to participate in electron transfer reactions. Flavodoxin has diverse roles in bacterial metabolism, which vary among bacterial species [13,14]. Identification of *Sp* genes essential for replication [11] and functional relationships have linked *Sp*-Fld to methionine synthesis, nucleotide synthesis, peroxide detoxification and fluorine export [15], among other pathways. Nevertheless, further work is needed to elucidate the specific functions of flavodoxin in bacterial physiology.

In this work, we have performed HTS assays on a chemical library of approved drugs and we have found that L-thyroxine binds to *Sp*-Fld. Subsequently, we have investigated the antimicrobial activity of L-thyroxine and a series of L-thyroxine derivatives and describe here their antimicrobial spectrum. Based on *in silico* docking studies and simple structure-activity relationship analyses, we have identified the structural determinants in L-thyroxine that are critical for anti-*Sp* activity. Previously reported associations of L-thyroxine with *Sp* infection, which support further exploration of L-thyroxine-based antimicrobial inhibitors of *Sp*-Fld against *Sp* infections, are briefly discussed.

2. Materials and Methods

2.1. Chemicals

L-thyroxine and 13 commercially available, structurally related compounds studied in this work are listed in Table 1. L-thyroxine (Cp1) was purchased from Merck (≥98% (HPLC)], whereas the structural analogues were obtained from Cymit Química.

2.2. Protein purification

Sp-Fld was expressed in *E. coli* BL21 (DE3) cells and purified as described [12]. The details are given in the **SI Material and Methods**.

2.3. Bacterial strains and MIC determinations

Sources and detailed culture conditions of the bacterial strains tested in this work are described in the **SI Material and Methods**. The minimum inhibitory concentration (MIC) of the compounds was determined through the broth microdilution (BMD) method (the details also appear described in the **SI Material and Methods**).

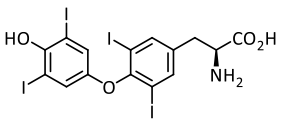
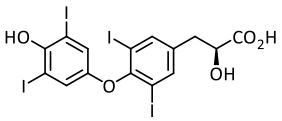
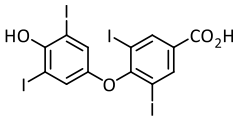
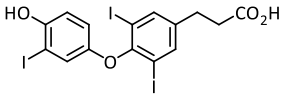
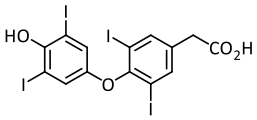
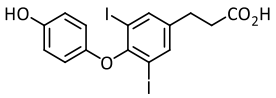
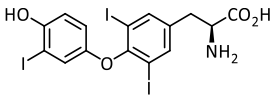
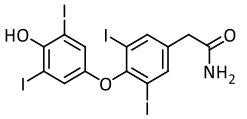
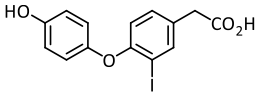
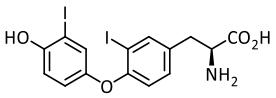
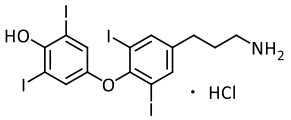
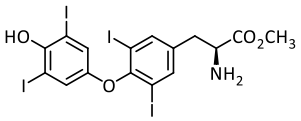
2.3.1. Toxicity assays

In vitro toxicity was evaluated using the resazurin assay to assess the cytotoxic effects of varying concentrations of **Cp1** (L-thyroxine), **Cp5** (3,5,3′,5′-tetraiodo thyroacetic acid), and **Cp4** (3,3′,5-triiodo thyropropionic acid) (ranging from 1000 μM to 64 μM) on HepG2 cells. The details of these assays are provided in the **SI Material and Methods**.

2.4. High-throughput screening

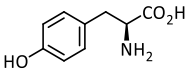
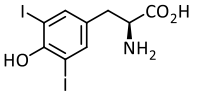
A Prestwick Chemical Library® encompassing 1240 FDA- and EMA-approved drugs dissolved in 100% DMSO at 10 mM was used for HTS assays designed to identify *Sp*-Fld binding compounds. The screening of the Prestwick library against *Sp*-Fld was done in 96-well plates, each containing 100 μL of 1 μM *Sp*-Fld with either 2.5 μL of compound or 2.5 μL of DMSO used as a control. The heating was done at 1 °C/min. The T_m of the protein in each well was determined using own scripts that have been described previously [12]. In our assay, tested wells were considered to contain a potential flavodoxin binding compound if the T_m recorded for the protein was higher

Table 1
Compounds used in this study to investigate growth inhibition of *Sp*.

Compound	Formula	CAS (PubChem CID)	Name	Net charge
Cp1		51-48-9 (5819)	L-thyroxine (3,3′,5,5′-Tetraiodo-L-thyronine)	0
Cp2		7069-47-8 (13120916)	(S)-3,5,3′,5′-tetraiodo thyrolactic acid	−1
Cp3		2055-97-2 (6451352)	3,3′,5,5′-tetraiodo thyroformic acid	−1
Cp4		51-26-3 (5804)	3,3′,5-triiodo thyropropionic acid	−1
Cp5		67-30-1 (65552)	3,5,3′,5′-tetraiodo thyroacetic acid	−1
Cp6		1158-10-7 (160565)	3,5-diiodo thyropropionic acid	−1
Cp7		6893-02-3 (5920)	3,3′,5-triiodo-L-thyronine	0
Cp8		176258-88-1 (71587580)	3,5,3′,5′-tetraiodo thyroacetamide	0
Cp9		60578-17-8 (91582103)	3-iodo thyroacetic acid	−1
Cp10		4604-41-5 (107564)	3,3′-diiodo L-thyronine	0
Cp11		788824-71-5 (not available)	3,3′,5,5′-tetraiodothyronamine hydrochloride	+1
Cp12		32180-11-3 (13911218)	thyroxine methyl ester	+1

(*continued on next page*)

Table 1 (*continued*)

Compound	Formula	CAS (PubChem CID)	Name	Net charge
Cp13		60-18-4 (6057)	L-tyrosine	0
Cp14		300-39-0 (9305)	3,5-diiodo-L-tyrosine	0

than that in drug-free control wells (12 control wells per plate) by at least twice the standard deviation ($\Delta T_m \geq 2 \times SD$) [16]. Additional details are given in the **SI Material and Methods**.

2.5. Isothermal titration calorimetry

The binding to *Sp*-Fld of the hits identified by HTS was tested using isothermal titration calorimetry (ITC) as a target engagement assay. The dissociation constant K_d was calculated as the inverse of the association constant (K_a), which was estimated along with the enthalpy of binding, ΔH_a from non-linear least squares regression of the experimental data (see additional details in the **SI Material and Methods**). Then, the Gibbs energy and the entropy of interaction were calculated using the basic relationships: $\Delta G_a = -RT \ln K_a = \Delta H_a - T\Delta S_a$.

2.6. Molecular docking

The *Sp*-Fld structure at 2.07 Å resolution (Protein Data Bank entry 5LJI) [12] was used in protein-ligand docking after some adjustment (see details in the **SI Material and Methods**). The three-dimensional structure of the modeled compounds are given in Table 1. Total charges and ionization states of the compounds were assumed to be those under solvating conditions of pH 7 (Table 1). Thus, the carboxylate and amine groups were settled to local negative (−1) and positive (+1) charges, respectively. The binding site prediction tools Fpocket [17] and P2Rank [18] (no docking) were run on the *Sp*-Fld structure to identify the most reliable binding sites of this protein. AutoDock Vina [19] and Ledock [20] tools (protein-ligand docking) were used to model the putative interaction between each compound and *Sp*-Fld. Additional information is provided in the **SI Material and Methods**.

3. Results and discussion

3.1. Discovery of binders to Sp-Fld and ITC validation

Initial screening of the Prestwick library identified 16 wells in which T_m for the thermal unfolding of *Sp*-Fld was higher than that in the control wells without any compound. The 16 compounds present in these wells were retested at a protein concentration of 2 μM, and only three of them increased the T_m by at least twice the standard deviation of the control T_m of 37.3 °C. The interaction of the three compounds (cefotaxime, cefixime and L-thyroxine) with *Sp*-Fld was then further tested by ITC. While cefotaxime and cefixime (Fig. 1a and **c**) did not produce a heat exchange pattern indicative of binding to the target protein, *Sp*-Fld titration with L-thyroxine clearly showed the binding of the hormone to the protein (Fig. 1b).

The fitting of the L-thyroxine thermogram indicated that, at 25 °C, L-thyroxine and *Sp*-Fld form a complex of a 1:1 stoichiometry ($n = 0.98 \pm 0.04$) that is mainly stabilized by the entropy change of binding ($\Delta G_a = -7.6 \pm 0{,}1$ kcal/mol; $\Delta H_a = -0.9 \pm 0.4$ kcal/mol; $-T \times \Delta S_a = -8.5 \pm 0.5$ kcal/mol), with a dissociation constant K_d of 2.5 ± 0.4 μM for the complex. Given the clear binding of L-thyroxine to *Sp*-Fld, 13 chemical analogues of the hormone (Table 1) were tested for their antimicrobial activity against *Sp* and a panel of Gram-positive and Gram-negative bacteria.

Antimicrobial activity of L-thyroxine and L-thyroxine derivatives against *Sp* and other Gram-positive and Gram-negative bacteria.

The MIC of L-thyroxine (**Cp1**) and 13 chemical analogues against *Sp* was determined using the broth microdilution (BMD) method [21] (Fig. 2a). According to their MICs, the tested compounds could be divided into four groups (Fig. 2b). The most active group, with MIC values of 64 μg/mL, included L-thyroxine (**Cp1**) and four derivatives (**Cp2**, **Cp3**, **Cp4**, **Cp5**), all of which have a carboxyl group at one end of the molecule, linked to the inner phenyl ring either directly or via one or two intervening carbon atoms. The four analogues in this group retain the two iodine atoms on the internal phenyl ring of L-thyroxine and at least one additional iodine atom on the distal phenyl ring. A second group with MIC values of 128 μg/mL was formed by two analogues (**Cp6**, **Cp7**) lacking iodine atoms on the distal ring. Two compounds (**Cp9**, **Cp10**) lacking one of the two iodine atoms on the internal L-thyroxine ring formed a third group with MIC values of 256 μg/mL. A fourth group with no or very low anti-*Sp* activity (MIC >512 μg/mL) consisted of three compounds (**Cp8**, **Cp11**, **Cp12**) lacking a carboxyl group and two compounds (**Cp13**, **Cp14**) lacking the distal phenyl ring.

To investigate the activity spectra of selected compounds, the most active inhibitors of *Sp* MIC group 1 and MIC group 2, with the exception of (S)-3,5,3′,5′-tetraiodo thyroformic acid (**Cp2**) and 3,5-diiodo thyropropionic acid (**Cp6**), were tested against two additional Gram-positive (*S. aureus* N305 and *E. faecalis* FA2-2) and three Gram-negative pathogenic bacterial species (*K. pneumoniae* 3025, *P. aeruginosa* MPAO1, and *S. maltophilia* K279a). It turned out that the lead compound, L-thyroxine (**Cp1**), was specifically active

against *Sp*, but did not inhibit growth of any of the other bacterial species tested (data not shown). Overall, the activity of the compounds against the three Gram-negative bacteria was low. None of the compounds showed any effect against *K. pneumoniae* 3025 at the highest concentration of 512 μg/mL, whereas compounds in group 1 resulted in some growth inhibitory effects against *P. aeruginosa* MPAO1 and *S. maltophilia* K279a in the concentration range between 256 μg/mL and 512 μg/mL (data not shown). Despite the lack of any activity of L-thyroxine (**Cp1**) on the growth of *S. aureus* and *E. faecalis*, the activity of the other compounds tested against these Gram-positive bacteria was higher than their activity against the Gram-negative species, which is consistent with the higher permeability barrier of the outer membrane of Gram-negative bacterial species. The most active compounds against the Gram-positive bacteria were 3,3′,5-triiodo thyropropionic acid (**Cp4**) and 3,5,3′,5′-tetraiodo thyroacetic acid (**Cp5**) (Fig. 3), showing a MIC of 32 μg/mL for *S. aureus,* which was lower than that for *Sp*.

3.2. Toxicity

Using HepG2 cells, the cytotoxicity of L-thyroxine (**Cp1**) and two of the most active L-thyroxine derivatives against *Sp* and the other Gram-positive bacteria, i.e., 3,3,5-triiodo thyropropionic acid (**Cp4**) and 3,5,3′,5′-tetraiodo thyroacetic acid (**Cp5**), was analyzed as described in **Materials and Methods**. None of the three compounds was toxic for these cells at concentrations of 500 μM (from 318 to 388 μg/mL) or lower (Fig. S1). At 1 mM (777 μg/mL), however, L-thyroxine (**Cp1**) was moderately cytotoxic, decreasing cell viability to 70 %. Notably, compound **Cp5** strongly increased cell viability in the concentration range between 0 and 500 μM (374 μg/mL) before it became cytotoxic at a higher concentration of 1 mM (748 μg/mL). The effect of compound **Cp4** was remarkable as it linearly increased the viability of HepG2 cells from 100% to 180% when its concentration was increased from 0 to 1 mM (636 μg/mL) without showing a cytotoxic effect.

3.3. Structure-activity relationships and the role of some substituents

Given the limited number of analogues tested, simple structure-activity analyses (qualitative and quantitative) were performed to determine relevant properties such as the effect of substituents on the aromatic rings, their size, charge, hydrophobicity, flexibility, surface area, and hydrogen bonds associated with the different anti-bacterial activity profiles of the compounds. Qualitatively, the presence of a carboxylic moiety (whether formic, acetic, or propionic acid) appears to be essential for activity against the Gram-positive bacteria tested including *Sp*. A carboxylic acid is indeed present in all active compounds, but absent in three of the non-active compounds (**Cp8**, **Cp11**, and **Cp12** of group 4). The two other non-active compounds (**Cp13**, **Cp14**) have a carboxylic moiety but lack one of the two phenyl rings that are present in the lead compound (**Cp1**), which is probably the cause of their lack of anti-*Sp* activity. This indicates that two phenyl rings are important for antibacterial activity. In addition, the docking study performed (see below) indicates that the interaction energies with *Sp*-Fld of the two smaller inactive compounds, **Cp13** and **Cp14** lacking one of the rings, are the lowest (absolute) values among all compounds studied. In contrast, the presence of the $-NH_2$ group in L-thyroxine (**Cp1**) or an analogous group appears to be of little relevance for the antibacterial activity (compare, for example, the activities of **Cp4** and **Cp7**). As for the number of iodine atoms in these compounds, pairwise activity comparisons (i.e., **Cp1**/**Cp7**, **Cp4**/**Cp6**, **Cp5**/**Cp9**) show that at constant scaffold, a gradual decrease in the number of iodine atoms decreases antibacterial activity.

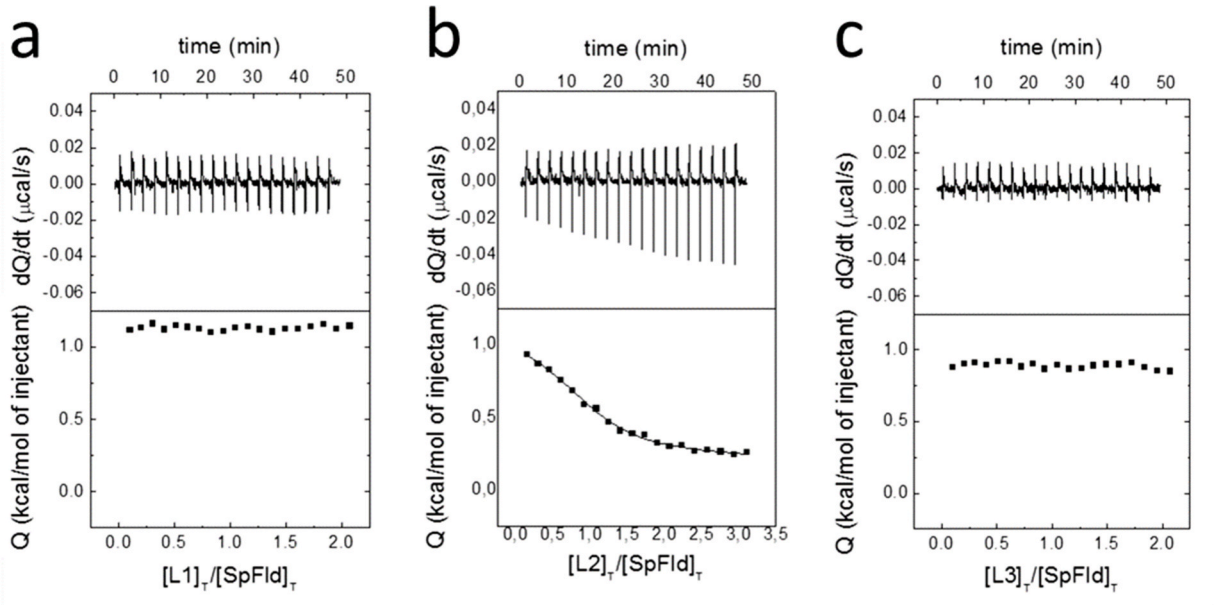


Fig. 1. ITC of *Sp*-Fld with three potential binders identified in a HTS assay: **a)** cefotaxime, **b)** L-thyroxine and **c)** cefixime.

On the other hand, some quantitative linear correlations were found between classic molecular descriptors of the compounds and their inhibitory activity on *Sp* growth. Some descriptors (e.g., the molecular weight (MW), the molecular volume (Vx) or the total surface area (SAtot), which are themselves highly correlated) show the best correlation with antibacterial activity (Fig. S2) when the three compounds lacking a carboxylate (**Cp8**, **Cp11**, **Cp12**) are considered as outliers. This suggests that size and associated properties have an impact on antibacterial activity, which is not surprising given the observed importance of the high atomic mass iodine atoms for antimicrobial activity. The only descriptor that did not correlate with the above descriptors but clearly correlated with antimicrobial activity is MLogP, the Moriguchi's octanol/water partition coefficient [22], suggesting that increasing lipophilicity increases activity of the compounds.

3.4. Predicted binding sites and relevant amino acids in the interaction

The series of compounds analyzed here includes L-thyroxine (**Cp1**) and a set of 13 analogues derived from it (**Cp2**-**Cp14**; Table 1). We performed receptor-based modelling using binding site predictors plus molecular docking aimed at predicting the putative binding sites and the interactions occurring between these ligands and flavodoxin. Fpocket [17] predicted six binding sites (Fig. S3), whose parameters and integrating amino acid residues are summarised in Table S1. The best-ranked binding sites according to Fpocket are those with the highest score (third column in Table S1). Thus, pockets F-Po-1, F-Po-2 and F-Po-3 are those that have the highest

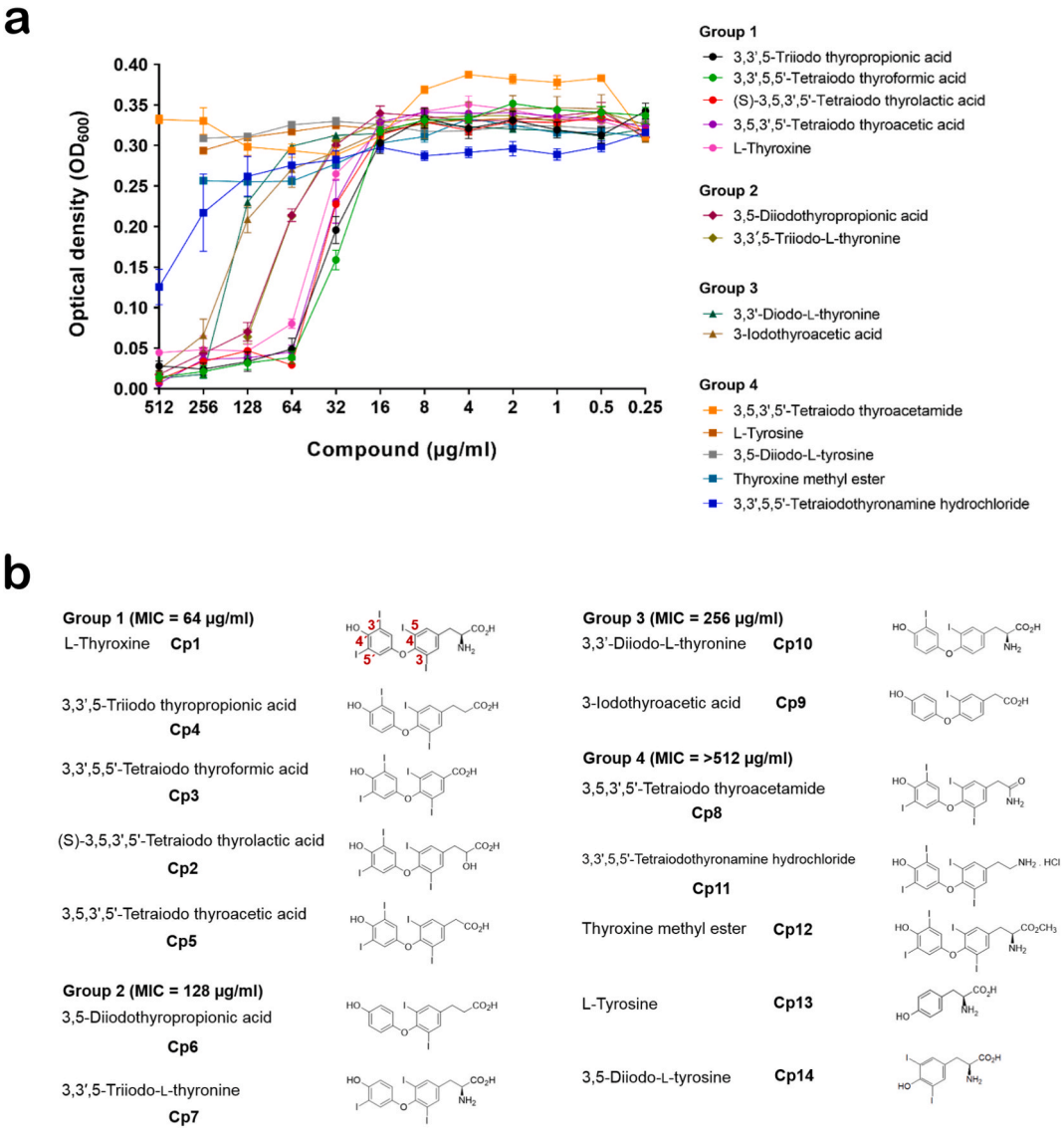


Fig. 2. Antimicrobial activity of L-thyroxine and 13 chemical analogues against *Sp*. **a)** Growth inhibition curves. **b)** MICs and structures of the tested compounds.

likelihood of binding these chemical compounds. Two binding sites were predicted by the P2Rank [18] predictor. The highest scoring one (here identified as P2R-Po-1, score = 5.93, probability = 0.29) includes residues S10, M11, T12, N14, Y57, T58, Y59, G60, S90, G91, D92, Y95, E97, and L125. Thus, P2R-Po-1 partially shares the binding pocket of the flavodoxin FMN cofactor. The second (P2R-Po-2, score = 1.07, probability <0.01) includes residues K5, D34, D36, T40, V41, D45, located between beta strands 1 and 2 (β1 and β2) and the short helix 2 (α2) (Fig. S3d).

Prediction of the most reliable binding sites of *Sp*-Fld was also performed by "blind" docking on the entire holoprotein using both AutoDock Vina [19] and LeDock [20]. The most frequent binding sites for the compounds corresponded to the three most reliable binding regions predicted by Fpocket, i.e., F-Po-1, F-Po-2 and F-Po-3 (not shown). The remaining binding sites predicted by Fpocket, as well as P2Rank-predicted P2R-Po-2, were much less represented in terms of the number of poses docked.

Altogether, this information prompted us to conduct more precise local docking by exploring exclusively the protein pockets F-Po-1, F-Po-2 and F-Po-3. The binding energy of the best-ranked poses (the more negative ones, see Tables S2–S4) obtained with AutoDock Vina (see Fig. 4 a–d and Fig. S4) and LeDock were correlated (Fig. S5) with the experimental MIC data (Fig. 2b). Only the poses obtained with AutoDock Vina on pockets F-Po-2 and F-Po-3 showed substantial correlation ($R^2 = 0.43$ and $R^2 = 0.32$, respectively, Fig. S5). For all compounds, poses in pocket F-Po-3 showed comparatively better affinities than those docked in F-Po-1 and F-Po-2 regardless the docking program used. This analysis indicates pocket F-Po-3 as the putative site where these compounds bind *Sp*-Fld, consistent with the highest number of docked poses obtained in the original "blind" docking to F-Po-3 (not shown).

Qualitatively, the best poses obtained with AutoDock Vina for compounds **Cp1** to **Cp14** on pocket F-Po-3 reveal that the most recurrent amino acid residues interacting with the compounds are V121, R134, K122 and C120 (Fig. 4 a–d). Val121 is forming part of the compound-protein interaction in all the best poses except for compounds **Cp9** and **Cp14**. Most of the interactions where Val121 participates involve one iodine atom in position 3 (meta) of the first aromatic ring (**Cp1** to **Cp5**, **Cp7**, **Cp8**, **Cp11**, and **Cp12**), which appears mostly buried, sometimes also interacting with Ser 118 (halogen bond donor in **Cp1**, **Cp2**, and **Cp12**, Fig. 4 a–d and Fig. S4). Arg 134 and Lys 122 interact mostly via the negatively charged carboxylate group present in the main substituent of the compounds (**Cp1** to **Cp5**, **Cp9**, **Cp12** to **Cp14**) by donating a hydrogen bond to it. Cys 120, is observed to receive a hydrogen bond from either the positively charged amino group of the main substituent (**Cp1**) or a hydroxyl group that substitute it in a compound (**Cp2**, **Cp11**, **Cp12**, and **Cp14**). Another element to consider here is the favourable interaction between the aromatic rings of the compounds and *Sp*-Fld via hydrophobic contacts (Fig. S4). This is indicated by the models when rings without substituents are present in positions 3, 3′, 5, and 5′ (e.g. in **Cp6** and **Cp13**) but also when only one of these positions has a substituent directed towards the solvent (e.g. in **Cp10**).

3.5. *L-thyroxine as the lead of a new family of antimicrobials against Sp*

Although the gene encoding *Sp*-Fld is not listed as essential in the Database of Essential Genes (DEG) for *Sp* [23], genes encoding the flavodoxins from *S. agalactiae A909* and *S. mutans UA159*, sharing 74 and 78 % sequence identity with *Sp*-Fld, respectively, were reported as essential in the DEG database. Therefore, we screened a chemical library of mainly FDA-approved drugs for compounds binding to *Sp*-Fld and inhibiting *Sp* growth, and identified L-thyroxine as a potential lead compound. L-thyroxine has been shown to bind to *Sp*-Fld by forming a complex with a stoichiometry of 1:1, the thermodynamic properties of which have been determined. The binding affinity is in the low micromolar range, and the interaction is entropically-driven. *In silico* docking analyses suggest, that the binding site is located in the C-terminal part of the protein, with the major interacting residues belonging to strand β5 and helix h5. L-thyroxine inhibits the growth of *Sp* with a MIC of 64 μg/mL. Thirteen commercially available compounds structurally related to L-thyroxine were also tested for their inhibitory effects on *Sp*. Structure-activity analysis indicated that the presence of a carboxylic group at one end of the molecule is required for the antibacterial activity of these compounds. The carboxylic group can be connected to the adjacent phenyl ring either directly or separated by one or two methylene groups. In contrast, the amine group of L-thyroxine does not appear to be important for antibacterial activity. Keeping the two phenyl rings, however, also appears to be important, as this keeps most of the iodine atoms in place. Docking analysis is consistent with these results and points to a greater importance of the

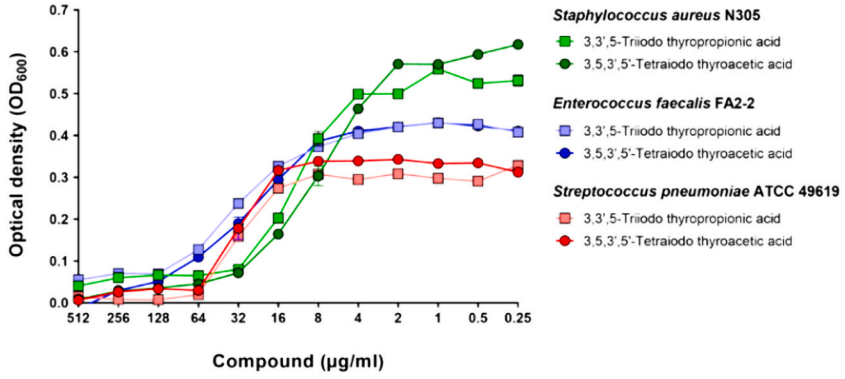
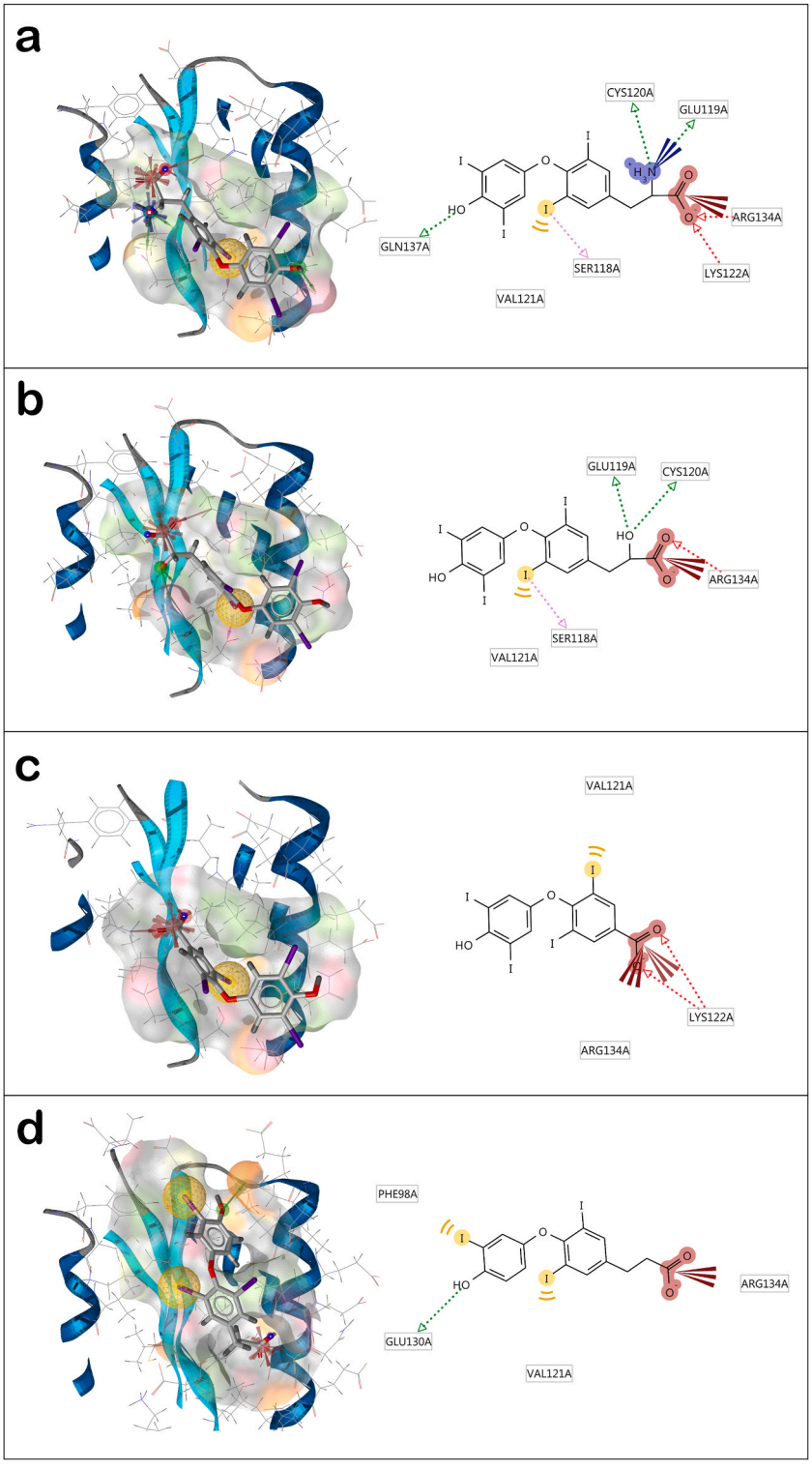


Fig. 3. Antimicrobial activity of 3,3′,5-triiodo thyropropionic acid (**Cp4**) and 3,5,3′,5′-tetraiodo thyroacetic acid (**Cp5**) against *Sp*, *S. aureus N305*, and *E. faecalis FA2-2*.



(caption on next page)

Fig. 4. Best ranked poses obtained with Autodock Vina on pocket F-Po-3 for the four most active compounds (lower MIC values). **a)** Compound Cp1, **b)** Cp2, **c)** Cp3, **d)** Cp4. At the left side of each panel the best pose (pose 1 in Table S4) is shown (sticks) in its binding site (semi-transparent surface coloured by atom binding affinity). The ligand environment is shown in the form of cartoons (secondary structure) and lines (residues of the side chain). The main ligand-protein interactions are shown in the same way as in the representation on the right side of each panel (red dashed arrows: hydrogen bond acceptor; green dashed arrows: hydrogen bond donor; magenta dashed arrows: halogen bond donor; yellow spheres: hydrophobic contact; red trident: negative ionizable group; blue trident: positive ionizable group). Interacting amino acid residues are indicated close to their partners in grey border boxes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

iodine atoms of the carboxyl-containing ring. Some of the L-thyroxine derivatives tested are also effective against other Gram-positive bacteria of the genus *Streptococcus*, which also have an essential flavodoxin. The cytotoxicity of this family of compounds is quite low. Interestingly, compound **Cp4**, 3,3′,5-triiodo thyropropionic acid, which has a slightly higher activity than L-thyroxine, and a lower molecular weight, shows no cytotoxicity at the high concentration of 1 mM (636 μg/mL), but rather a strong protective effect on HepG2 cells. While the available antibacterial and toxicity data suggest that these compounds have a useful therapeutic window, their activity should be further improved.

There are several previous reports of markedly altered host metabolism and significantly decreased serum thyroid hormone levels during *Sp* infection in rhesus monkeys and rats [24–27]. More recent studies of the mechanisms leading to changes in the metabolism of thyroid hormones in mice and zebrafish embryos have shown that reduced serum levels of thyroxine and 3,3′,5-triiodothyronine during acute *Sp* infections are most likely associated with significant changes in the expression of iodothyronine deiodinases [28,29], which play an important role in regulating thyroid hormone homeostasis by deiodinating the inactive prohormone L-thyroxine into the biologically active 3,3′,5-triiodothyronine. While deficiency of type 2 deionidase (D2) has been demonstrated to lead to impaired *Sp* clearance *in vivo* and significantly increased mortality in zebrafish embryos, this effect was cured by the addition of 3,3′,5-triiodothyronine, the reaction product of D2 [29]. Nutter et al. observed enhanced resistance of mice to pneumococci as well as *Mycobacterium tuberculosis* upon treatment with 3,3′,5-triiodothyronine [30]. Already in 1946, Izzo and Ricardo reported that thyroidectomized guinea pigs were more susceptible to experimental *M. tuberculosis* infection, whereas those treated with thyroxine showed a prolonged survival time [31]. There is also recent evidence that decreased L-thyroxine production increases the risk of developing tuberculosis [32,33]. The direct antimicrobial effect of L-thyroxine and derivatives of L-thyroxine on bacteria of the genus *Streptococcus* reported here for the first time represent an additional biological activity of this hormone that deserves attention in future studies of thyroid function and infectious diseases caused by pathogenic streptococci, including community acquired pneumonia. The growth-inhibiting effects on *Sp* are in good agreement with earlier findings on the thyroid hormone-mediated growth inhibition of other, but exclusively Gram-positive bacteria such as *Bacillus cereus*, *B. licheniformis*, or *Staphylococcus epidermidis*. However, we are aware that the antibacterial MIC values determined in this study are far above the physiological thyroid hormone concentrations in the human body, with the risk of causing hyperthyroidism and serious associated health problems. Nevertheless, the hormonal potency of L-thyroxine analogues may be well below that of L-thyroxine. This is at least the case of 3,3′,5-triiodo thyropropionic acid (**Cp4**) and 3,5,3′,5′-tetraiodo thyroacetic acid (**Cp5**), which were reported to have a potency of 5 % or less than that of L-thyroxine [34]. Further studies to develop thyroid hormones as antibacterial agents against *Sp* will have to attenuate their hormonal effects while improving their antibacterial activity. One such strategy has been recently described for the development of an antibacterial agent against *Hp* based on the steroid hormone progesterone as a lead structure.

4. Conclusions

The human hormone L-thyroxine interacts with the flavodoxin of *S. pneumoniae,* an essential protein for *Sp* replication during experimental meningitis in mice. L-thyroxine inhibits *Sp* growth at concentrations at which it is not cytotoxic for HepG2 cells. Certain functional groups in L-thyroxine appear to be of greater importance for its antimicrobial activity. While L-thyroxine appears to be quite specific for *Sp*, some L-thyroxine analogues are also active against other Gram-positive bacteria. L-thyroxine and its analogues represent a new class of antimicrobials that, once improved, could make an important contribution to combat infections caused by drug-resistant *Sp* where broad-spectrum antibiotics fail.

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Ethical approval statement

The authors declare no ethical issues in this research.

Data availability statement

All data associated with this study is included in the article/Supp. Material.

CRediT authorship contribution statement

Juan José Galano-Frutos: Writing – original draft, Investigation. **Ritwik Maity:** Writing – original draft, Investigation. **Verónica Iguarbe:** Data curation. **José Antonio Aínsa:** Writing – review & editing, Supervision. **Adrián Velázquez-Campoy:** Writing – review & editing, Supervision, Investigation, Formal analysis, Conceptualization. **Ulrich E. Schaible:** Writing – review & editing, Supervision, Conceptualization. **Uwe Mamat:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation. **Javier Sancho:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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