2-Thiouracil is a selective inhibitor of neuronal nitric oxide synthase antagonising tetrahydrobiopterin-dependent enzyme activation and dimerisation

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Abstract 2-Thiouracil (TU), an established antithyroid drug and melanoma-seeker, was found to selectively inhibit neuronal nitric oxide synthase (nNOS) in a competitive manner ($K_i = 20$ μM), being inactive on the other NOS isoforms. The drug apparently interfered with the substrate- and tetrahydrobiopterin (BH_4)-binding to the enzyme. It caused a 60% inhibition of H_2O_2 production in the absence of L-arginine and BH_4, and antagonised BH_4-induced dimerisation of nNOS, but did not affect cytochrome *c* reduction. These results open new perspectives in the understanding of the antithyroid action of TU and provide a new lead structure for the development of selective nNOS inhibitors to elucidate the interdependence of the substrate and pteridine sites and to modulate pathologically aberrant NO formation.

Key words: nNOS inhibition; Thiouracil; Tetrahydrobiopterin

1. Introduction

Nitric oxide synthase (NOS) is a haem-containing enzyme that catalyses the NADPH-dependent formation of nitric oxide (nitrogen monoxide, NO) from L-arginine and O_2 [1–3]. NOS occurs in at least three distinct isoforms, a Ca^{2+}/calmodulin-dependent neuronal isoform (nNOS, type I NOS), a Ca^{2+}-independent, inducible isoform (iNOS, type II NOS), and a Ca^{2+}/calmodulin-dependent, endothelial isoform (eNOS, type III NOS). The physiological activation of these isoforms ensures supply of NO to fulfil a variety of signalling functions, e.g. in neurotransmission, macrophage-dependent immune response and vascular relaxation. However, overactivation of certain NOS isoforms has been implicated in several pathological states, including septic shock [4] and tissue damage secondary to inflammatory conditions [5]. The modulation of NO synthesis through selective inhibition of the various NOS isoforms is therefore a promising therapeutic strategy and a valuable means to define the relative involvement of these enzymes in biological systems [6]. The research and clinical utility of this approach is well apparent in the case of neurological conditions, especially ischaemia/reperfusion and vasospasm secondary to subarachnoid haemorrhage, where recognition of the pathophysiological role of nNOS has warranted considerable interest toward novel selective inhibitors of this isoform [7].

Functionally active nNOS is homodimeric and each subunit contains a reductase domain that binds NADPH, FAD and FMN, and an oxygenase domain, that binds haem, the substrate L-arginine and tetrahydrobiopterin (BH_4) [2,3]. Electron flow from the reductase domain to the haem in the oxygenase domain is triggered by the binding of calmodulin and results in L-arginine oxidation and NO synthesis. NOS can catalyse two additional NADPH-dependent reactions, namely reduction of cytochrome *c*, requiring only the reductase domain, and activation of molecular oxygen, leading to the production of superoxide and H_2O_2.

The role of BH_4 as cofactor is still not entirely elucidated, but seems to involve allosteric activation of the enzyme with promotion of L-arginine binding, shift of the haem from the low-spin to high-spin state and stabilisation of the homodimeric form [2,3,8,9]. The major outcome of these BH_4-induced changes is the coupling of NADPH oxidation to NO synthesis. Whether BH_4 controls this process by being chemically involved as a redox active agent is unclear [2], though recent evidence suggests that BH_4 is consumed during catalysis in nNOS [10] and may be involved in redox cycling processes [11].

The increasing interest in the role(s) of BH_4 as NOS cofactor has drawn considerable attention toward BH_4 antagonists as useful tools to probe the pteridine binding pocket and its allosteric interactions with the L-arginine binding site, as well as to control aberrant NO production [12–14]. We report herein that 2-thiouracil (TU), an established antithyroid drug [15] and an extensively investigated agent for the early detection and targeting of metastatic melanotic melanoma [16–18], is a highly selective inhibitor of nNOS which specifically antagonises BH_4-dependent enzyme activation.

2. Materials and methods

2.1. Materials

TU, uracil, thiourea were from Aldrich (Italy). Calmodulin, NADPH, FAD, FMN, BH_4 were from Sigma (Italy). Radiolabelled

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Abbreviations: TU, 2-thiouracil; NOS, nitric oxide synthase; nNOS, neuronal NOS; iNOS, inducible NOS; eNOS, endothelial NOS; BH_4, tetrahydrobiopterin

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L-[U-^{14}C]arginine monohydrochloride (317 mCi/mmol) was obtained from Amersham Italia (Italy). Recombinant rat nNOS and mouse iNOS were from Alexis Italia (Italy). Recombinant bovine eNOS was from Cayman (USA).

2.2. Enzyme preparation

Rat cerebellum extract was prepared as previously described [19].

2.3. NOS activity assays

2.3.1. L-[^{14}C]Citrulline formation. NOS activity was determined by monitoring the conversion of L-[U-^{14}C]arginine to L-[U-^{14}C]-citrulline. In the case of rat cerebellum extract, the reaction mixtures were prepared as previously described [20]. For nNOS, aliquots (2 μl, 0.18 μg protein) of commercial recombinant enzyme were incubated for 15 min at 37°C in 0.1 ml of 20 mM Tris–HCl, pH 7 containing 0.5 mM NADPH, 250 μM calcium chloride, 1 μM BH_4, 10 μg/ml calmodulin, 5 μM FAD, 5 μM FMN and 1.56 μM L-[U-^{14}C]arginine. In all cases, each reaction was stopped by the addition of 0.9 ml of ice-cold 100 mM HEPES buffer containing 4 mM EDTA, pH 5.5 and passed through 1 ml Dowex 50 (Na^+ form) column that retains arginine. [^{14}C]citrulline generated was eluted with water and quantified by liquid scintillation counting.

For inhibitor selectivity experiments toward NOS isoforms, comparable units of the three isoforms were assayed under the same experimental conditions.

Reversibility of NOS inhibition by TU was tested according to Garvey et al. [21]. Briefly, nNOS was preincubated at 4°C for 10 min in the presence of cofactors with or without 500 μM TU. The reaction was then started by the addition of L-arginine and portions of the mixture were removed at various intervals of time and assayed for NOS activity.

2.3.2. NO formation. In some experiments NOS activity was also assessed by measuring NO metabolites, nitrate and nitrite. Aliquots (3 μl, 2.7 μg protein) of commercial recombinant nNOS were incubated for 15 min at 37°C with 50 μl of 20 mM Tris–HCl, pH 7 containing 0.5 mM NADPH, 250 μM calcium chloride, 1 μM BH_4, 10 μg/ml calmodulin, 5 μM FAD, 5 μM FMN and 100 μM L-arginine in the presence or in the absence of 1 mM TU. NO formation was determined by quantitative analysis of nitrate/nitrite in the incubation medium with the NO colourimetric test from Boehringer Mannheim Italia, Italy.

2.3.3. H_2O_2 formation by nNOS. Hydrogen peroxide was determined by the ferrous thiocyanate assay [22].

2.3.4. Cytochrome c reductase activity of nNOS. NADPH-dependent reduction of cytochrome *c* in the absence of calcium and calmodulin was determined spectrophotometrically as previously described [23].

2.4. HPLC analysis

The stability of TU was determined by HPLC on a Gilson model 302 instrument, using a Sphereclone 5 μm ODS2 column (250×4.60 mm) (Phenomenex Chemtek Analytica, Bologna, Italy). The mobile phase was 0.05 M sodium citrate buffer, pH 2.5, containing 5% acetonitrile. The flow rate was maintained at 1 ml/min. Detection was carried out at 280 nm with a Gilson model 116 UV detector.

2.5. PAGE

Formation of stable nNOS dimers was analysed by low-temperature SDS–PAGE as described [9]. Recombinant nNOS was incubated for 10 min at 37°C in 20 μl of 50 mM triethanolamine–HCl buffer (pH 7) in the absence and presence of compounds as indicated. Incubations were terminated by the addition of chilled Laemmli buffer [24] containing 0.125 M Tris–HCl (pH 6.8), 4% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 20% (w/v) glycerol and 0.02% (w/v) bromophenol blue. Samples containing 2.25 μg of proteins were subjected to SDS–PAGE for 110 min at 150 V on discontinuous 6% SDS slab gels. Gels and buffers, prepared according to Laemmli [24], were equilibrated at 4°C and the buffer tank was cooled during electrophoresis in an ice bath. Gels were stained for protein detection with silver using the silver stain plus kit from Bio-Rad.

3. Results

Incubation of recombinant nNOS with TU resulted in a marked, concentration-dependent inhibition of the enzyme activity ($IC_{50} = 50 \pm 5$ μM), as determined by the L-[^{14}C]-arginine conversion assay (Fig. 1). The inhibitory effect was confirmed by determination of the main NO metabolites, nitrate and nitrite, accumulated in the reaction mixture [2]. With 100 μM L-arginine and 54 μg/ml of recombinant enzyme, 51 ± 2% (average of three separate experiments) inhibition of NO formation was observed with 1 mM TU. The high enzyme, substrate and TU concentrations were warranted by the relatively low sensitivity of the colourimetric assay used (see experimental). Comparable inhibitory effects of TU were observed on the NOS activity in rat cerebellum extracts. At 500 μM concentration the parent thioureylene compound, thiourea, and uracil, which lacks the sulphur atom, were ineffective in inhibiting nNOS activity.

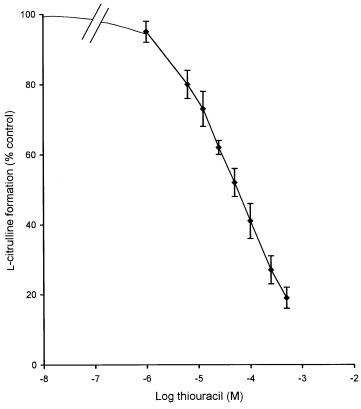

Fig. 1. Concentration-dependent inhibition of nNOS by TU. NOS activity was measured as the production of L-[^{14}C]citrulline from L-[^{14}C]arginine as described in Section 2. Results are means ± S.E.M. from four experiments.

Interestingly, TU proved to be highly selective for nNOS, being poorly active toward iNOS (less than 25% inhibition) and eNOS (less than 15% inhibition) at a concentration as high as 500 μM.

The progress curve for inhibited nNOS was linear, the onset of inhibition was rapid and citrulline formation increased almost linearly during a 15 min incubation time (data not shown), suggesting that inhibition by TU was reversible. In accordance with these results, preincubation of nNOS with

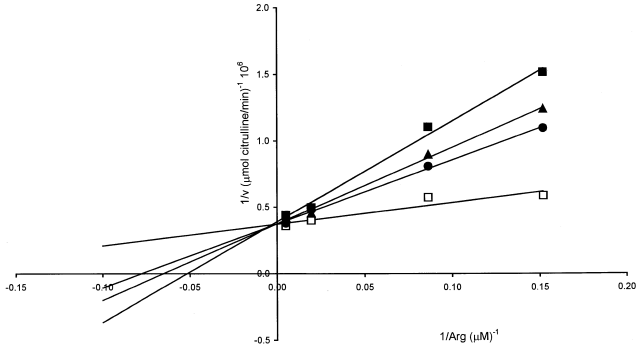

Fig. 2. Double reciprocal plot of nNOS activity as a function of L-arginine concentrations, in the presence of various concentrations of TU. The rate of L-[^{14}C]citrulline formation was measured as described in Section 2 in the absence (□) or in the presence of 12.5 (●) or 25 (▲) or 50 μM (■) TU. The plot shown is representative of three.

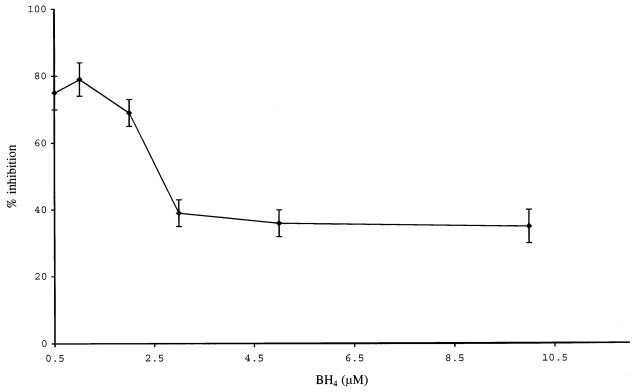

Fig. 3. Effect of BH_4 concentration on nNOS inhibition by TU. The enzyme was incubated with increasing BH_4 concentrations in the presence of 500 μM TU and NOS activity was measured as described in Section 2. Results are means ± S.E.M. from four experiments.

500 μM TU for 10 min prior to initiation of the reaction with 1.56 μM L-arginine did not cause additional inhibition, ruling out any irreversible effect.

Initial-rate kinetic studies were carried out over a range of L-[^{14}C]arginine and TU concentrations to determine the nature of nNOS inhibition. Double-reciprocal plots of the nNOS activity as a function of L-arginine concentration showed that TU inhibits L-citrulline formation in an essentially competitive manner (Fig. 2).

Replots of reciprocal rates of L-citrulline formation against TU concentration at a series of fixed arginine concentrations gave straight lines intersecting on the ordinate axis, which allowed determination of a K_i value of 20 μM. Consistent with a competitive-type mechanism, inhibition of nNOS by 500 μM TU was decreased by ca. 50% with saturating L-arginine concentrations (e.g. 200 μM).

At 100 μM concentration, TU did not affect cytochrome *c* reduction in the absence of Ca^{2+}/calmodulin, but caused ca. 60% inhibition of H_2O_2 production in the absence of L-arginine and BH_4. In control experiments, HPLC analysis of TU and peroxide determination ruled out any direct reaction of TU with H_2O_2 under the specific conditions of the assay.

The inhibitory effect of TU on L-citrulline formation could be reverted by BH_4 in a concentration-dependent manner (Fig. 3). In particular, nNOS inhibition by 0.5 mM TU was decreased to a constant value (ca. 36%) at BH_4 concentrations ≥3 μM. The possibility that this effect reflected decomposition of TU by BH_4 was ruled out by HPLC analysis, demonstrating that the drug (500 μM) is fairly stable even when incubated with as much BH_4 as 100 μM under the specific conditions of the assay.

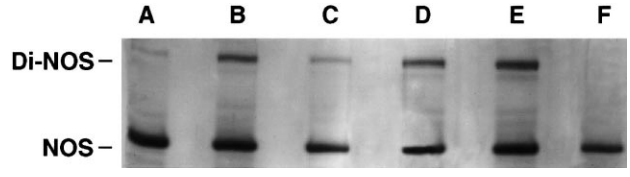

Fig. 4. Effect of TU on BH_4-induced nNOS dimerization. nNOS was incubated, as described in Section 2, with or without BH_4 in the absence or presence of TU (500 μM), followed by low-temperature SDS–PAGE analysis. From left to right: lane A, no BH_4; lane B, 1 μM BH_4; lane C, 1 μM BH_4+TU; lane D, 10 μM BH_4; lane E, 10 μM BH_4+TU; lane F, TU. NOS and Di-NOS refer to nNOS monomer and dimer with apparent molecular masses of approximately 160 and 320 kDa, respectively. The gel shown is representative of three separate experiments.

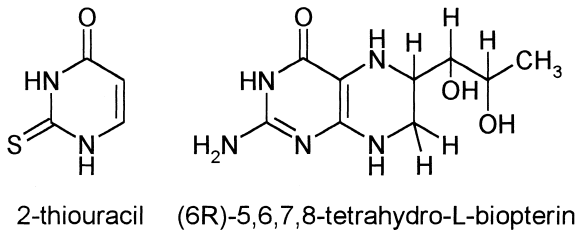

Fig. 5. Structures of TU and BH_4.

The plateau effect in Fig. 3 is difficult to explain based on available data. Apparently, it would denote a tight and complex interaction of TU with the BH_4 binding site, affecting BH_4-dependent enzyme activation at various levels and with different modalities [2].

In this connection, separate experiments showed that TU affected BH_4-dependent nNOS dimerisation, that is critical for enzyme activity [2,3]. Low temperature SDS–PAGE analysis [9], displayed in Fig. 4, showed that at 500 μM concentration TU markedly decreased the amount of nNOS dimer (320 kDa band) formed in the presence of 1 μM BH_4 (lane C versus lane B). Notably, the inhibitory effect of 500 μM TU on enzyme dimerisation was virtually suppressed when BH_4 concentration was raised to 10 μM (lane E versus lane D). No dimer formation was induced by TU in the absence of BH_4 (lane F).

4. Discussion

The antithyroid and melanoma targeting properties of TU have been related to its inhibitory effects on distinct enzymatic activities, including thyroid iodide peroxidase [25], myeloperoxidase, eosinophil peroxidase [26] and tyrosinase [27]. Our finding that TU is also a selective inhibitor of nNOS preventing BH_4-dependent dimerisation discloses a hitherto unrecognised property of this drug of potential relevance to the mechanism of the antithyroid action, in view of the recent demonstration of NOS activity in the thyroid gland [28]. TU may also represent a useful prototype for the rational design of novel neuroprotective agents for the treatment of cerebral ischaemia and other neurological disorders, with an advantage over other nNOS inhibitors derived from having a well known pharmacological profile.

The competitive pattern of inhibition and the antagonising effects of BH_4 would be consistent with TU interacting with a site located proximal to the BH_4 and L-arginine binding sites, possibly the haem or its periphery. In accord with this interpretation, the drug inhibited haem-catalysed oxygen activation and H_2O_2 production, but not cytochrome *c* reduction, indicating that it did not affect the reductase domain. In addition, TU has been reported to perturb the haem environment of another enzyme, horseradish peroxidase [29].

The inhibitory properties of TU can be ascribed to the presence of the thioureylene moiety, a feature shared also by the haem-binding NOS inhibitor L-thiocitrulline [23]. However, this moiety per se is unlikely to account for nNOS inhibition, as indicated by the lack of effects of thiourea, although the sulphur centre seems essential, since uracil was inactive. Similarly different effects of TU and uracil have been de-

scribed in the case of horseradish peroxidase [29]. Since at physiological pH, TU is in equilibrium with the anionic form (p*K*a = 7.75), it can be engaged in strong ionic-type interactions and/or H-bonding with polar residues within the BH_4 binding site, while being able to maintain contact with flat aromatic moieties through π-electron stacking and related interactions [12]. Thus, because of its loose structural resemblance to the pyrimidine moiety of BH_4 (Fig. 5) and its redox stability, TU may replace BH_4 at the pteridine binding site without however being able to sustain redox processes and/or prevent enzyme monomerisation during L-arginine turnover [10].

The action profile of TU is peculiar and reminiscent of that of 7-nitroindazole [14,30]. TU may therefore provide an attractive lead structure for novel selective nNOS inhibitors aimed at probing the delicate interplay between the tightly coupled pteridine- and substrate-binding domains. Moreover, given the different dependence of the various isoforms of NOS on BH_4 activation, TU would represent a superior biochemical tool to differentiate the physiopathological roles of the various isoforms in vivo.

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