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RESEARCH

Tyrosine inhibits the *Mycobacterium tuberculosis* protein tyrosine phosphatase MptpA

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Abstract

The catalytic action of the low-molecular weight protein tyrosine phosphatase (MptpA), expressed by *Mycobacterium tuberculosis*, is known to be essential for the pathogenicity of the bacterium. Candidate MptpA inhibitors are, therefore, actively sought and evaluated, and important work has been dedicated to the identification of competitive inhibitors. Here, we show that the activity of MptpA at the expense of phosphotyrosine (pTyr) is promptly inhibited by tyrosine, whereas the *p*-nitrophenol generated from *p*-nitrophenyl phosphate antagonizes the enzyme to a limited extent. We also report here a K_i of 512 ± 26 µM of MptpA for tyrosine, a value approximately one order of magnitude lower than the corresponding K_i for orthophosphate. Remarkably, the inhibition of MptpA observed when pTyr was used as substrate was effectively relieved by tyrosinase, the action of which was responsible for maintaining zero-order kinetics up to the generation of 200–400 µM of reaction product. Overall, the different sensitivity of MptpA towards tyrosine and *p*-nitrophenol suggests a significant inhibitory role of the α-aminocarboxylic acid moiety of the aromatic amino acid. This may, therefore, suggest the design of MptpA competitive inhibitors targeting the portion of the enzyme active site which is distal from the phosphate-binding region.

Keywords *Mycobacterium tuberculosis* · Tyrosine · Tyrosine phosphatase · Competitive inhibition · Tyrosinase

Introduction

Among the enzymes known to control a number of cell functions, kinases and protein phosphatases are of great relevance because of their specific and rapid action. Target proteins are phosphorylated by kinases at serine, threonine, or tyrosine loci, dephosphorylation of which is exerted by phosphatases with different specificities. In particular, when phosphotyrosine (pTyr) is considered, phosphatases strictly specific for pTyr and enzymes exerting a dual action (e.g., towards both phosphothreonine and phosphotyrosine) have been recognized (Tautz et al. 2013). Furthermore, pTyr-specific phosphatases are sub-grouped according to their molecular mass, being accordingly classified into High-Molecular-Weight (HMW, > 20 kDa) and Low-Molecular-Weight (LMW, ≤ 20 kDa) enzymes. Remarkably, the action of pTyr-specific phosphatases (PTPases) is of importance for the onset of many human diseases (Tonks 2006; Verma and Sharma 2018). In the case of infectious diseases, PTPases from *Yersinia pseudotuberculosis* and *Mycobacterium tuberculosis* are reported to be essential for these bacteria to escape the host immune system (Bölin and Wolf-Watz 1988; Bliska et al. 1991; Bach et al. 2008; Armstrong and Hart 1971; Castandet et al. 2005; Wong et al. 2011). In particular, the *Mycobacterium tuberculosis* enzyme MptpA is a LMW-PTPase (17.9 kDa) competent in the dephosphorylation of the host VPS33B (Vacuolar Protein Sorting 33B) protein. This implies the inactivation of VPS33B (Bach et al. 2008), which in turn inhibits phagosome–lysosome fusion (Armstrong and Hart 1971; Castandet et al. 2005), and therefore, confers virulence to the pathogen (Wong et al. 2011). Remarkably, it was also shown that *M. tuberculosis* strains bearing a deletion of the gene coding for MptpA feature a modest, if at all, competence in long-term infection (Bach et al. 2008). MptpA is, therefore, an appealing target for the search of anti-tuberculosis drugs, and not surprisingly quite a number of potential MptpA inhibitors have been proposed (Manger et al. 2005; Chlaradia et al. 2008; Silva and Tabernero 2010; Fanzani et al. 2015).

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Phosphatases featuring strict specificity for pTyr select their substrates by means of a hydrophobic pocket, the structure of which is tailored to interact with the tyrosine aromatic ring (Stuckey et al. 1994; Zhang et al. 1994; Barford et al. 1994). This interaction translates into the positioning of the pTyr phosphate in line with the nucleophilic cysteine residing at the bottom of the hydrophobic pocket. Importantly, when the phosphate group is bound to a shorter amino acid (i.e., serine or threonine) the distance between the catalytic cysteine and the scissile bond is long enough to imply very unfavourable geometry for the nucleophilic attack. These structural properties of the active sites of PTPases are, therefore, one of the main factors considered when designing competitive inhibitors directed against these enzymes. Indeed, quite a number of inhibitors of PTPases contain a phosphate-mimicking group bound to an aromatic moiety (Bialy and Waldmann 2005). Moreover, several covalent inhibitors sharing these functional determinants have been synthesized and tested (Ruddraraju and Zhang 2017). Besides this common theme, a higher degree of chemical divergence can be used to design parts of the inhibitors that target regions of PTPases distal from the substrate binding pocket.

The activity of PTPases is conventionally assayed using *p*-nitrophenyl phosphate (pNPP) as substrate, the hydrolysis of which can be conveniently observed by determining the increase in Absorbance (e.g., at 405 nm) coupled with the release of *p*-nitrophenolate. Alternatively, pTyr can be used as substrate, with the corresponding assay exploiting the absorption difference between pTyr and tyrosine (Stefan et al. 2020). We recently studied substrate activation of *M. tuberculosis* MptpA, using pNPP and pTyr at concentrations ≤ 1 mM (Stefan et al. 2020). Curiously enough, quite different reaction kinetics were observed at substrate concentrations > 1 mM, depending on the substrate used. Indeed, in the presence of pNPP the generation of product was found to obey a linear dependence on time up to *p*-nitrophenol concentrations of 80 µM, whereas an initial non-linear burst of tyrosine release was detected when pTyr was used as substrate (Stefan et al. 2020). A plausible explanation of these outcomes could be that MptpA has different sensitivity to inhibition by the reaction products *p*-nitrophenol and tyrosine. It is important to note that the K_m values of MptpA do not differ significantly for pNPP and pTyr, being equal to 6.1 ± 0.7 and 5.4 ± 0.2 mM, respectively (Stefan et al. 2020). It should also be considered that MptpA is rather insensitive to the inhibition exerted by orthophosphate, the K_i of which was determined to be 5.9 mM (Stefan et al. 2020). Remarkably, this value is in reasonable agreement with those previously determined (2–6 mM) for bovine PTPases (Davis et al. 1994; Bornadata et al. 1996; Zhang et al. 1997).

Overall, our previous observations on MptpA suggest that tyrosine triggers a pronounced inhibition of this enzyme, whereas the inhibition exerted by *p*-nitrophenol and orthophosphate on MptpA is much weaker. We, therefore, tested the effect of tyrosine on the catalytic performance of MptpA, and we report here on activity assays performed in the absence or in the presence of this reaction product, along with observations obtained by coupling the generation of tyrosine by MptpA with the reaction catalyzed by tyrosinase.

Materials and methods

Reagents

L-tyrosine (L-Tyr) and L-phosphotyrosine (L-pTyr) were dissolved in 100 mM NaOH to prepare a 50 mM stock solution. L-dihydroxyphenylalanine (L-DOPA) was solubilized at pH 7 in TBE universal buffer, which contains Tris–HCl, Bis–Tris (25 mM each), and 2 mM EDTA. A stock solution (25 U/µL) of mushroom tyrosinase was prepared in 50 mM Tris–HCl, pH 7.5. All reagents were purchased from Merck Millipore (Burlington, MA, USA).

Protein expression and purification

The expression and purification of MptpA W152F was performed as previously described (Stefan et al. 2020). The purified protein was stored at − 20 °C in 50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, pH 8. Protein concentration was determined according to Bradford (Bradford 1976).

MptpA activity assays

Reaction mixtures (1 mL final volume) contained 420 nM enzyme and 10 mM pTyr or pNPP in TBE buffer. Absorbance changes at 282 nm were observed using a Cary 300 Bio UV–VIS spectrophotometer. A $\Delta\varepsilon$ of 0.96 mM^{-1} cm^{-1} between tyrosine and phosphotyrosine at 282 nm (Stefan et al. 2020) and a ε of 18.3 mM^{-1} cm^{-1} for *p*-nitrophenol at 405 nm were used (Hriscu et al. 2013).

Inhibition of MptpA by L-tyrosine

To test the effect of L-tyrosine on phosphatase activity, reaction mixtures (1 mL final volume) were prepared using 5 mM pTyr, 420 nM MptpA, and variable concentrations of L-Tyr, ranging from 0.25 to 3 mM. Assays were performed at pH 7.5 using the universal TBE buffer. The dephosphorylation of pTyr was monitored at 286 nm, and a value equal to 0.48 mM^{-1} cm^{-1} for the $\Delta\varepsilon$ between tyrosine and phosphotyrosine at this wavelength was used (Stefan et al. 2020). A control reaction mixture devoid of L-tyrosine was also considered. The inhibition of MptpA by tyrosine

was quantitatively determined using the following equation (Burlingham and Widlanski 2003):

$$\frac{1}{v} = \frac{[S] + K_m}{V_{max}[S]} + \frac{K_m}{V_{max}[S]K_i}[I] \quad (1)$$

where K_i is the inhibition constant, and $[I]$ indicates the concentration of the inhibitor.

Absorption spectra of L-tyr and L-DOPA

The absorption spectra of 1 mM and 0.5 mM L-tyrosine and L-DOPA, respectively, were recorded between 220 and 320 nm.

Enzyme-coupled assay

Assays were performed at 20 °C in the presence of 10 mM pTyr, 50 U tyrosinase and 420 (840) nM MptpA in TBE buffer at pH 7. The generation of L-DOPA was detected at 295 nm. The absorbance changes occurring in control reactions devoid of tyrosinase were determined spectrophotometrically at 282 nm. One Unit of tyrosinase is defined as the amount of enzyme inducing an increase of Absorbance equal to 0.001 per minute at pH 6.5 and 25 °C, in 3 mL of a reaction mixture containing L-tyrosine.

Results and discussion

As already mentioned, we previously observed that the release of tyrosine by MptpA deviates from linearity as a function of time when pTyr is used as substrate, whereas *p*-nitrophenolate is generated from pNPP at a constant rate up to 80 µM (Stefan et al. 2020). Here, we report the outcome of a similar experiment, performed at pH 7.5 (our previous data were obtained at pH 8) in the presence of 420 nM MptpA, using pTyr or pNPP as substrate (Fig. 1). When pTyr was used, the reaction kinetics is early subjected to a significant slowdown (Fig. 1a, c), in sharp contrast to the output detected when the substrate was pNPP (Fig. 1a, b). Importantly, deviation of the kinetics of pTyr hydrolysis from linearity occurred when a relatively low product concentration was generated, i.e., approximately 6 µM (Fig. 1c,

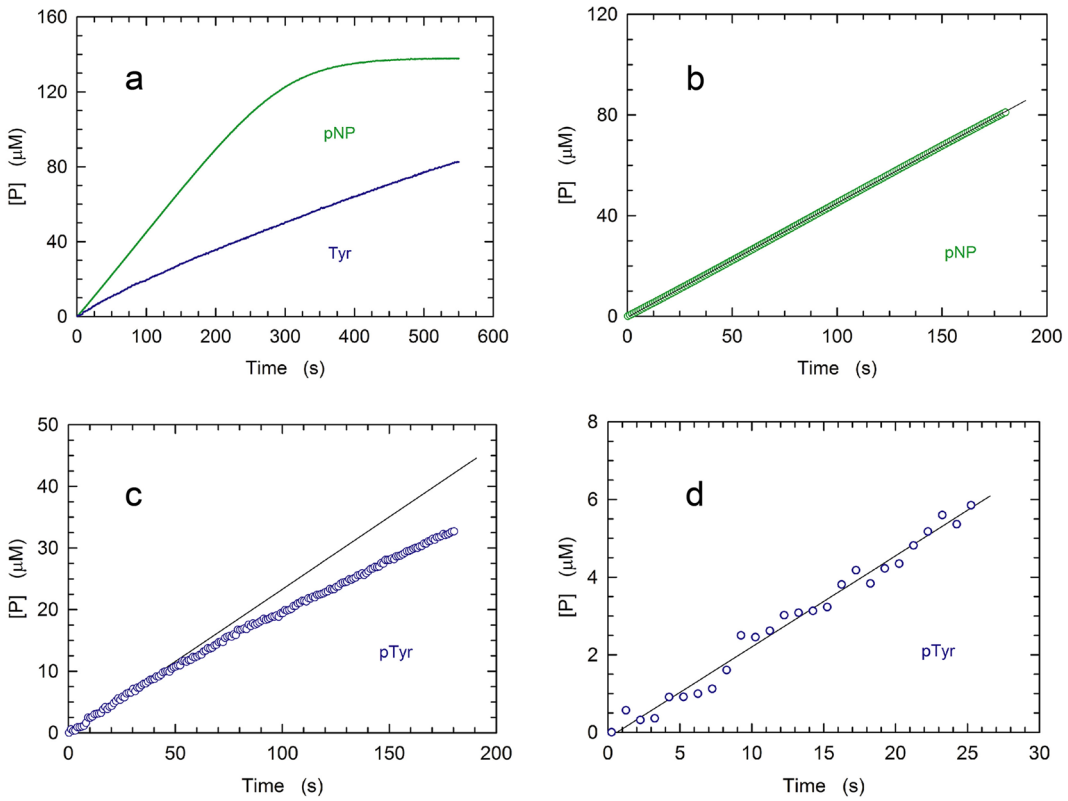

Fig. 1 **a** Kinetics of reactions catalyzed by 420 nM MptpA at the expense of pNPP (green curve) or pTyr (blue curve). Both substrates were used at 10 mM final concentration in TBE buffer, pH 7.5. **b** Initial phase of the *p*-nitrophenolate generation by MptpA reported in **a**. The continuous line is the best fit of a linear equation to the experimental observations (green circles). **c** Initial phase of the tyrosine release by MptpA reported in **a**. The continuous line is the best fit of a linear equation to the experimental observations (blue circles). **d** Detail of the catalytic breakdown of pTyr shown in **a** and **c**

d). We, therefore, tested whether or not tyrosine significantly inhibited the catalytic action of MptpA. To this aim, we performed activity assays, monitoring changes in Absorbance at 286 nm triggered by the addition of 5 mM pTyr to MptpA, in the absence or in the presence of increasing concentrations of tyrosine (up to 3 mM). The wavelength of 286 nm was chosen to contain the initial Absorbance of the reaction mixtures (it should be noted that the extinction coefficients of tyrosine over the 250–300 nm wavelength interval are much higher when compared to those of pTyr, Stefan et al. 2020). In addition, the containment of the initial Absorbance of reaction mixtures was obtained lowering the pTyr concentration from 10 to 5 mM. By reporting the reciprocal of initial reaction velocities as a function of tyrosine concentration, we obtained a linear dependence (Fig. 2). Applying the equation (see Methods) previously proposed (Burlingham and Widlanski 2003) to quantitatively estimate the inhibition of MptpA by tyrosine, we obtained a value of K_i equal to 512 ± 26 µM. Surprisingly, therefore, our observations indicate that the K_i of MptpA for tyrosine is one order of magnitude lower than the corresponding previously determined K_i for orthophosphate (Stefan et al. 2020).

To further test the inhibition of MptpA by tyrosine, we reasoned that the use of a secondary enzyme coupled to the primary reaction catalyzed by MptpA could be a meaningful strategy. In particular, we selected tyrosinase as the secondary enzyme because it acts on L-tyrosine and generates L-dihydroxyphenylalanine (L-DOPA). To design the enzyme-coupled assay, we first recorded the absorption spectra of tyrosine and L-DOPA. Fortunately, the two spectra were sufficiently distinct, featuring maxima at 275 and 280 nm, when tyrosine and L-DOPA are considered, respectively (Fig. 3a). Moreover, at wavelengths ≥ 290 nm significant absorption by L-DOPA was observed, whereas the extinction coefficients of tyrosine were extremely

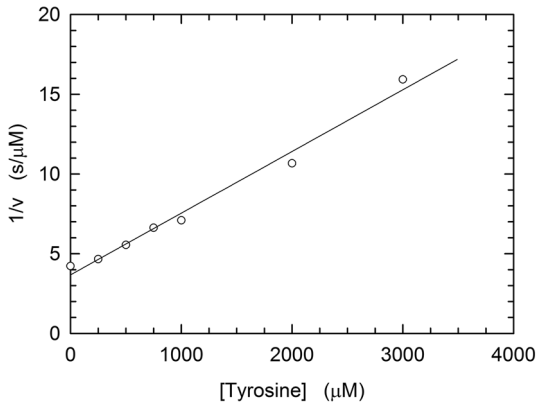

Fig. 2 Dependence of the initial velocity of the catalytic breakdown of 5 mM pTyr by MptpA on the presence of tyrosine. Assays were performed using 420 nM MptpA in TBE buffer, pH 7.5

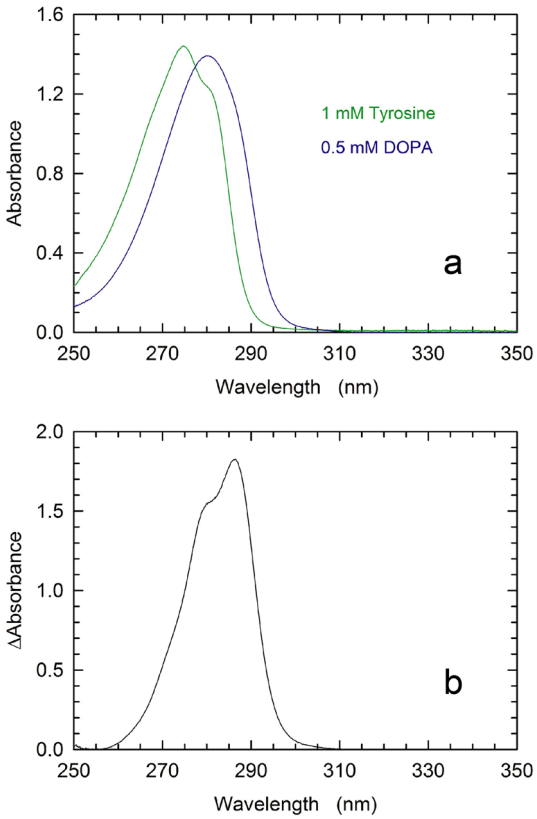

Fig. 3 **a** Absorption spectra of 1 mM Tyrosine (green curve) and 0.5 mM L-dihydroxyphenylalanine (L-DOPA, blue curve) in TBE buffer, pH 7.0. **b** Difference spectrum (L-DOPA minus tyrosine) obtained with the Absorbance values reported in **a**

modest (Fig. 3a). Using the difference spectrum we, therefore, selected 295 nm as an appropriate wavelength for detecting the generation of L-DOPA, with a minimal interference by tyrosine (Fig. 3b). In particular, at this wavelength we determined a $\Delta\varepsilon$ of 0.297 mM^{-1} cm^{-1} between L-DOPA and Tyrosine (Fig. 3b). We then tested the hydrolysis of pTyr exerted at pH 7 by 420 or 840 nM MptpA, by direct assay (detecting tyrosine at 282 nm) and enzyme-coupled assay (detecting L-DOPA at 295 nm). The direct assay showed kinetics essentially in line with those obtained at pH 7.5 (cf. Figs. 1a, 4a, c), suggesting that MptpA is rather pH-insensitive over the 7.0–7.5 interval. Remarkably, when tyrosinase was coupled to the primary reaction catalyzed by MptpA the time-course of L-DOPA release showed: (i) an initial lag phase, which is a signature of enzyme-coupled assays (McClure 1969), lasting for 250–300 s (Fig. 4b, d); (ii) a subsequent phase, that remained linear up to 200 and 400 µM of reaction product in the presence of 420 and 840 nM MptpA, respectively (Fig. 4d). The observations obtained with the enzyme-coupled assay, therefore, confirm that tyrosine is quite an effective inhibitor of MptpA.

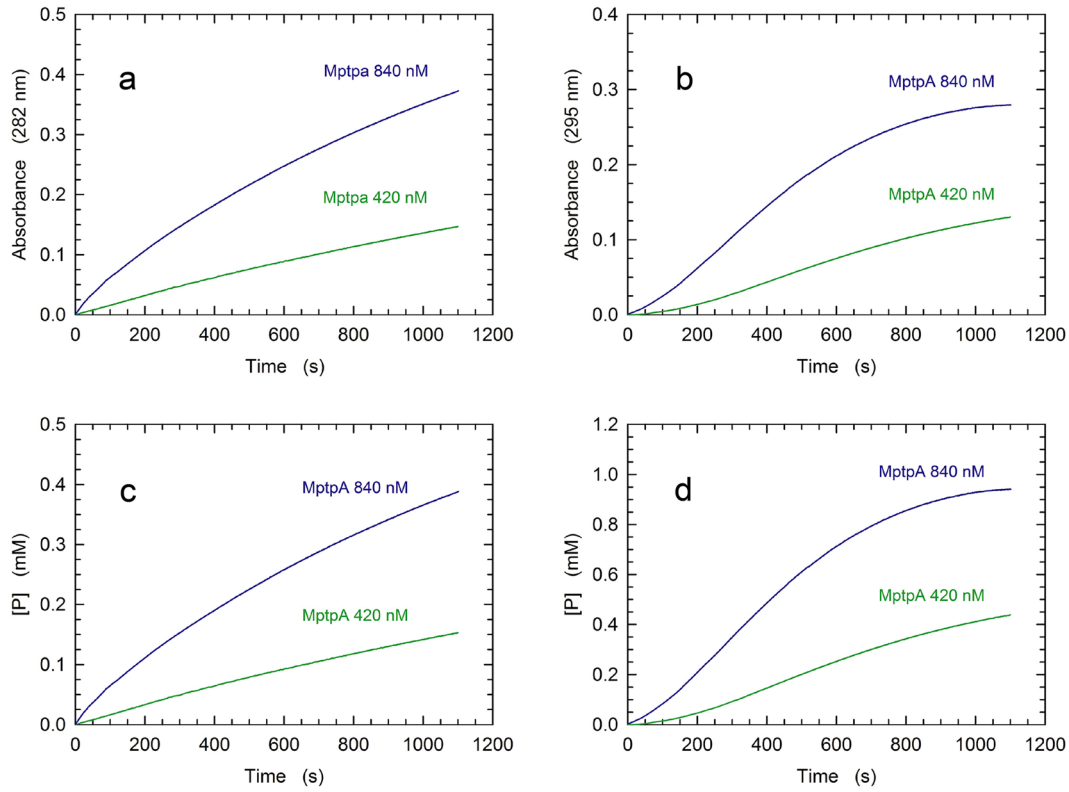

Fig. 4 **a** Time-course of the hydrolysis of pTyr catalyzed by 420 (green curve) or 840 nM (blue curve) MptpA in TBE buffer, pH 7.0. The assays were performed in the presence of 10 mM substrate. **b** Kinetics of the generation of L- dihydroxyphenylalanine (L-DOPA) by tyrosinase (50 U/mL) acting on the tyrosine generated by 420 (green curve) or 840 (blue curve) MptpA at the expense of 10 mM pTyr. **c** Conversion of the Absorbance values shown in **a** using a Δε of 0.96 mM^{-1} cm^{-1} between tyrosine and phosphotyrosine at 282 nm. **d** Conversion of the Absorbance values reported in **b** using a a Δε of 0.297 mM^{-1} cm^{-1} between L-DOPA and tyrosine at 295 nm (cf. Fig. 3b)

Conclusions

Here, we report on the inhibition exerted by tyrosine on the *Mycobacterium tuberculosis* protein tyrosine phosphatase MptpA. Activity assays showed that tyrosine is more effective than *p*-nitrophenol in antagonizing the catalytic action of MptpA. The K_i of MptpA for tyrosine was also found to be one order of magnitude lower than the K_i we previously determined for orthophosphate (Stefan et al. 2020). Overall, our observations suggest that the α-aminocarboxylic acid moiety of tyrosine is responsible for the inhibitory competence of this amino acid. It should also be noted that the methylene group of tyrosine could also confer inhibitory effectiveness to this amino acid, by spacing apart the α-aminocarboxylic acid moiety and the aromatic ring. Accordingly, it is our hope that the observations of the present work could help in the design of inhibitors targeting MptpA, which is an essential determinant of *M. tuberculosis* pathogenicity.

Authors contributions AS, CP and AH performed the experiments. AH designed the study and wrote the manuscript.

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Data availability The data generated during the current study are available from the corresponding author.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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