Concentration-dependent HAT/ET mechanism of the reaction of phenols with 2,2-diphenyl-1-picrylhydrazyl (dpph·) in methanol†

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The reaction of a 2,2-diphenyl-1-picrylhydrazyl radical (dpph·) with phenols carried out in alcohols is a frequently used assay for estimation of the antiradical activity of phenolic compounds. The rates of reactions of dpph· with five phenols (ArOH: unsubstituted phenol, 4-hydroxyacetophenone, two calix[4]resorcinarenes and baicalein) measured in methanol indicate the different kinetics of the process for very diluted phenols compared to their non-diluted solutions. This effect was explained as dependent on the ratio [ArO·]/[ArOH] and for diluted ArOH corresponds to an increased contribution of much faster electron transfer (ET, ArO·/dpph·) over the Hydrogen Atom Transfer (HAT, ArOH/dpph·). Simplified analysis of the reaction kinetics resulted in estimation of $k_{ET}$/$k_{HAT}$ ratios for each studied ArOH, and in calculation of the rate constants $k_{ET}$. Described results are cautionary examples of how the concentration of a phenol might change the reaction mechanism and the overall kinetics of the observed process.

Introduction

The stable 2,2-diphenyl-1-picrylhydrazyl radical, abbreviated as DPPH or dpph·, is broadly employed for quick assessment of the radical scavenging abilities of natural and synthetic compounds. The methods are based on the monitoring of dpph· decoloration:

$$\text{ArOH} + \text{dpph}^· \rightarrow \text{ArO}^· + \text{dpph-H}; \text{overall } k^S$$  \hspace{1cm} (1)

In hydrocarbons and other non-polar solvents, reaction 1 proceeds as Hydrogen Atom Transfer (HAT). Since dpph· radicals are about 3 orders of magnitude less reactive than peroxyl radicals, reaction 1 can be easily employed for studies on reactivity of phenols and the kinetic solvent effect (KSE)\textsuperscript{1,2} (see Chart 1A), and even for estimation of O–H bond strengths. However, in ionization supporting solvents (water and alcohols) the HAT mechanism is “contaminated” by electron transfer (ET) from the ionized fraction of ArOH. This two-step mechanism is described as Sequential Proton-Loss Electron Transfer (SPLLET)\textsuperscript{1,4} with $k_{ET} >> k_{HAT}$, see Chart 1A, and even traces of ArO· causing an enormous increase of overall rate of reaction 1.\textsuperscript{5} Mixed mechanism in alcohols excludes dpph· as mimetic to peroxy radicals but, regardless of this controversy,\textsuperscript{6} reaction 1 is one of the most frequently used colorimetric assays employed for quick assessment of the antiradical abilities of natural and synthetic compounds, with more than 40 thousand papers.

Chart 1  (A) General scheme of reaction of phenol (ArOH) with dpph· including kinetic solvent effect (KSE, left), HAT (central) or SPLLET mechanisms (right, shown in red). (B) Structures of phenols 1–5, with crown conformation of 3 and 4.
published during the last decade (SCOPUS, keywords “dpff” and “antioxidant”).

In contrast to simple kinetic measurements, typical non-kinetic assays are based on a titration of dpff solution with phenol (or mixture of phenol extracts) in order to determine IC_{50} parameter, i.e., the concentration of phenol sufficient to scavenge 50% of the radicals present in the sample. However, reaction 1 can be (and usually is) reversible,\(^\text{6}\) because the bond dissociation enthalpy, BDE, in dpfp-H (78.9 kcal mol\(^{-1}\)) is lower than BDE_{O-H} for majority of phenols, and IC_{50} measurements can be misleading. Another problem was noticed by Foti et al. who reported that for some phenols reaction 1 exhibits non-integer order in [ArOH].\(^{4,7}\) Such confusing phenomena needs to be further explored, and we selected series of ArOH accommodating two opposing features: enhanced acidity and a measurable reactivity (within stopped-flow time scale) toward dpff. The proper selection of compounds was not trivial because the introduction of an electron-withdrawing group into ArOH enhances its acidity but also causes an increase in the strength of the O–H bond. Here we present the results obtained for: phenol (1), 4-hydroxyacetophenone (2), C-methylcalix[4]-resorcine (3), C-undecylcalix[4] resorcine (4), and baicalin (5), the only natural compound within this series, being also an interesting example of ArOH with a strongly acidic catechol moiety, see Chart 1B.

Results and discussion

The reaction was monitored in neat methanol or, in order to suppress phenol ionization, in methanol acidiﬁed with acetic acid (AcOH). Experimental, pseudo-ﬁrst-order rate constants, \(k_{\text{exp}}\), determined for series of increasing [ArOH] being always in stoichiometric excess over dpff\(^{-}\), and bimolecular rate constant (\(k^{3}\)) was obtained from the straight-line equation:

\[
k_{\text{exp}} = k^{3} \left[ \text{ArOH} \right] + \text{const} \quad (2)
\]

where the intercept (sometimes also denoted as \(k_0\))\(^{4}\) includes the self-decay of the radical which is not dependent on the ArOH concentration. In order to avoid the effect of reversibility of reaction 1 \((\text{vide supra})\), the very initial rates of reaction were measured (conversion of dpff\(^{-}\) was less than 5–10%).

Fig. 1 indicates that 1 is the only phenol for which a straight-line dependence of \(k_{\text{exp}}\) on [ArOH] was obtained within the whole [ArOH] range. For 2-5, after the initial linear increase in \(k_{\text{exp}}\) against increasing [ArOH] there is a break in the trend, and the overall plot is not linear. This means that the rate law is more complex and the species other than ArOH are involved in the rate determining step. Foti et al.\(^{4,6}\) studied the reaction of quercetin (QH\(_{2}\)) with dpff\(^{-}\) in methanol/water, and interpreted non-integer order \((k_{\text{exp}} \sim [\text{QH}\(_{2}\)]^{0.4})\) as resulting from the reversible formation of π-stacked pre-reaction complex of quercetin anion with dpff\(^{-}\), followed by fast ET:\(^{4,6}\)

\[
\text{QH}^{-} + \text{dpff}^{-} \overset{k}{\rightarrow} [\text{QH}^{-}/\text{dpff}^{-}] \rightarrow \text{QH}^{-} + \text{dpff}^{-} \quad (3)
\]

A similar mechanism was also described for curcumin/dpff\(^{-}\) pair in ethanol\(^{4,6}\) whereas for phenols with OH group internally H bonded to the N-base a formation of the contact ion pair (ET to dpff\(^{-}\)) was proposed:*

\[
\text{ArOH} + \text{dpff}^{-} \leftrightarrow [\text{ArOH}^{+}/\text{dpff}^{-}] \rightarrow \text{ArO}^{+} + \text{dpff-H} \quad (4)
\]

Non-linear ﬁtting of our data to a function \(k_{\text{exp}} = d[\text{ArOH}]^{b}\) gave reaction orders 0.38–0.60 with respect to concentration of 2–5, however, the goodness of ﬁtting for 3 is rather moderate, with residuals as high as up to ±10%, see Fig. 2, S13, S25 and S30.\(^{\dagger}\) After addition of AcOH, the linear relationships \((\text{eqn} \ (2))\) were obtained\(^{1}\) for the whole concentration range, see Fig. 2 and ESI,\(^{\dagger}\) proving the mixed HAT/SPLLET mechanisms. Therefore, we limited our calculations of \(k^{3}\) for apparently straight line sectors of \(k_{\text{exp}}\) vs. [ArOH] plots, below the inflection point, as presented in Fig. 1, and the results are listed in Table 1.

The value of \(k^{3}\) for reaction of 1 with dpff\(^{-}\) is in a reasonable agreement with our previously published 0.04 M\(^{-1}\) s\(^{-1}\),\(^{4,6}\) with some deviations that can be ascribed to a great sensitivity of the kinetics of the processes carried out in neat, non-buﬀered systems.\(^{8}\) BDE_{O-H} for 1 is 87.2 kcal mol\(^{-1}\) (ref. 6) or 88.2 kcal mol\(^{-1}\),\(^{14}\) and for 2 is 90.3 kcal mol\(^{-1}\),\(^{14}\) and \(\Delta\text{BDE} = \text{BDE}_{\text{ArO-H}} - \text{BDE}_{\text{PhOH}}\) suggests that \(k^{\text{HAT}}\) for 2 should be 10–25 times smaller than for 1, as it can be predicted from eqn \((5)).\(^{\text{¶}}\)

\[
\log k_{\text{ArOH/dpff}} = -0.33 + 0.35(\Delta\text{BDE}) \quad (5)
\]

\(\dagger\) Kinetic data from the inset can be also fitted for non-integer order model \(k_{\text{exp}} = [\text{ArOH}]^{b}\), giving artiﬁcial (in our opinion) \(b = 0.76 \pm 0.10\). For reaction of 5 with dpff\(^{-}\) in methanol containing 100 and 1000 mM acetic acid the calculated \(b\) are scattered from 0.63 to 0.36 because even very small deviation of single experimental point produce substantial decrease of \(b\), than can be either an error or an effect of mixed HAT/ET mechanism. Therefore, for acidified systems we did not push the reaction order into noninteger orders in [ArOH]. Moreover, data for non-acidiﬁers 1 \((\text{straight line in Fig. 1A})\) can also be ﬁtted to non linear function, giving reaction order 0.83.

\(\ddagger\) To remove traces of phenols/stabilizers and other compounds that could contaminate the kinetics, we distilled methanol over a small amount of dpff\(^{-}\) and a few beads of ion-exchange resin.

\(\text{¶}\) This empirical equation is valid for non-hindered phenols reacting with dpff\(^{-}\) radical in hydrocarbons (pure HAT mechanism, with no KSE). In other solvents some small differences can be observed due to different ability of ArOH and PhOH to form H bond with solvent, see discussion in footnote 34 in ref. 1. Predicted differences in \(k^{3}\) can be even bigger, because 2, with strong EW substituent, will be better HB donating agent than unsubstituted phenol 1.
Both compounds react faster than in neat methanol, con-
ing the role of deprotonation in the reaction mechanism.

For reactive phenols this effect is kinetically not signif-
icant, but for a slowly and reversibly reacting electron defi-
cient 2, the transfer of H⁺ from AcOH to dpph drives the reaction to the products (2⁺/dpph-H). We also measured k5 for 1 and 2 reacting with dpph⁺ in buffered methanol/water (1:1) at pH 5.4 and 7.4. Both compounds react faster than in neat methanol, confirming the role of deprotonation in the reaction mechanism. Surprisingly, at pH 7.4 both phenols, 1 and 2, react with almost the same rate, k5 is 0.74 ± 0.07 M⁻¹ s⁻¹ for 1 and 0.66 ± 0.12 M⁻¹ s⁻¹ for 2, whereas at pH 5.4 phenol 2 is a bit more reactive (k5 = 0.25 ± 0.01 M⁻¹ s⁻¹) than 1 (k5 = 0.19 ± 0.03 M⁻¹ s⁻¹). At pH 5.4 the plots of kexp vs. [ArOH] are linear, but some deviations from linearity occur at pH 7.4 (see ESI†).

Phenols 3–5 are 10² times more reactive than 1 and 2, therefore, much smaller concentrations (<1 mM) were used for measurements, and the presence of 10 mM AcOH (large excess) causes a 100-fold suppression of k8 for 5, and ca. 20-fold decrease for cyclic tetramers 3 and 4. A 10% better reactivity of 4 over 3 can be explained as an effect of a crown conformation of 4, with internal hydrogen bonds within the upper rim, see Chart 1B, facilitating the stabilization of a radical [although we cannot exclude other effects related to different conformations and causing small differences in acidity of both compounds].

Table 1 Bimolecular rate constants, kexp for reactions of dpaph⁺ with phenols 1–5 in neat and acidi-
cifiedalcohols

| phenol | 1 | 2 | 3 | 4 | 5 |
|--------|---|---|---|---|---|
| pKₐ¹⁰,MetOH | 9.99 | 7.87 | 8.82/10.8/11.7 | — | 5.4/9.8/11.3 |
| pKₐ¹⁰,MeOH | 14.4 | 12.2 | 13.2 | — | 9.5 |

Table 1. Values of the reaction order for each system and the concentration dependence of the rate of reaction.}

Fig. 2 Plots of kexp against [3] and [5] for reaction with dpaph⁺ in neat methanol with non-linear fit. Inset: plots of kexp versus [M] and kexp for reaction carried out in acidi-
cified methanol (10 mM AcOH) with linear fit.

However, only 5-fold (instead of 25-fold) difference in k⁵ indicates that HAT is partially compensated with a greater participation of the SPLLET mechanism for 2 than for 1. Interestingly, addition of AcOH causes a decrease in k⁵ for 1, but not for 2 (even in methanol acidi-
cified with 1 M AcOH the parameter k⁵ was the same as in neat MeOH). This observation does not exclude SPLLET because 2 is a relatively strong acid and its concentra-
tion is moderately high. Using Kₐ for 2 in methanol (footnote e in Table 1), we obtained [2] = 0.56 × 10⁻⁷ M and 1.8 × 10⁻⁷ M for 5 mM and 50 mM solution of 2, respectively (this concentration range was used for calculation of k⁵). Furthermore, in this particular case acetic acid can slightly accelerate the completion of SPLLET by fast protonation of dpaph⁺ formed after ET from 2⁻ to dpaph⁺ (pKₐ of dpaph-H is 8.54 (ref. 15) or 8.59 (ref. 16) in methanol/water 1:1). Therefore, the equilibrium 2⁺ + dpaph⁺ ⇌ 2⁺ + dpaph⁺ will be shifted to the right. For reactive phenols this effect is kinetically not significant, but for a slowly and reversibly reacting electron defi-
cient 2, the transfer of H⁺ from AcOH to dpph drives the reaction to the products (2⁺/dpph-H). We also measured k5 for 1 and 2 reacting with dpph⁺ in buffered methanol/water (1:1) at pH 5.4 and 7.4. Both compounds react faster than in neat methanol, confirming the role of deprotonation in the reaction mechanism. Surprisingly, at pH 7.4 both phenols, 1 and 2, react with almost the same rate, k⁵ is 0.74 ± 0.07 M⁻¹ s⁻¹ for 1 and 0.66 ± 0.12 M⁻¹ s⁻¹ for 2, whereas at pH 5.4 phenol 2 is a bit more reactive (k⁵ = 0.25 ± 0.01 M⁻¹ s⁻¹) than 1 (k⁵ = 0.19 ± 0.03 M⁻¹ s⁻¹). At pH 5.4 the plots of kexp vs. [ArOH] are linear, but some deviations from linearity occur at pH 7.4 (see ESI†).

Phenols 3–5 are 10² times more reactive than 1 and 2, therefore, much smaller concentrations (<1 mM) were used for measurements, and the presence of 10 mM AcOH (large excess) causes a 100-fold suppression of k⁸ for 5, and ca. 20-fold decrease for cyclic tetramers 3 and 4. A 10% better reactivity of 4 over 3 can be explained as an effect of a crown conformation of 4, with internal hydrogen bonds within the upper rim, see Chart 1B, facilitating the stabilization of a radical [although we cannot exclude other effects related to different conformations and causing small differences in acidity of both compounds].

Equation (6) can be solved after introducing [ArO⁻] = [ArO⁻] + kET[ArO⁻]/dpaph⁺ for reactions carried out in methanol containing 1 M AcOH. Obtained values kET/kHAT ratio listed in Table 2. Such estimation gives the opportunity to compare the overall ET/HAT rates, without any additional knowledge about a formation of dpaph⁺/ArO⁻ complexes.

Values of kET listed in second column of Table 2 were calculated assuming that ET is eliminated (k⁸ ≈ kHAT) for reactions carried out in methanol containing 1 M AcOH. Obtained values kET for 1⁻, 3⁻, and 5⁻ (anions) are in reasonable agreement with 1 × 10¹ M⁻¹ s⁻¹ calculated by Foti et al. (for
whereas for law of dilution. For reaction order close to 0.5 in a small problem with the dpph electron de allow to obtain a nice linear correlation of is stronger acid than quercetin). The presented data do not perhaps, because of large error of estimation of within the whole concentration range (Fig. 2 and S43). This is the upper value, the lower kET can be 35 M⁻¹ s⁻¹, see explanation in the text. Measured in water with Britten–Robinson buffer vs. HgHg₂SO₄/K₂SO₄ electrode and recalculated into NHE.† From ref. 18. This value is very close to E° for quercetin at pH 7.²

| phenol | kET/kHAT | kET | E°  |
|--------|----------|-----|-----|
| 1      | 1.5 × 10⁶ | 8.8 × 10⁴ | 970  |
| 2      | 1.4 × 10⁴ | 8.9 × 10⁻² | 1060 |
| 3      | 5.4 × 10⁴ | 3.1 × 10⁴ | 617  |
| 5      | 1.6 × 10⁸ | 3.7 × 10⁻⁶ | 290  |

From ref. 14 In the same work redox potentials (at pH > 12) for 1/1⁻ and 2/2⁻ are 790 mV, and 1000 mV, respectively.¹ This is the upper value, the lower kET can be 35 M⁻¹ s⁻¹, see explanation in the text. Measured in water with Britten–Robinson buffer vs. HgHg₂SO₄/K₂SO₄ electrode and recalculated into NHE.† From ref. 18. This value is very close to E° for quercetin at pH 7.²

QH²/dpph⁻ in methanol⁴⁶ and with 3.5 × 10⁶ M⁻¹ s⁻¹ for ET from electron rich anion of 4-methoxyphenol derivative to dpph⁻ in acetonitrile. Table 2 presents also the reduction potentials for studied phenols taken from literature (E° for dpph⁻/dpph⁻H⁺ in water at pH 7 is 545 mV).²⁰ E° for 5 is 290 mV⁸ making this flavonoid stronger reducing agent than quercetin (E°(dpph⁻H⁺) = 330 mV),⁸ which can be reasoned also as an effect of greater participation of ET than QH⁻ in ET at pH 7 (5 is stronger acid than quercetin). The presented data do not allow to obtain a nice linear correlation of kET versus E°, perhaps, because of large error of estimation of kET values. For electron deficient 2, the upper value of kET was estimated assuming that kHAT for 2 and for 1 are the same (that can be reasoned also due to similar values of E°, see Table 2), however, basing on eqn (5), kHAT for 2 should be ca. 25 times smaller than for 1, thus, kET for 2 might be as low as 35 M⁻¹ s⁻¹. Such a small kET is not surprising because for 2 this process is the most endergonic among all five ArO⁻/dpph⁻ pairs.

The presented kET values are not strictly quantitative and include propagation of experimental errors (especially for 2) but they allow to estimate the concentration-dependent contribution of HAT and ET mechanisms to the overall rate of reaction, see Fig. S41–S43 and graphical abstract. Reaction of dpph⁻ with the most acidic and most reactive 5 is dominated by ET within the whole concentration range (Fig. 2 and S43) with the reaction order close to 0.5 in [5] as a consequence of the Ostwald law of dilution. For 1 (the weakest acid) HAT dominates over ET, whereas for 2 there is an inversion of dominating mechanism at 2 ≈ 50 mM.

Conclusions

There are many well-documented shortcomings of the dpph⁻ assay, including poor or no correlation with the antioxidant activity of phenols measured under physiologically relevant conditions (reactivity toward peroxyl radicals).² Another problem with the dpph⁻ assay is that IC₅₀ parameter reflects the position of redox equilibrium between dpph⁻ and the tested compound, established after the incubation time, and gives no information about the kinetics and stoichiometry of the reaction.⁵b,d

Kinetic measurements (stopped flow technique, initial rates of reaction) can provide information on the structure-reactivity relationship of putative antioxidants reacting via HAT in non-polar solvents.⁸ However, in polar solvents much faster electron transfer is competitive or dominating mechanism and we demonstrated that contribution of HAT/ET to the overall rate is strongly dependent on the concentration of a tested phenol. A careful interpretation of both, kinetic and quasi-kinetic (IC₅₀) results, has to be always performed, and mixed HAT/ET mechanism of reaction 1 might produce false results not only in the kinetic experiments but also in IC₅₀ assay, as IC₅₀ is a parameter strongly related to the concentration of tested phenols (and even expressed in phenol concentration units !). IC₅₀ parameter is frequently used for comparison of “antioxidant properties” of phenols and other phytochemicals but our findings indicate a serious limitation of such methodology.

General experimental procedures

Commercially available phenols 1, 2, and 5 were of the highest purity and were used as received. Macrocyclic polyphenols: C-methylcalix[4]resorcinarene (3) and C-undecylecalix[4]resorcinarene (4) were prepared following the method proposed by Weinelt and Schneider¹⁴ by condensation of resorcinol and appropriate aldehyde in ethanol containing aqueous HCl as in our previous work.¹¹ ¹H NMR of C-alkyl[4]resorcinarenes were recorded on a Varian spectrometer at 300 MHz and 298 K and were compared with literature.²² 3 was obtained by condensation of resorcinol with ethanol. 0.5 mol of resorcinol dissolved in 500 mL of ethanol/water (1/1, v/v) was immersed in an ice bath, then 125 mL of concentrated hydrochloric acid was added. Subsequently, 0.5 mol of ethanol was added dropwise. Then, the reaction mixture was kept at rt and the reaction was carried out for 96 hours with continuous stirring under nitrogen. The mixture was cooled and concentrated under reduced pressure. The obtained precipitate was washed several times with cold ethanol, crystallized form ethanol/water (1 : 1), and dried (yield 40–50%)¹¹ ¹H NMR (300 MHz, acetone-d₆) δ 8.48 (s, 8H), 7.64 (s, 4H), 6.21 (s, 4H), 4.52 (q, J = 7.3 Hz, 4H), 1.76 (d, J = 7.4 Hz, 12H), see Fig. S1. ⁴ was obtained by condensation of resorcinol (0.69 mol) with dodecanal (0.69 mol). The compounds were dissolved in 690 mL of ethanol and cooled in the ice bath to temperature close to 0 °C. Subsequently, 111 mL of concentrated hydrochloric acid was added dropwise to the mixture. Then, the temperature was increased to 70 °C and kept for 12 hours with continuous stirring and nitrogen flow. The mixture was cooled and concentrated under reduced pressure. The obtained precipitate was washed several times with cold ethanol, crystallized from methanol, and dried (yield 20–30%).¹¹ ¹H NMR (300 MHz, CDCl₃) δ 9.56 (t, J = 20.0 Hz, 4H), 9.28 (t, J = 25.0 Hz, 4H), 7.20 (s, 4H), 6.11 (s, 4H), 4.29 (t, J = 7.7 Hz, 4H), 2.21 (s, 8H), 1.27 (s, 72H), 0.88 (t, 12H), see Fig. S2. Since rate of ArOH/dpph⁻ reaction in neat methanol is highly sensitive to traces of acids and bases, prior to the use, methanol was fractionally distilled over a small amount of dpph⁻ and a few beads of an acidic ion-exchange resin. Measurements were made following the procedure described previously.⁵c,d
of dpph’ (ε ~ 11 000 M−1 cm−1) were monitored 517 nm on an Applied Photophysics SX 20 stopped flow spectrometer, equipped with a xenon arc lamp source and photodiode array detector. The mixing cell (10 mm optical path length, dead-time of mixing 1.1 ms) and the tubes delivering the reactants were thermostated at temperature 25 °C. Initial concentrations of dpph’ were 6–12 × 10−5 M for reactions with 1 and 2 and 1–15 × 10−5 M for reactions with much more reactive 3–5, i.e., always in the presence of a stoichiometric excess of ArOH. Measurements were made in neat and acidified methanol (with 10, 100 and 1000 mM AcOH) and in mixed 1 : 1 (v/v) methanol–water with pH adjusted to 5.4 (acetate buffer) and pH 7.4 (phosphate buffer). In all experiments, the initial rates (usually determined for 5–10% of dpph’ conversion) were taken for calculations of k_{exp}, for example, the conversion of dpph’ 0.5 s after mixing was 1%, 0.38%, 17%, 29% and 31% for 1–5, respectively, and after 2 seconds the conversion was 3.2%, 0.8%, 50% and 71% for compounds 1–4. The pseudo-first-order rate constants, k_{exp}, were calculated as average values from at least two independent sets of measurements. Values of bimolecular rate constants, k_2, were calculated as a slope of the straight-line eqn (2). EPR measurements by Staśko et al.4 for mixed ethanol/water systems indicated that dpph’ behaves as typical solute for a lower water ratio of 0–60% (v/v) but at a higher water content (above 60%) dpph’ forms microaggregates (still without precipitation). We assume, therefore, that dpph’ forms a homogeneous system with water/methanol (1 : 1, v/v) during our experiments at pH 5.4 and 7.4.

Author contributions
Conceptualization (GL and PP), funding acquisition (GL), investigation (PP, AK, PL), methodology, supervision and project administration (GL), visualization (KJP and GL), writing (all authors).

Conflicts of interest
There are no conflicts to declare.

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