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Murburn Concept: A Molecular Explanation for Hormetic and Idiosyncratic Dose Responses

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Abstract

Recently, electron transfers and catalyses in a bevy of redox reactions mediated by hemeproteins were explained by murburn concept. The term "murburn" is abstracted from "*mured burning*" or "*mild unrestricted burning*" and connotes a novel "*molecule-unbound ion–radical*" interaction paradigm. Quite unlike the genetic regulations and protein-level affinity-based controls that govern order and specificity/selectivity in conventional treatments, murburn concept is based on stochastic/thermodynamic regulatory principles. The novel insight necessitates a "reactivity outside the active-site" perspective, because select redox enzymatic activity is obligatorily mediated via diffusible radical/species. Herein, reactions employing key hemeproteins (as exemplified by CYP2E1) establish direct experimental connection between "additive-influenced redox catalysis" and "unusual dose responses" in reductionist and physiological milieu. Thus, direct and conclusive molecular-level experimental evidence is presented, supporting the mechanistic relevance of murburn concept in "maverick" concentration-based effects brought about by additives. Therefore, murburn concept could potentially explain several physiological hormetic and idiosyncratic dose responses.

Keywords

murburn concept, hemeprotein, hormetic dose response, idiosyncratic dose response, drug metabolism, cytochrome P450

Introduction

In order to maintain the "order" of life processes, several stochastic routes are tided over within a cell to spatiotemporally generate a particular molecular product in desired quantities. To achieve such outcomes, the order-seeking cellular machinery regulates catalysis by controls at genetic and protein levels.[1,2] Important cell pool components such as carbohydrates, amino acids, and nucleotides are usually maintained at $\sim 10^{-4}$ to 10^{-2} M, although some key metabolites may be at lower concentrations ($\leq 10^{-6}$ M). The low flux enzymes are maintained at $\sim 10^{-9}$ to 10^{-8} M, and the high flux enzymes are between 10^{-6} and 10^{-5} M. Although the distribution varies widely, the average cellular concentration for a given protein is $\sim 10^{-6}$ M, and the net concentration of cell metabolites is in the range of $\sim 10^{-1}$ M. In spite of several such checks and balances, life forms living under the most optimal sustenance conditions eventually lose cellular order and function. Loss of control at the genetic level is being extensively investigated.[3,4] Regulation is mostly stringent at the protein level, but loss of control is also noted at certain instances. Usually, increasing the concentration of a molecule gives a unidirectional physiological effect, which is best captured by a sigmoid dose–response curve for activations and inhibitions. This is because enzymes or receptors binding their substrates or ligands usually abide by simple "1-site, 1-ligand" stoichiometry (explained by "active-site" treatments like Fisher's "lock and key" or Koshland's "induced fit" classical considerations), which lead to Michaelis-Menten or Hill equations. Further, small molecules are rather "unintelligent" to have choices in selecting a particular concentration or concentration ranges for

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optimum or increased activity. Therefore, greater the concentration of the molecule, the functions of enzymes and receptors are raised to higher levels to give concomitantly enhanced downstream effects/signals. Generally, a bell-shaped curve with an optimal value is seen when the ligand is toxic or produces substrate inhibition. Else, even a unidirectional decrease or increase in response is noted with respect to a substrate's/ligand's/metabolite's concentration. However, an intriguing phenomenon called hormesis has been documented by experimentalists, wherein a molecule shows a particular effect only at a dilute or discrete concentration and not at a higher concentration.[5] Recently, some explanations based on self-aggregation were available[6] and even dilute solutions were also shown to affect gene expression.[7] It is interesting to note that the Proteasome, Endoplasmic Reticulum and Mitochondria (PERM) hypothesis, a comprehensive interactive scheme of cellular metabolism, has been posited to elucidate the role of xenobiotics and hormesis in cell survival and homeostasis.[8] However, though hormesis is known as a fact and is deemed to be a revolution in pharmacology and toxicology, its explanation has remained a long-standing conundrum.[9-12] To our knowledge, this is because a simple and direct "activity" based molecular mechanistic explanation is lacking. Therefore, several "mainstream scientists" deem hormesis to be a "marginal science" in cellular physiology, owing to its "proximity to homeopathic ideas."[13]

Over the last decade, we discovered some rather unusual activations and inhibitions in heme-enzyme catalyzed reactions in reductionist systems.[14-33] Recently, a bevy of heme and flavin enzyme reaction outcomes were explained by invoking upon "murburn concept," a new paradigm of "molecule-unbound ion–radical" interactions. This perception entails catalysis by diffusible species generated in the milieu, owing to enzyme activity.[27-31] These ideas helped explain the diverse substrate specificities of hemeperoxidases and cytochrome P450s (CPY), because we now had a reaction mechanism that did not occur at a specific active site alone. These pursuits culminated in a radically new mechanistic perspective for the fundamental process of cellular respiration.[34] Essentially, murburn concept is a stochastic paradigm of interactions that occur at a nanoscale, within subnano to submilli seconds. When one-electron transfers are involved, charge transfer between donor and acceptor does not necessarily involve an affinity-based interaction. The overall outcome is rather governed by thermodynamics, mobility of the species, dynamic partitioning, concentrations, and so on. Contrary to aesthetic perceptions that such a scheme could potentially lead to rather chaotic outcomes, it was found that the reactions afforded significant selectivity, specificity, and, at times, varying stoichiometry. These findings motivated us to think beyond the active-site limited mechanisms and kinetic explanations. Thus, the promiscuous and beneficial nature of "unavoidable" one-electron reactions in physiological milieu was advocated by murburn concept.

In several of the manuscripts cited above, we had professed that murburn concept could explain hormetic and idiosyncratic dose responses, because radicals are stabilized at unique concentrations in diverse milieu. To investigate and demonstrate our projections, this work uses the hepatocyte enzyme CYP2E1 to serve as a "murzyme" (an enzyme that works with murburn mechanism). We hypothesized that a murzyme could sponsor unusual dose responses because it could enable "reaction outside the active site," sponsored by a diffusible reactive species produced or stabilized or modulated by the enzyme. This simple consideration would afford the scope to explain for higher enzyme activities at dilute concentrations of additives because radicals are better stabilized at dilute concentrations. Further, radicals could also show varying preferences for optimal activity, which could be contingent upon the evolution of concentration terms in the mixture and be subjected to change with respect to environmental conditions. Thus, with murburn concept, we aimed to provide a direct and tangible molecular explanation to select hormetic/idiosyncratic dose responses.

Materials and Methods

Materials

All chemicals employed were of analytical grade. Nicotinamide adenine dinucleotide phosphate (NAD[P]H), para-nitrophenol (pNP), para-nitrocatechol (pNC), 7-ethoxy-4-trifluoromethylcoumarin (7EFC), 4-methylpyrazole, dibromohydroxybenzoic acid (DBHBA), dibromomethylphenol (DBMP), dibromophenol (DBP), diiodotyrosine (DIT), isoquinoline were procured from Sigma Aldrich(Sigma Aldrich, India and Sigma Aldrich, USA), Alfa-Aesar (Alfa-AesarUSA), or Lancaster (Lancaster,USA). The benzarone sources and their derivatives' preparation and purification were described earlier.[24] The procurement/preparation of enzymes and certain chemicals employed in the current study has been presented in manuscripts published earlier.[18,19,22,24,35] The structures and acronyms of small molecules employed/probed in the current study are listed in Supplementary Material, Item 1.

Overall Strategy

Herein, we employ CYP2E1, a heme-enzyme with a rather occluded distal site, for tracing interesting reaction outcomes in a minimalist setup. We aimed to probe CYP2E1 reactions through the incorporation of additives belonging to several diverse categories of molecules, some of them perceived as "heme-binders." Furthermore, the effect of incorporation of additive molecules and overexpression of select hemoproteins (such as cytochrome *c* and CYP3A4) on growth of *Escherichia coli* DH5α was used as a methodological tool for correlating the murburn mechanism with hormetic/idiosyncratic responses.

Preliminary and Extended Studies of Inhibition of CYP2E1 Activity by Diverse Molecules

Preliminary inhibition studies were carried out by a slightly modified spectrophotometric assay[36,37] using CYP2E1

baculosomes from Invitrogen. It was established in the laboratory that pNC thus estimated correlated to the high-performance liquid chromatography determination method,[18] which was used for extensive analysis of inhibitions. The 7EFC assay was as per the protocol we have earlier reported.[18]

In Silico (Docking) Studies

The details of procedures adopted are available from our recently published works.[24,27] The 3E6I pdb file was used as the rigid protein molecule for CYP2E1.[38]

Cross-Reactivity Studies With CYP2E1 (and Chloroperoxidase)

All enzymatic reactions were carried out (1 mL total reaction mixture) at 27°C ± 1°C in 100 mmol/L potassium phosphate buffer at pH 7.4. In these reactions, the final concentrations of the reactants were as follows: substrate (pNP) at 200 μmol/L, enzymes CYP2E1 at 0.6 μmol/L, and chloroperoxidase (CPO) at 0.3 μmol/L. Nicotinamide adenine dinucleotide (NADH) and H_2O_2 were taken at 360 μmol/L and 7.14 mmol/L, respectively. When employed, dilauroyl phosphatidyl choline (DLPC) was at 0.5 μg DLPC/pmol P450. The reactants were added in a 1-mL vial from the respective 10× stock concentrates, and the volume was made up to 1 mL with double distilled ultrapure water. The reaction was terminated by adding a chilled stopper solution (40% of the reaction volume) consisting of 6% glacial acetic acid in acetonitrile.

Simulation of CYP2E1-Cytochrome P450 Reductase Activity With Diffusible Reactive Oxygen Species

Initial concentrations of the reaction components were [pNP] = 25 μmol/L, [DLPC] = 20 μmol/L, and [CYP2E1] = 100 nmol/L. When present, the initial concentration of the following components was [NADPH] = 200 μmol/L, cytochrome P450 reductase [CPR] = 200 nmol/L, cytochrome b_5 [Cyt b_5] = 200 nmol/L, [H_2O_2] = 32 μmol/L, and [KO_2] ≤16 μmol/L. Superoxide stock for this reaction was prepared as follows: ∼5 mg of KO_2 was weighed and dissolved in 700 μL of 50:50 dimethyl sulfoxide (DMSO) and 18-crown ether to give a superoxide stock solution of ∼100 mmol/L concentration. From this solution, 2 μL was taken and added to 300 μL of 50:50 DMSO and 15-crown ether to give a mixture with ∼667 μmol/L concentration of superoxide. 10 μL of this substock was added to 400 μL of the reaction. Therefore, the reaction also had ∼1% DMSO and 15-crown ether. It is important to note that the actual initial concentration of superoxide may have been much lower than 16 μmol/L, because superoxide readily absorbs moisture (while weighing and from DMSO). The water molecules provide sufficient protons for the formation and stabilization of peroxide from superoxide; further reactions between them could lower the effective concentration significantly.[39]

Bacterial Cell Culture and Transformation

Luria-Bertani (LB) medium was purchased from HiMedia Co, India. Ampicillin was procured from Sigma Aldrich, India. The MTCC 428 cell line (*Bacillus megaterium*, same as ATCC 14581)[40] was purchased from IMTECH, Chandigarh, India. *E coli* DH5α [endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1 Δ(lacZYA-argF) U169deoR (φ80dlac Δ(lacZ) M15)][41] was a kind gift from Dr Aradhyam Gopala Krishna, IIT Madras, Tamil Nadu, India. A stab culture of *E coli* DH5α-bearing pBTR1 (Cyt *c*—a human mitochondrial electron transfer protein; Addgene plasmid 22468)[42] was purchased from Addgene, Cambridge, Massachusetts. The bacterial (heterologous) expression vector for human cytochrome P4503A4, pCWori+3A4,[43] was a kind gift from Dr Gary Yost, University of Utah. The type II binders azide (Azd) and amitrol were included at different concentrations in bacterial broth cultures in order to assess their effects on bacterial growth (OD_{600}) on native *E coli* DH5α (as an example for Gram-negative organism) and *B megaterium* MTCC 428 cells (to represent Gram-positive bacteria). Similar studies were also carried out using *E coli* DH5α cultures transformed with the heterologous heme/redox protein expression vectors, pBTR1 (cytochrome *c*—a human mitochondrial electron transfer protein) and pCWori+3A4 (CYP3A4—a human liver microsomal cytochrome P450 isozyme). The plasmids were freshly transformed into *E coli* DH5α cells using $CaCl_2$-mediated transformation.[44] Transformants were selected on super optimal broth (or LB) agar plates with 100 μg/mL ampicillin. Before being used in the culture, the transformants were screened for the presence of the plasmid of interest, by extraction using the alkaline lysis method[45] and identified using agarose gel (0.8% agarose) electrophoresis. The differences in bacterial growth between hemeprotein-induced (+ IPTG) and noninduced cells (no IPTG) helped determine the synergic effect, if any, of the additives (in the presence/absence of the overexpressed redox protein). The controls lacked Azd/amitrol and contained only ampicillin (100 μg/mL). Overnight transformed cultures of *E coli* were inoculated into autoclaved LB broth and cultured to obtain fresh inoculum of 1.0 OD_{600} or lesser, from which 1 mL was added to 98 mL sterile LB broth in 250 mL Erlenmeyer flasks; 1 mL ampicillin (from 10 mg/mL stock, 0.22 μm filter sterilized) was added in all the culture flasks, except in the ampicillin-lacking control. Additives were incorporated into the cultures at mmol/L, μmol/L, nmol/L, and pmol/L concentrations. The *E coli* transformants were subsequently cultured in LB broth overnight and diluted to ∼OD_{600} of 1.0 with freshly autoclaved LB broth (corresponding to ∼1.5×10^8 to 1×10^9 CFU/mL). All flasks had exactly 100 mL volume before the start of the experiment. The experiment was commenced with addition of 1% inoculum, which was carefully spaced by 2 minutes for each flask; OD_{600} was monitored initially and thereafter, inoculated flasks were maintained on a temperature controlled incubator shaker at 37°C (120 rpm), and growth of the cultures was monitored at periodic intervals. Experiments were performed in duplicates; IPTG was not

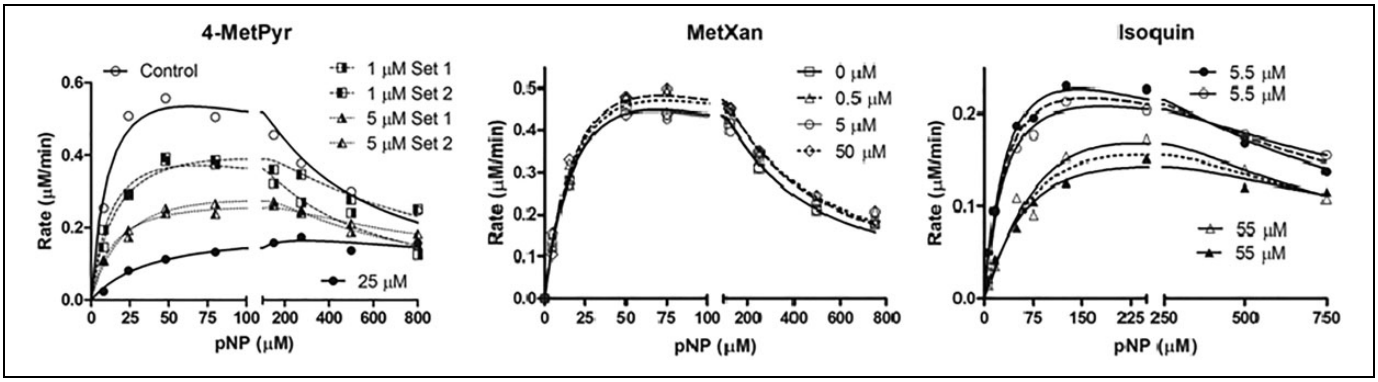

Figure 1. Profiling of CYP2E1 inhibitions over wide substrate and additives' concentration ranges. The initial concentrations of reactants were 25 nmol/L CYP, 100 nmol/L CPR, 50 nmol/L Cyt b_5, 0.5 mmol/L $NADP^+$ in regenerating system, and 0.02 mmol/L DLPC. Assay details have been described in our earlier works. CYP indicates cytochrome P450; CPR, cytochrome P450 reductase; Cyt b_5, cytochrome b_5; NADP, nicotinamide adenine dinucleotide; DLPC, dilauroyl phosphatidyl choline.

added to 1 set of flasks (containing mmol/L, μmol/L, nmol/L, pmol/L, and fmol/L additive). To another set, 0.5 mmol/L IPTG was added to induce expression from respective plasmids after the OD_{600} of ~0.3 was attained (barring a few cases, where IPTG was included at the start of the experiment). One milliliter of culture broth was withdrawn intermittently at specific time intervals for OD_{600} assessment and another 1 mL was taken and immediately refrigerated (for assaying protein content). At the end of the experiment, the chilled samples were centrifuged at 6000 rpm (rotor prechilled to 4°C). To the pelleted cells, 1 mL of ice-cold potassium phosphate buffer (50 mmol/L) containing 50 mmol/L NaCl and 10 mmol/L $MgCl_2$ was added (after washing twice with the same buffer). The cells were lysed by sonication, and the supernatants were further clarified by centrifugation at 4°C at 6000 rpm. Protein content in the lysate was assayed using Bradford method[46] by adding 20 μL of clarified supernatant to 1 mL of freshly diluted 1× Bradford reagent. The OD at 600 nm at initial time frames (1.5-6 hours) was experimentally found to correlate to cell density and total cellular protein concentration (within an experimental error margin of 10%-15%).

Statistical Analysis

All experiments were performed in triplicates; results were represented as mean (standard deviation). Significant differences were calculated using a GraphPad Prism, version 5.1 software. A 2-way analysis of variance was performed for all the experiments. Statistical significance were accepted at a level of $P < .001$ (***), $P < .01$ (**), and $P < .05$ (*), while $P > .05$ denotes statistical insignificance.

Results

Effect of the Incorporation of Select Additives in CYP2E1 Reactions

For the results of preliminary studies, please refer Item 2 of Supplementary Material. The discussion therein would facilitate a better understanding of the data and findings presented herein.

N-Heterocyclics

The left panel of Figure 1 demonstrates the effect of incorporating 4-methylpyrazole, a well-known CYP2E1 inhibitor.[47] The inhibition profile of this molecule was exhaustively studied as a function of enzyme, substrate, and inhibitor concentrations. The results were plotted into the erstwhile models of competitive, noncompetitive, uncompetitive, mixed inhibitions, and so on by linear and nonlinear regression plots (note 1). Although the plots mostly had acceptable R^2 and standard error values (just like the profiles peroxidases proffered with cyanide as inhibitor[25]), a visual examination showed that the erstwhile models did not do justice to the data. We found that the values of half maximal inhibitory concentration (IC_{50}), and as a consequence, K_i, measured at lower concentration ranges of inhibitors end up being much lower than the values at higher inhibitor concentrations (Supplementary Material, Item 3, points 2 and 3). The "heme-pocket–binding" hypothesis cannot explain why 3-methylxanthine (center panel of Figure 1) did not significantly perturb/inhibit pNP reactions even at 50 μmol/L concentration. The control reactions and the ones containing 3-methylxanthine acquire a negative slope after 50 to 75 μmol/L pNP. In reactions containing high amounts of isoquinoline (right panel of Figure 1), though the actual rates were lowered, the curve did not acquire a negative slope until the concentration of pNP was >200 μmol/L.

Halophenolics

We incorporated various dihalophenolics at equimolar concentrations of the substrate (100 μmol/L), equimolar concentration of the enzyme (10 nmol/L), and an intermediary concentration (1 μmol/L) to study the pNC formation at an early time (10 minutes) and later time (30 minutes) points. The results are displayed in Figure 2. At high concentrations of additives, all the molecules inhibited the reactions at earlier time frames. While the control rates remained constant with respect to time (quite akin to some

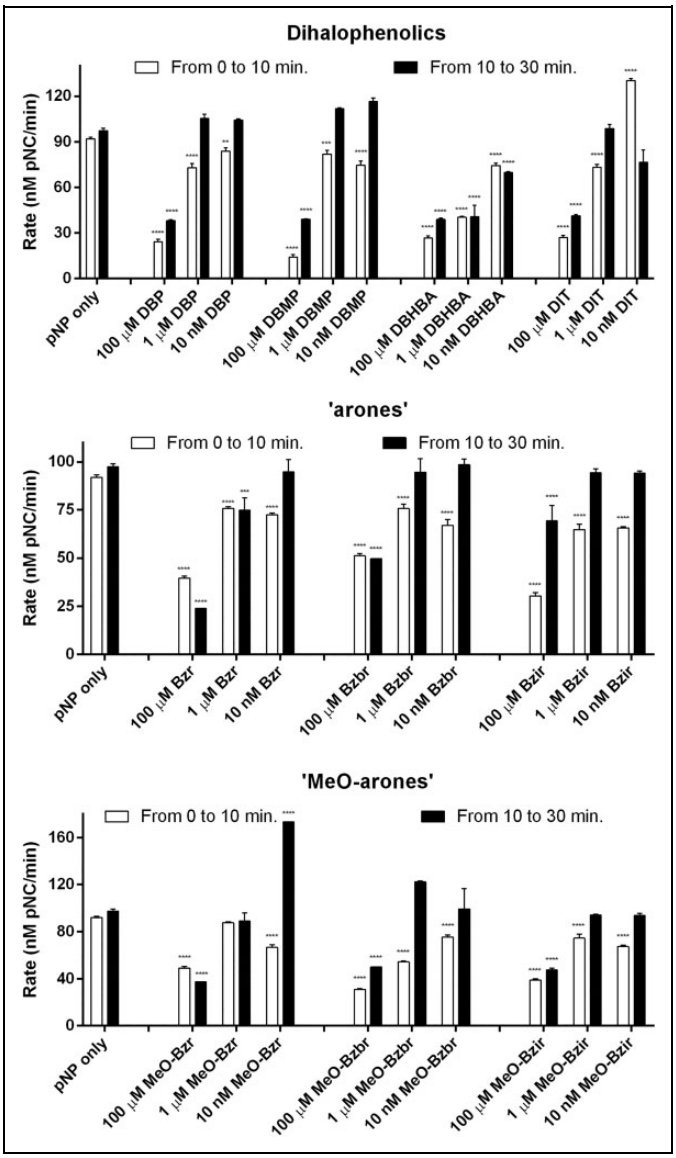

Figure 2. Variation of specific hydroxylated product formation rate as a function of additive concentration and sampling time. Inhibitor concentrations varied from 0 nmol/L, 10 nmol/L, 1 μmol/L, and 100 μmol/L. Substrate (pNP) = 100 μmol/L, 2E1 = 10 nmol/L, NADPH = 200 μmol/L, 2E1-pNP KM = 14 μmol/L. The velocity of reaction measured at 2 different time points was statistically analyzed by ANOVA. The mean rate (SD; n = 3) is represented as bars. Asterisks correspond to the respective significance herein: **** $P < .0001$; *** $P < .001$; ** $P < .01$; * $P < .05$; else $P > .05$. pNP indicates para-nitrophenol; NADPH, nicotinamide adenine dinucleotide phosphate; ANOVA, analysis of variance; SD, standard deviation.

test samples incorporating 1 μmol/L levels of additives like DBHBA, benzarone, and MeO-benzarone), the incorporation of additives gave maverick concentration- and time-dependent enhancement or lowering of rates of pNC formation. For example:

(i) while 10 nmol/L levels of DIT enhanced reactions at earlier time frames, higher amounts of the same molecule inhibited pNC formation for the same time period,
(ii) the data for 10 nmol/L of DBMP, benzarone, benzbromarone, MeO-benzarone, and MeO-benziodarone showed greater extent of inhibition at initial sampling time, when compared to 1 μmol/L levels of the respective additives in the same time period, and
(iii) while 10 nmol/L levels of DBMP and MeO-benzarone inhibited pNC formation at earlier times, they were found to activate pNC formation at later time points.

The dose–response curves plotted for many of these molecules do not fall within the purview of classical models, as shown in Figure 3. Moreover, a comprehensive analysis of IC_{50} and K_i with multiple approaches gave values spread over an unacceptable range (Table 1 and Supplementary Material, Item 3, Point 6), with either acceptable or nonacceptable standard deviations or R^2 values. These findings suggest a nonadherence to "active-site–centered activity" and "affinity-binding–based" theories and mechanisms. Else, we must accommodate irrational ideas (note 2) in the following ways:

(i) DBHBA binds relatively inefficiently at 100 μmol/L concentration (wherein it has a K_i of 5 μmol/L) whereas it binds effectively at a concentration of 10 nmol/L (wherein it has a K_i of 5 nmol/L)
(ii) DBMP binds better than DBHBA at 100 μmol/L concentration, whereas DBHBA binds better than DBMP at 10 nmol/L concentration.

Binding and Docking of Ligands to CYP2E1

With a CYP2E1 preparation (taken at ~1 μmol/L), the Soret absorption of the control was a mixture of low- and high-spin iron species. Upon the addition of the CYP2E1-specific substrate chlorzoxazone (200 μmol/L, at 0.2% acetonitrile), the Soret absorption maximum at ~412 nm was still seen to be unchanged (note 3). We could not observe any significant increase in the classical high-spin marker band at ~650 nm in this system upon the addition of chlorzoxazone at several hundred-fold concentrations. This implied that there was no significant enhancement in heme spin shift (if substrate binding had occurred). Since pNP was not "absorbance silent" in the visible spectrum, its binding study was not carried out.

The results of in silico docking of several substrates and additives are shown in Table 2. For the scope and limitations of such in silico exploration, please refer our earlier work.[27] Efficient substrates like pNP or diclofenac bound to the heme distal pocket with comparable energy terms as a nonsubstrate like diclofenac. Effective inhibitors, such as isoquinoline, benzothiadiazole, DBMP, 1-methyl imidazole, and pyridine, all showed varying binding energies (−3.5 to −6.4 kcal/mol), distances (4.6-11.8 Å), and associations with different amino acids within the distal pocket, suggesting that there was no common "logic" for their effects, as inferred from the heme distal pocket binding. At the heme distal pocket, quinoline, a poor inhibitor shows better binding energy, docking distance,

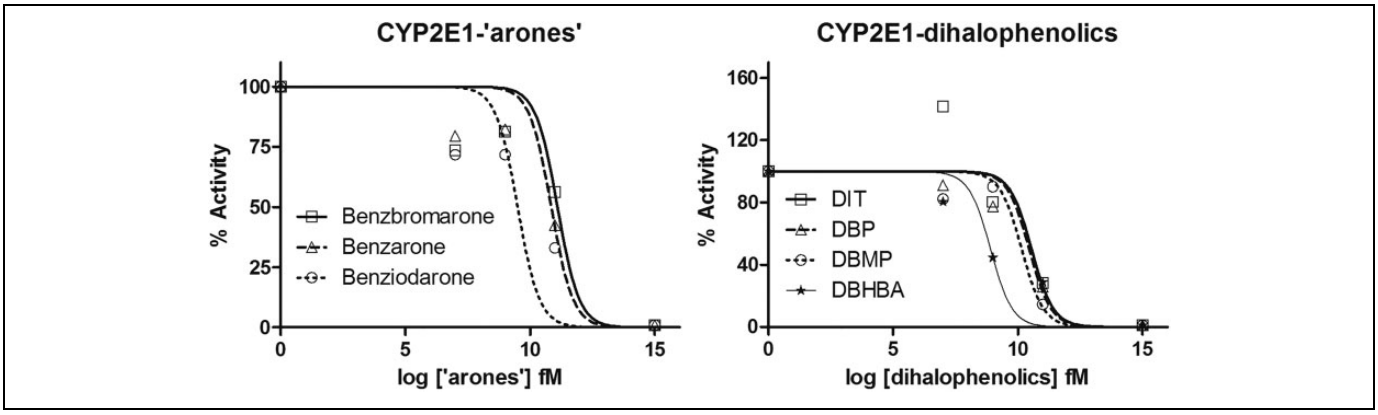

Figure 3. Dose–response curves for baculosome preparations of CYP2E1-pNP system. Experimental conditions are as detailed in legends to Figure 2. Rate data within 10 minutes was used for relative activity calculation, with respect to the reaction mixture that lacked any additive. Since activations were also observed, the plots were fitted weighing 1 fmol/L as 99.99% and 1 M as 0.01%. (The error bars are not seen because of low standard deviations.). CYP indicates cytochrome P450; pNP, para-nitrophenol.

Table 1. Classical Kinetic Constants Calculated for CYP2E1 Inhibitions.[a,b]

Serial Number	Molecule	IC_{50}	R^2	K_i Competitive	K_i Uncompetitive	K_i Noncompetitive	K_i (Linear)	R^2
1	Bzbr	126 (26)	−2.11	15.5 (3.1)	110.5	126	785055 (478883)	0.39
2	Bzr	71 (12)	0.31	8.7 (1.4)	62.3	71	195749 (64597)	0.67
3	Bzir	3 (0.8)	−0.71	0.4 (0.1)	2.6	3	126736 (31684)	0.75
4	MeOBzbr	2 (0.4)	−0.24	0.2 (0.04)	1.8	2	60534 (605)	0.99
5	MeOBzr	115 (20)	0.18	14.1 (2.5)	100.9	115	1232197 (443591)	0.64
6	MeOBzir	74 (15)	−0.27	9.1 (1.9)	64.9	74	321218 (173458)	0.46
7	DIT	–	–	–	–	–	–	–
8	DBP	29 (4)	0.81	3.5 (0.5)	25.4	29	14588 (584)	0.96
9	DBMP	14 (1.7)	0.91	1.8 (0.2)	12.3	14	13521 (5408)	0.60
10	DBHBA	1 (0.2)	0.19	0.1 (0.02)	0.9	1	34041 (1702)	0.95

Abbreviations: CYP, cytochrome P450; Bzbr, benzbromarone; Bzr, benzarone; Bzir, benziodarone; IC_{50}, half maximal inhibitory concentration; MeOBzbr, methoxybenzbromarone; MeOBzr, methoxybenzarone; MeOBzir, methoxybenziodarone; DIT, diiodotyrosine; DBP, dibromophenol; DBMP, dibromomethylphenol; DLPC, dilauroyl phosphatidyl choline; DBHBA, dibromohydroxybenzoic acid.

[a]All values are reported in μmol/L.

[b]Assay conditions are described in legends to the pertinent Figure 2. The IC_{50} values were derived with rates from early reaction time (10 minutes) data and only the experimental values were used for plotting/calculation. IC_{50} was calculated by nonlinear regression analysis from a log plot of inhibitor concentration versus percentage activity. The IC_{50} values thus obtained were used to determine K_i (for competitive inhibition) using Cheng-Prusoff equation (where [S] is the substrate concentration and K_M is the Michaelis-Menten constant, taken to be 14 μmol/L): $K_i = IC_{50}/(1 + [S]/K_M)$. K_i uncompetitive and K_i noncompetitive were calculated from the website (http://botdb.abcc.ncifcrf.gov/toxin/kiCalES.jsp). A linear regression method with a double logarithmic plot is also available for the calculation of K_i, as originally developed by Britton Chance. This equation, which does not use a K_M value, is: $\log [Ri/(Ru - Ri)] = \log K_i - \log [I]$, where Ri and Ru are inhibited and uninhibited rates, respectively, obtained with the inhibitor concentration [I].

and orientation compared to the efficient inhibitor, isoquinoline. Similarly, in the small halophenolic group, the efficient inhibitor of DBMP shows relatively unfavorable binding energy, docking distance, and orientations compared to the lesser efficient inhibitor, DBP. With the erstwhile hypothesis, we cannot reason why 3-methylxanthine, which has relatively better binding energy and proximity terms, is incapable of inhibition whereas DBMP is a very efficient inhibitor. The larger molecules ("arones" and their derivatives) would rather not be located in the heme distal pocket, as suggested by the positive binding energy terms. The effective inhibition by imidazole or its 4-bromo substitution is not explained by the binding energy or distances/orientation at the heme center, in comparison to the inefficient inhibitions proffered by 4-hydroxymethyl imidazole or 4-imidazole carboxylic acid. The heme distal pocket-binding hypothesis cannot explain why 1-aminopropylimidazole, with a good binding term and N-atom positioning, is experimentally observed to be a poorer inhibitor than some of the other imidazolics. Benzbromarone, which has no affinity for the heme distal pocket of CYP2E1 (Table 2), is also a decent functional inhibitor.

Changing Redox Partnering Systems to Mimic CYP2E1 Activity

Chloroperoxidase is 321 amino acids long, heme-thiolate extracellular protein secreted by the fungus *Caldariomyces fumago*. This pH-dependent enzyme is stable at acidic pH from 2.5 onward and denatures above pH 7. CPO has a polar distal heme pocket with Glu183, Phe186, His105, Phe103, Val67, and

Table 2. Docking of Ligands to CYP2E1.[a]

		Best Binding Parameters			
Number	Flexible Ligand	ΔG (kcal/mol)	Distance (Å)	Amino Acids	Orientation
1	Para-nitrophenol (pNP)	−5.6	5.7	VAL 364, LEU 368	+
2	Chlorzoxazone	−6.0	21.4	GLN 216, ASN 219	−
3	Diclofenac	−6.9	17.2	SER 472	+
4	Quinoline	−6.6	4.9	THR 303	+
5	Isoquinoline (Isoquin)	−6.4	6.6	ALA 299	−
6	3-Methylisoquinoline	−5.4	6.8	ILE 115	−
7	4-Methylpyrazole (4-MetPyr)	−4.3	6.9	ILE 115, THR 303	−
8	3-Methylxanthine (MetXan)	−5.8	8.8	LEU 133, ALA 438, GLY 441	−
9	Benzothiadiazole	−5.9	4.6	THR 303	−
10	Diiodotyrosine (DIT)	−6.2	19.2	SER 74, GLN 75, ASP 102, ASN 220	+
11	Dibromophenol (DBP)	−5.1	5.8	ILE 115, LEU 368	+
12	Dibromomethylphenol (DBMP)	−4.5	11.8	LEU 133, ASN 143	−
13	Dibromohydroxybenzoic acid (DBHBA)	−5.7	28.8	ASP 470, LYS 486	−
15	Benzarone (Bzr)	4.8	10.4	ASP 295	−
16	Benzbromarone (Bzbr)	2.6	10.4	ASN 143, ILE 183, LEU 442	−
17	Methoxybenzbromarone (MeOBzbr)	4.0	9.6	LEU 130, ASN 143	−
18	Imidazole	−3.1	15.1	THR 169, MET 200, SER 305	+
19	1-Methylimidazole	−3.5	5.4	ALA 299	+
20	1-Aminopropylimidazole	−4.9	6.2	PHE 298	−
21	4-Bromoimidazole	−4.0	4.0	THR 303	+
22	4-Hyroxymethylimidazole	−4.2	6.2	PHE 298, ALA 299, THR 303	−
23	4-Imidazole carboxylic acid	−4.7	4.2	PHE 298, THR 303	−
24	Pyridine	−4.5	5.4	THR 303	+
25	Pyrazine	−3.5	5.4	ILE 115, ALA 299	+
26	Piperazine	−3.6	26.3	PHE 153, ASP 190	−
27	Tiron	−6.5	13.7	GLN 216, ASN 219, ASN 220	−
28	Ascorbic acid	−5.4	12.6	GLN 216, ASN 219, ASN 367	+
29	3-Aminobenzotriazole (ABT)	−6.2	6.1	THR 303	−

[a] + indicates that the pertinent reactive/binding moiety is oriented toward the heme Fe, whereas − indicates that the same is oriented away from it.

Asn74 in the active site and extensive N-linked and O-linked glycosyl chains.[48] In contrast, CYP2E1 is a larger membrane-bound globular protein, 593 amino acids long and the hydrophobic heme distal pocket has Ala299, Thr303, Phe106, Val364, Ile115, Leu368, and Phe478.[38] A basic local alignment search tool (BLAST) study gave only 15% "query coverage" (note 4) and 47% "maximum identity" (note 5). We used (CPR + [O_2 + NADH/H_2O_2]) as the (partner + [activator/reductant]) system for CYP, instead of the traditional mixture of (CPR + Cyt b_5 + O_2 + NADPH). For CPO, when the enzyme usually gets activated with an H_2O_2 system alone, we used (CPR + O_2 + NADH/H_2O_2) for catalysis. The results are shown in Figure 4. In both systems, the enzymes were able to form pNC whereas the controls lacking the heme-thiolate enzymes did not afford significant amounts of the specific hydroxylated product. This qualitative finding suggests that specific recognition of the substrate at the heme distal pocket is not necessary for oxygen activation and also that electron transfer between CPR and CYP2E1/CPO need not be mediated through protein–-protein complexations either. Furthermore, attempts were made to derive CYP2E1-specific reaction with a reductionist system. Table 3 summarizes the results obtained. The negative control reaction (pNP with CPR + NADPH alone) gave negligible or trace amount of pNC. Positive control reaction of CPR + NADPH gave efficient hydroxylations with CYP2E1, which was further augmented by the presence of Cyt b_5, when Cyt b_5 is incapable of supporting hydroxylations on its own merit. When we used peroxide alone with CYP2E1, very small amounts of hydroxylation could be seen, which was enhanced by the addition of Cyt b_5. Superoxide alone or the same in conjunction with CYP2E1 (without CPR) afforded efficient pNC formation. The "specific" hydroxylation of pNP by CYP2E1 + superoxide + Cyt b_5 approached the efficient enzymatic catalytic yields of the positive control.

Effect of Incorporation of Azide on Gram-Positive and Gram-Negative Bacteria

The growth inhibition for both cultures at mmol/L concentration of azide may be attributed to hitherto understood toxicity of azide by "heme-pocket binding" based effects (Figure 5). Although azide was very effective in curbing the growth of *B megaterium* at mmol/L ranges, the effect was much less harsh on *E coli*. Azide did not impact *B megaterium* at lower concentrations (10^{-6} to 10^{-12} M), as the growth profile was relatively similar to that of the control (Figure 5A). However, for

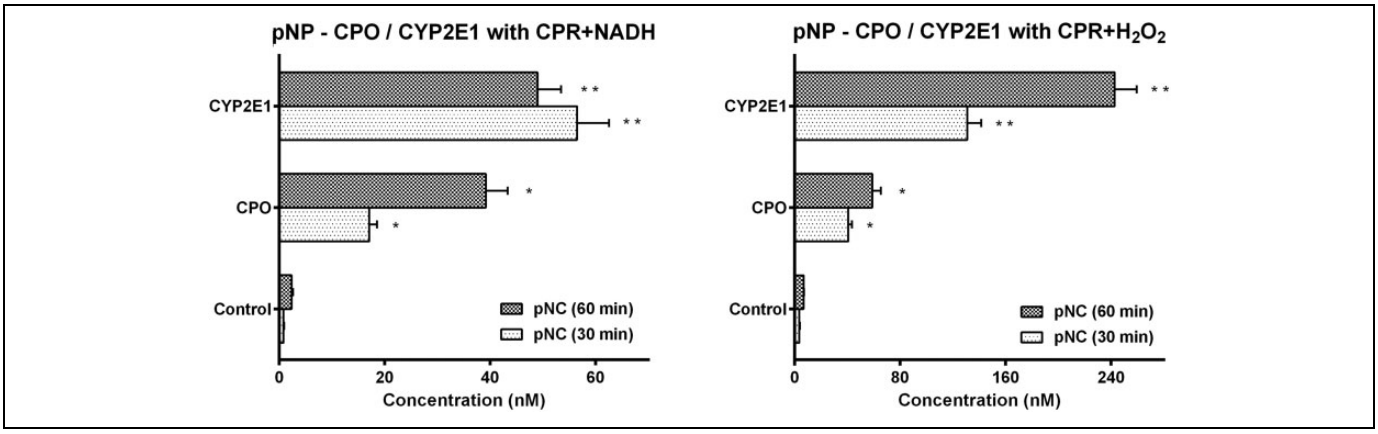

Figure 4. Demonstration of nonspecific partnering with diverse combinations of hemeproteins, reductases, auxiliary redox partners, substrates, and electron donors. Head labels of each subfigure represent the constituents of control reactions. All the reactions were carried out at 27°C ± 1°C in 100 mmol/L potassium phosphate buffer (pH 7.4). The initial concentration of substrate employed was 200 μmol/L. Initial concentrations were as follows: [CPO] = 0.3 μmol/L, [CYP2E1] = 0.6 μmol/L, [NADH] = 360 μmol/L, and [H_2O_2] = 7.14 mmol/L. The levels of pNC at different time points were statistically analyzed by ANOVA. The mean pNC (SD; n = 3) is represented as bars. Significant differences for all CYP2E1 reactions were $P < .01$ (**) and for all CPO reactions were $P < .05$ (*). pNC indicates para-nitrocatechol; ANOVA, analysis of variance; SD, standard deviation; CPO, chloroperoxidase.

Table 3. Simulation of Hydroxylating Activities of CYP2E1 With DROS.[a]

	pNP		Cyt b_5 + pNP	
Reaction	CYP2E1	Control	CYP2E1	Control
CPR+NADPH	450 (51)	Trace	786 (36)	Trace
H_2O_2	<10	Nil	~74	Nil
$O_2^{\cdot-}$	~104[b]	~88[b]	~499[b]	~70[b]

Abbreviations: CYP, cytochrome P450; DROS, diffusible reactive oxygen species; pNP, para-nitrophenol; Cyt b_5, cytochrome b_5; CPR, cytochrome P450 reductase; NADPH, nicotinamide adenine dinucleotide phosphate.
[a]Concentrations of hydroxylated products, formed after 10 minutes are in nmol/L. The experimental details are provided in "Methods" section.
[b]These reactions also showed nonspecific hydroxylations or side reactions.

Table 4. Effect of Redox-Active Small Molecules and Enzymes on Growth of *Escherichia coli* DH5α.[a]

	Azide-CYP3A4		Amitrol-Cyt *c*	
Concentration (M)	− IPTG	+ IPTG	− IPTG	+ IPTG
Control	100	95 (6)	100	102 (5)
10^{-3}	12 (5)	24 (3)	94 (4)	60 (9)
10^{-6}	97 (7)	79 (5)	103 (10)	70 (6)
10^{-9}	41 (6)	29 (6)	103 (3)	63 (5)
10^{-12}	107 (8)	32 (3)	50 (2)	51 (4)

Abbreviations: CYP, cytochrome P450; Cyt *c*, cytochrome *c*.
[a]Data shown are relative growth densities with respect to control (lacking both the additive and IPTG). For the controls of azide–CYP3A4 and amitrol–Cyt *c* cultures, the absolute OD values were 6 hours = 0.264 and 4 hours = 0.556. Other aspects are detailed in experimental descriptions.

E coli DH5α (Figure 5B), even nmol/L concentration of azide was inhibitory, whereas pmol/L concentration of azide gave ~35% to 40% enhancement. Very clearly, inhibitory effects at nmol/L concentration of azide (for *E coli* DH5α) cannot be owing to binding-based effects, as μmol/L concentration showed no inhibition whatsoever. Therefore, both activation and inhibition of growth (non-unidirectional; not as a single bell-shaped curve) was observed with the same molecule in a concentration domain where "binding-based" effects are not expected.

Involvement of a Nonspecific Heme-Enzyme in Hormesis, With Azide and Amitrol as Probes

To test the hypothesis that heme enzymes could be involved in hormetic dose responses, *E coli* DH5α was transformed with plasmids-harboring CYP3A4 and cytochrome *c* genes, and the effects of protein induction were traced by the addition of IPTG. Table 4 shows the results obtained. As seen, the controls were quite reproducible and therefore, the data can be used for comparison across the particular experiment. As expected, azide produced hormetic effects, which was altered to various extents by the expression of CYP3A4. Particularly, at low concentrations, the inhibitory effect was pronounced. In the amitrol-cytochrome *c* experiment, very clearly, this inhibitory effect is pronounced at mmol/L to nmol/L concentrations.

Discussion

CYP2E1 reductionist system presents direct evidence for "outside the active-site" reactions, sponsoring hormetic/idiosyncratic dose responses: earlier, we had dissected several erroneous assumptions in estimations of substrate-binding–associated spin shifts based on differential Soret measurements.[27] These ideas could explain our current binding/docking results and others' intriguing results where a K_d of 0.005 μmol/L for indazole with CYP2E1 was reported, when the enzyme

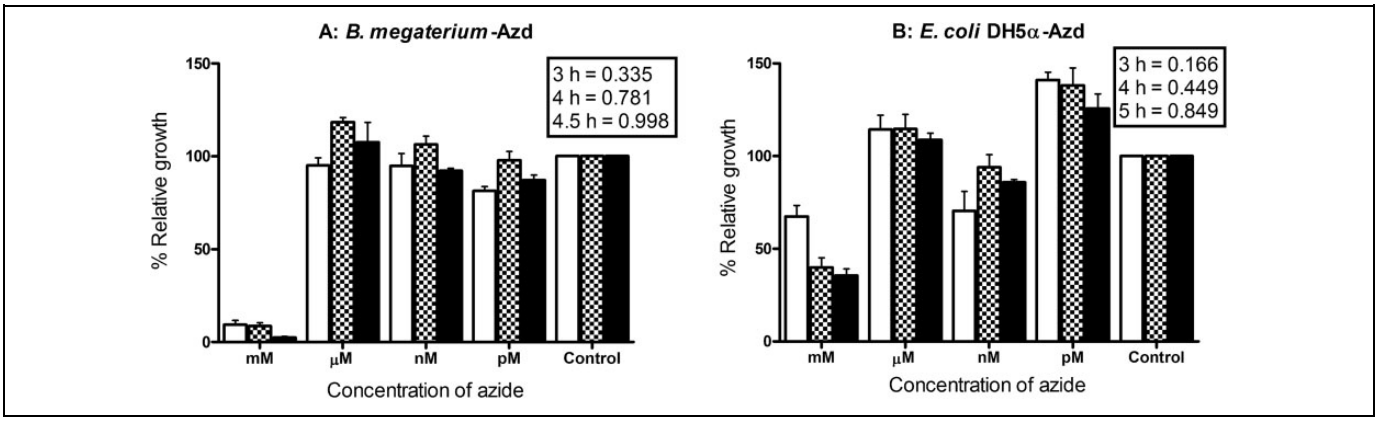

Figure 5. Effect of azide (Azd) concentration on growth of a Gram-negative and Gram-positive bacteria. The data are represented as % OD (or relative growth) wrt the control sample at the corresponding time. The values in boxes above the control stand for the OD at 600 nm for the respective time periods. Averages for % control growth were within 10% of the absolute control values (test values normalized to control values in the figures). In (A), control value was 100 (9), 100 (7), and 100 (5) for 3 hours, 4 hours, and 4.5 hours, respectively. In (B), control value was 100 (9), 100 (5), and 100 (5) for 3 hours, 4 hours, and 5 hours, respectively.

was taken at 0.1 μmol/L.[49] Further, we have also brought forth the simple argument that at nmol/L levels of enzymes taken in the reaction vial, the kinetic viability of micromolar levels of diverse substrates (or even a small molecule like peroxide) reaching the occluded heme center through some channels would be very low.[25,29] Therefore, access to heme-iron cannot be "taken for granted" (note 6) The "low-mobility" scenarios of lipid membranes would only compound this effect. Further, the pK_a of the 4 dihalophenolics studied range between 6.9 and 8.6. The molecules show greater extents of inhibitions with increase in their pK_a and this finding goes against the idea advocated by researchers that the negative charge on phenolate is required[50] for binding to amino acid residues (or heme, as can be speculated) in the heme distal site. It was seen here that highly polar radical modulators (cytochrome *c* or vitamin C) could also affect CYP2E1 activity. Since these molecules pose little scope for access to the active site (or protein–protein interactions), we are forced to think beyond the active-site (heme distal pocket) criteria for explaining our observations.

Using diverse molecules of varying topographies and functional groups, elaborate experimentation meticulously points out the nonadherence of the CYP2E1 system to Michaelis-Menten kinetics (or any model of inhibitions based thereof). Therefore, the obvious message is that the currently adopted approach (of determining IC_{50} and K_i with a fixed enzyme and substrate concentration, with a few of varying additive concentrations) may have little relevance for in vivo considerations. We have demonstrated herein and elsewhere (for CYP2C9 and several other heme-enzyme reactions) that smaller IC_{50} and K_i values were noted at lower concentrations of inhibitor, and the effects also depended on environmental conditions.[24,29] Since binding would only be inefficient at dilute concentrations, the enhanced inhibition at dilute inhibitors' concentration cannot be attributed to binding site blockage. These findings render the classical mechanistic approach untenable. It must also be noted that CYP2E1 reactions do not show significant size- or topography-dependent effects in catalysis, as seen from SuperCYP database.[51] It lists at least 260 diverse drugs as substrates or inducers or inhibitors of CYP2E1. Further, the current communication and our early works[24,27] clearly vouch that CYP2E1's occluded heme centers cannot mediate such a plethora of reactions at the heme distal site. The fact that the crystal structure of CYP2E1 does not show any channel with "bottleneck dimensions" larger than a couple of Angstroms on the distal pocket side is ample support for the consideration that benzbromarone would have very low probability to gain access to the heme distal pocket. Therefore, the inhibition by benzbromarone must be an "outside the active-site" phenomenon, and this is a deduction supported by the fact that even CYP2C9 is inhibited by benzbromarone.[24] If CYP2E1's F and G loops are highly mobile and their movement could make way for diverse substrates and inhibitors to bind at the heme center, the subsequently bound molecules (wherein the F and G loops have closed in) should have some preferences or antagonisms to explain for the selectivity of the substrates or modulation ability of the additives. As found in our earlier study,[27] the current in silico explorations also failed to arrive at such anticipated observations. Therefore, inferences and predictive protocols that discount (1) the effects of cosolvents and environmental features (as exemplified by[49,52,53]) and predict in vivo CYP kinetics based on purely CYP–drug binding[54] and (2) vouch for elaborate schemes of protein–protein complexes (homo/hetero dimmers)[55] stand to be questioned. When minutes to hours of preincubation of proteins mixes can significantly enhance reaction efficiency in CYP + CPR reactions, it is not viable to suppose that protein complexations hold sway over kinetics in such systems. This is particularly the case because in several in vitro assays, the proteins are in nmol/L ranges. The collision frequencies of such proteins (embedded in heterogeneous membranes) would fail to explain the high reaction rates observed in the systems.[27,30]

The current observations necessitate a paradigm shift in interpretation, and murburn concept offers the scope for explaining the experimental observations. For example, in the data presented herein, a question that could bog a keen observer is, How can 3-methylxanthine and isoquinoline (and higher concentrations of 4-methylpyrazole, as shown in Figure 1) alleviate substrate inhibition (which is clearly seen in the controls lacking the inhibitor)? The erstwhile mechanistic and kinetic treatments fail to explain these findings. Murburn concept (which invokes upon obligatory roles of diffusible radicals/reactive species and reactions occurring outside heme distal pocket) could potentially come to our aid for the same. If isoquinoline (and 4-methylpyrazole) effectively vied for the diffusible reactive intermediate or intermediates formed in milieu, the outcome could be justified. The clear inference drawn from the current work is that activation or inhibition in a CYP reaction can be brought about even if the inhibitor does not belong to the type I or type II binder class. In fact, inhibition at lower enzyme and additive concentration is inferred to be owing to a radical stabilizing ability/characteristic of the additive/milieu. The point we want to stress on is that such examples are not the major representatives CYP–substrate dynamics. Drug–drug interactions and idiosyncratic dose responses to diverse drugs may not result primarily because "CYPs bind with some active-site compatible molecules for a protracted time and therefore, they stop acting on others." Drug interactions could mostly result in vivo primarily because the concerned drugs have comparable or preferred redox-active specifics, electronic density distribution within the molecular framework, and partitioning (log *P*/log *D*) aspects. Dimensional constraints, topography, and electrostatics (factors that exert critical roles in pharmacodynamics) also play roles, but to a much lesser extent in pharmacokinetics.[28] The qualitative results we derived with "alternate redox partnering" and "CYP + diffusible reactive oxygen species (DROS) simulations" of native enzyme activities are in accordance with our earlier findings on CYP2C9.[30] The findings suggest that the CYPs use diffusible ROS generated by CPR and Cyt b_5 merely serves as an interim "electron-buffering relay." As per the murburn concept, the CYP reactions could use/release DROS, whose buildup and interactions within the milieu could afford the effects obtained in Figure 2. (Disclaimers: There can also be some cases where a synthetic drug could have excellent molecular-binding complementarity with a given CYP's distal pocket and channel, as exemplified by bromoergocryptine and CYP3A4.[56] We do not challenge the established fact that metabolic rate [in vivo] of several drugs is inhibited by molecules like ketoconazole[57] or furanocoumarins [of grapefruit juice fame[58]] and some of these effects may be brought out by alternate mechanisms too. Also, a drug like bufuralol could go through an active-site enantioselective hydroxylation of the carbon atom, by the isozyme CYP2D6. To us, such examples are salient exceptions to the overall paradigm. Please refer note 6 for further clarifications.) Figure 4, in conjunction with Table 3, allows us to make some very important and forthright deductions. CYP2E1 with peroxide alone is inefficient at pNP hydroxylation. However, incorporation of CPR (along with peroxide) gives effective pNP hydroxylation by the CYP. The CPR is known to break down peroxide via a radical pathway.[19] So, CPR + NADPH or CPR + peroxide combinations serve to provide CYPs the radicals (superoxide/hydroxyl, etc) for stabilization at its heme center distal pocket, for finally reacting with the substrate in itself or around its vicinity. The same argument is corroborated by the data with CPO, which cannot hydroxylate pNP with peroxide. The lower yield of hydroxylation with CPO can be explained by the polar active site of CPO, which contains a distal glutamate[48] adjacent to the heme Fe. Both these facets would destabilize a 1-electron charged ligand like superoxide.[28,29] Therefore, electron transfer from CPR to CYPs does not need protein–protein complexation. Cyt b_5 (Table 3) can now be seen as a transient electron buffer, rather than as a direct electron transfer agent to CYP2E1.

Inhibitory and Activating Hormetic and Idiosyncratic Dose Responses in Bacterial Growth

Classical interaction paradigms only afforded a unidirectional correlation for activity (enzyme/receptor vis-à-vis activator or inhibitor) or the highly fastidious multibinding scenarios for a given ligand for multiple effects like mixed inhibition. However, the binding-based explanations fall flat in terms of Ockam razor and also because of the dilute nature of the additives. Sodium azide has been used routinely in buffers as a bacteriostatic agent,[59] and this is reported to be due to its ability to inhibit Cyt *c* oxidase (complex IV of the electron transfer chain) of Gram-negative bacteria.[60,61] Recent revelations employing murburn mechanistic viewpoints challenge such inferences.[32,33] Azide is also known to show "positive effects"—it enhances the growth bacterial cells and also breaks seed dormancy.[62] The effects of azide-mediated growth enhancements in eukaryotic cells have been attributed to nitric oxide generation and concomitant signaling events.[63] Amitrol has been demonstrated to improve vase life of carnation flowers.[64] These 2 molecules were probed for a heme-assisted hormetic effect on the growth curve of bacterial culture. The findings of this work afford a molecular basis for hormesis. If dilution of certain molecules could potentially enhance their biological activity by increasing or decreasing the rate of production of any specific and important metabolite, it could result in a physiologically and morphologically observable outcome. The incorporated heme/redox enzymes could act on a metabolite/molecule of interest, and this reaction could be (in real space/time, within the cellular pool) enhanced or inhibited by the "hormetic probe" (such as azide or amitrol, in conjunction with a heme redox enzyme). Apart from activation through cellular signaling (which leads to nitric oxide–mediated heme-enzyme affects/effects), our studies clearly reveal that maverick azide-sponsored dose responses is more directly due to the physiological catalysis mediated by azidyl radical (duly generated by heme enzymes presence with trace amounts of azide).[22]

Conclusions

Using CYP2E1 reaction milieu, we have provided several evidences and arguments (both experimental and theoretical) for the obligatory involvement of diffusible radicals, particularly at low-concentration scenarios of the heme-thiolate proteins and additives. Further, we have also demonstrated that it is a very misplaced endeavor to derive kinetic constants from *in vitro* data and use it for analogy with liver microsomes, owing to inapplicability of the erstwhile hypothesis and the complications of the *in vivo* system. Herein, several projections of murburn concept are reconfirmed and further consolidated. The current communication advocates murburn concept as a probable mechanistic/molecular explanation for idiosyncratic and hormetic physiological dose responses. We caution that the rationale borne out of this work should not be taken for justifying the "dilution principle" of homeopathy. This is because: (1) little influence is seen below dilutions of 10^{-18} M and (2) the dilution is not unidirectional in correlation with activity enhancement. We reason that some activity effects are noted at ultra-dilute concentrations (<pmol/L or fmol/L) only because trace amounts of the substance could still be carried into the milieu by the dispensing pipette, owing to surface activity of the solute. Homeopathic preparations are purported to "work" at greater potency with dilutions going way beyond the physical feasibility of finding even one molecule of the drug within milieu, by virtue of solvent retaining "memory" of the solute. This work does not support such effects. But one could definitely say that some drugs might work only at dilute concentrations due to the effects shown in the current study (and owing to other rationale already established, as discussed in "Introduction" section). However, the concentration range at which a molecule would bring out a desired effect would be very difficult to predict, as it may be too specific to generalize or standardize. It is now evident that idiosyncratic and hormetic dose responses are seen owing to the functional (obligatory) involvement of diffusible reactive species/radicals. In some individuals, a particular biomolecule might accumulate in certain cells or could even get covalently attached to some membrane moieties. These stochastic events could mediate radical mediated reactions when a small molecule "allergen" or "drug" is presented, invoking an immune or secondary response. Such influences could also explain why ascorbate (vitamin C) could be beneficial (for the treatment of membrane inflammation symptoms/diseases such as coryza) to some individuals, whereas in other individuals (particularly with poorer health of "mitochondria or hepatocytes," wherein large amounts of redox enzymes are concentrated), it could aggravate the symptoms.

Therefore, it is now opportune to explore the details that "regulate" the outcomes in murburn mechanism -

A. Constitutive controls: Dynamic partitioning of reactants, products and intermediates, dielectrics of the reaction ambiance, concentration effects, proton availability, spin controls (of the metal and oxygen species), charge transfers and stabilizations of the metal species (eg, Fe II vs Fe IV), etc.
B. Charting the space and time scales: Stability and migration of 1-electron species in real space and time within a given reaction microenvironment, factors determining overall selectivity/specificity, and so on.
C. Dynamic tracing of the fates of intermediates (collapses with ionic species and other additives) and stoichiometry within the particular reaction milieu.

It is envisaged that pursuits of the operative principles in the reductionist milieu could offer us a greater understanding of the physiological outcomes. With increasing receptivity and awareness, we envisage that the extremely complex theoretical and experimental paradigm can be explored and detailed.

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Supplemental Material

Supplementary material for this article is available online.

Notes

1. Please refer to Supplementary Material, Item 3, points 1 through 4, for a more detailed discussion on data acquisition and analysis. We have presented it to demonstrate that though a high level of precision was obtained many times, accuracy for the value of "kinetic constants" was unobtainable, even with the most stringent conditions.
2. Please refer Item 3, point 6 of Supplementary Material for a contextual discussion.
3. Results are not shown, owing to redundancy.
4. This value does not take into account the length of the hit, but only the percentage of the query that aligns with the hit.
5. This is the extent to which 2 amino acid sequences have the same residues at the same positions in an alignment; greater values mean greater similarities.
6. Several mechanistic perceptions in heme enzymology were driven by binding studies and spectroscopy carried out at unrealistic (nonphysiological or noncontextual) concentrations of the enzyme and substrate. If nmol/L concentrations of chloroperoxidase and μmol/L levels of peroxide are taken (as is usual in enzyme assays), the

peroxide does not get depleted. But if we take µmol/L levels of enzyme and µmol/L-mmol/L levels of peroxide, the peroxide gets depleted quickly. In the former case, peroxide is unable to access the distal pocket whereas in the latter case, the peroxide gets depleted going through an active-site process. The thermodynamic control exercised in peroxide depletion within the former system can be demonstrated by the addition of a 1-electron donor substrate. It would be seen that even in the kinetically limited regime (when peroxide has low probability of accessing the distal heme pocket), peroxide goes through a rapid depletion through a non–active-site process.[29]

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