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Effect of structural modulation of polyphenolic compounds on the inhibition of *Escherichia coli* ATP synthase

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Abstract

In this paper we present the inhibitory effect of a variety of structurally modulated/modified polyphenolic compounds on purified F₁ or membrane bound F₁F₀ *E. coli* ATP synthase. Structural modulation of polyphenols with two phenolic rings inhibited ATP synthase essentially completely; one or three ringed polyphenols individually or fused together inhibited partially. We found that the position of hydroxyl and nitro groups play critical role in the degree of binding and inhibition of ATPase activity. The extended positioning of hydroxyl groups on imino diphenolic compounds diminished the inhibition and abridged position enhanced the inhibition potency. This was contrary to the effect by simple single ringed phenolic compounds where extended positioning of hydroxyl group was found to be effective for inhibition. Also, introduction of nitro group augmented the inhibition on molar scale in comparison to the inhibition by resveratrol but addition of phosphate group did not. Similarly, aromatic diol or triol with rigid or planar ring structure and no free rotation poorly inhibited the ATPase activity. The inhibition was identical in both F₁F₀ membrane preparations as well as in isolated purified F₁ and was reversible in all cases. Growth assays suggested that modulated compounds used in this study inhibited F₁-ATPase as well as ATP synthesis nearly equally.

Keywords

F₁F₀ ATP synthase; *E. coli* ATP synthase; ATPase; Polyphenolic compounds; Enzyme inhibition; ATP synthase inhibition

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Introduction

Cellular energy production in animals, plants, and almost all microorganisms is carried out by ATP synthase through oxidative or photophosphorylation. The simplest form of F_1F_0 -ATP synthase is found in *Escherichia coli* where eight subunits can be divided into water soluble F_1 ($\alpha_3\beta_3\gamma\delta\epsilon$) and membrane embedded F_0 (ab_2c_{10}). The total molecular size of *E. coli* ATP synthase is ~530 kDa. ATP hydrolysis and synthesis occurs on three catalytic sites in the F_1 sector, whereas proton transport occurs through the membrane embedded F_0 [1–2]. Separation of water soluble F_1 sector from the F_0 sector does not affect the ATP hydrolysis function of F_1 and proton conduction in F_0 sector. ATP synthesis occurs through the energy supplied by electrochemical transmembrane proton gradient. A unique “rotary system” couples the proton flow through F_0 down the gradient to ATP synthesis on F_1 . This unique “rotary system” is composed of γ , ϵ , and a ring of c subunits. Rotation of γ -subunit induces conformational changes in nucleotide binding catalytic sites of the β -subunit resulting in formation and release of ATP. Proton gradient-driven clockwise rotation of γ (as viewed from the membrane) leads to ATP synthesis and anticlockwise rotation of γ results from ATP hydrolysis. Subunit b_2 and δ make up the “stator”. The function of the stator is to prevent co-rotation of catalytic sites as well as the a subunit with the rotor [3–4]. Detailed reviews of ATP synthase structure and function may be found in references [5–14].

ATP synthase is critical to human health. Malfunction of this complex has been implicated in a wide variety of diseases including Alzheimer’s, Parkinson’s, Leigh syndrome, neuropathy, Batten’s disease, and the class of severely debilitating diseases known collectively as mitochondrial myopathies ([15] and reference therein). ATP synthase is also a likely target for the treatment of diseases like cancer, heart diseases, cystic fibrosis, diabetes, ulcers and tuberculosis [15–19]. Thus, a better understanding of this enzyme will greatly aid patients with these diseases and will have a broad impact on biology and medicine.

A wide range of natural and synthetic products including polyphenols are known to bind and inhibit ATP synthase. Polyphenols are naturally occurring plant based phyto-chemicals which possess antioxidant, chemo-preventive, and chemotherapeutic properties [20–23]. Foods such as apples, berries, cantaloupe, cherries, grapes, pears, plums, broccoli, cabbages, and onions are rich in polyphenols [24]. Some polyphenols are known to block the action of enzymes and other substances that promote the growth of cancer cells [25–28]. Furthermore, the beneficial effects of dietary polyphenols are, in part, linked to the blocking of ATP synthesis in tumor cells thereby leading to apoptosis [20]. Polyphenols are also known to have antimicrobial activity through inhibitory actions on ATP synthase. Thus, understanding the mechanism of polyphenol actions may lead to the development of better strategies for combating the pathogenic effects of bacteria. Biological activity against *Streptococcus mutans* is one example. *S. mutans* is a primary microbial agent in the pathogenesis of dental caries. It was shown that polyphenols can inhibit biofilm formation and acid production of *S. mutans* by inhibiting its proton-translocating F_1 -ATPase activity [29–31].

Lately we have shown that polyphenols, resveratrol (IC_{50} ~94 μ M), piceatannol (IC_{50} ~14 μ M), quercetin (IC_{50} ~33 μ M), quercetrin (IC_{50} ~20 μ M), or quercetin-3- β -D glucoside

(IC₅₀ ~71 μ M) inhibit *Escherichia coli* ATP synthase partially or maximally in a reversible manner [32]. Apparently the inhibitory concentrations on molar scale are much higher than desired for therapeutic purposes at physiological range. Hence, in order to reap the full benefits of polyphenols physiologically, it's vital to identify potent polyphenol inhibitors on molar scale. The position of the hydroxyl groups, along with two or more phenolic structures of polyphenols, appears to be critical in exerting the inhibitory effect on ATP synthase [33]. Therefore, we embarked on the structural modification of polyphenols to develop and identify the potent *E. coli* ATP synthase inhibitors on molar scale. Structural modulations of polyphenols will also facilitate the development of polyphenols usage as antimicrobial and chemo-preventive agents. By and large, inhibitory studies of the wild-type and mutant *E. coli* ATP synthase by natural or structurally modified polyphenol compounds will divulge a wealth of information which could provide basis to develop new therapies for diseases like cancer and set ways to contend with pathogenic bacteria.

In this paper we present the inhibitory effect of a variety of structurally modulated/modified polyphenolic compounds on *E. coli* ATP synthase using both purified F₁-ATPase and membrane bound F₁F₀ ATP synthase preparations.

Materials and Methods

Measurement of growth yield in limiting glucose medium; preparation of *E. coli* membranes; purification of *E. coli* F₁; assay of ATPase activity of membranes or purified F₁; measurement of proton pumping in membrane vesicles

The wild-type *E. coli* strain pBWU13.4/DK8 was used throughout this study [34]. Measurement of Growth yield in limiting glucose was as in [35]. F₁F₀ *E. coli* membrane preparations were as in [36]. It should be noted that this procedure involves three washes of the initial membrane pellets. The first wash is performed in buffer containing 50 mM TES pH 7.0, 15% glycerol, 40 mM 6-aminohexanoic acid, 5 mM p-aminobenzamidine. The following two washes are performed in buffer containing 5 mM TES pH 7.0, 15% glycerol, 40 mM 6-aminohexanoic acid, 5 mM p-aminobenzamidine, 0.5 mM DTT, 0.5 mM EDTA.

Membranes were washed twice more by resuspension and ultracentrifugation in 50 mM TrisSO₄ pH 8.0, 2.5 mM MgSO₄ prior to the experiments. Isolated F₁ was purified as described in Ref [37]. F₁ samples (100 μ l) were passed twice through 1-ml centrifuge columns (Sephadex G-50) equilibrated in 50mM TrisSO₄ pH 8.0, to remove catalytic site bound-nucleotide prior to the experiments. ATPase activity was measured in 1 ml assay buffer containing 10 mM NaATP, 4 mM MgCl₂, 50 mM TrisSO₄, and pH 8.5 at 37 °C. Reactions were started by addition of 1 ml assay buffer to the purified F₁ or membranes and stopped by addition of 1 ml 10% SDS. Taussky and Shorr reagent was used to assay the Pi release as in [38]. ATPase assay on membranes required (30 – 50 μ g protein) with reaction times from 20 to 30 minutes while purified F₁ required 20 μ g protein with reaction times between 2–5 minutes. All reactions were shown to be linear with time and protein concentration. Membranes were also tested for ATP-driven proton pumping activity which is measured using acridine orange and expressed as percent quench of acridine orange fluorescence in membrane vesicles upon addition of 1 mM MgATP. Measurements were performed as described in [39]. Purity, integrity, and composition of protein subunits were

checked by SDS-gel electrophoresis on 10% acrylamide gels as in [40]. Immunoblotting with rabbit polyclonal anti-F₁- α and anti-F₁- β antibodies was as in [41].

Source of Polyphenols and other chemicals

Synthesis of Polyphenolic compounds—Detailed synthesis, purity and spectral properties of modified polyphenols used in this study are presented in ref [42] while dihydrothymoquinone (¹ PC01) was prepared by catalytic hydrogenation of thymoquinone over 10% Pd/C catalyst. Aldimine polyphenols were synthesized by either one of the two general methods listed below. All physical and spectral data of the products were consistent with literature data. Method 1 (to prepare salicylaldimine derivatives PC05, PC06, PC09, PC12, or PC13): Equimolar amounts (10 mmole) of each of salicylaldehyde and amine were mixed in a small glass flask and heated in a kitchen-style microwave oven for 3–5 minutes. After cooling the crude product was recrystallized from methanol to afford 85–95% yield. Method 2 (to prepare aldimine derivatives PC07, PC08, PC10, or PC11): Equimolar amounts (0.1 mole) of each of aldehyde and amine were dissolved in absolute ethanol (150 mL) in a round-bottom flask equipped with a Dean-Stark receiver and a condenser. The solution was refluxed while allowing the water that is formed in the reaction to distill out, heating continued for 2 hours or until no more water was observed in the distillate. The remaining solution was concentrated to 50 mL under reduced pressure; pure products were allowed to crystallize from the solution upon cooling. Reaction yield ranged from 45% to 65% [43]. For inhibitory effects polyphenol stock solutions were resuspended in DMSO simply by weighing out. All other compounds or chemicals used in this study were ultra pure analytical grade purchased either from Sigma –Aldrich Chemical Company or Fisher Scientific Company.

Inhibition of F₁F₀ or purified F₁ ATPase activity by structurally modified polyphenolic compounds PC01-PC17

Membranes or purified F₁ (0.2–1.0 mg/ml) were preincubated with varied concentrations of polyphenol compounds, PC01-PC17 (to maintain clarity seventeen polyphenolic compounds used in this study are designated as PC01-PC17, see figure 2) for 60 min at room temperature, in 50 mM TrisSO₄ pH 8.0. Then 1 ml ATPase assay buffer was added to measure the activity.

Reversal of purified F₁ or membrane bound enzyme ATPase activity from PC01- PC17

Reversibility of inhibition was assayed by dilution of the membrane enzyme or by passing the inhibited purified F₁ through centrifuge columns. Membranes were first reacted with inhibitory concentration of PC01- PC17 for 1 hour at room temperature. These concentrations were used based on the maximal inhibition of the ATP synthase (see figure 3–7). Then 50 mM TrisSO₄ pH 8.0 buffer was added to decrease the concentrations to non inhibitory levels and incubation continued for 1 additional hour at room temperature before ATPase assay. Reversibility was also tested by passing the PC01- PC17 inhibited purified F₁

¹Abbreviations used: PC, Polyphenolic compounds; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1, 3-diazole; TES, N,N,N',N'-Tetraethylsulfamide; Mbr, membrane containing ATP synthase; IC₅₀ corresponds to the concentration of inhibitor where 50% of maximal inhibition was observed.

enzyme twice through 1 ml centrifuge columns before measuring the ATPase activity. Control samples without PC01- PC17 were also incubated for the same time periods as the samples with PC01-PC17. Two consecutive passages through centrifuge columns were previously found to decrease the concentration of small molecules bound to ATP synthase and other proteins to non-detectable levels. Thus, after passage through centrifuge columns, reactivation is likely a first-order kinetic process that is a function of release of bound inhibitor.

Results

Inhibition of ATPase activity of purified F_1 or F_1F_0 ATP synthase in membranes by simple phenolic compounds dihydrothymoquinone (PC01), hydroquinone (PC02), resorcinol (PC03), or catechol (PC04)

Figure 1 shows the X-ray crystal structure of polyphenol binding pocket and speculative binding of some of the structurally modulated polyphenols. The polyphenol binding pocket for resveratrol, piceatannol, and quercetin was shown to lie between the β_{TP} -subunit and the c-terminal region of γ -subunit [20].² Earlier it was shown that bound polyphenols generate hydrophobic interactions with γ Gln274 (γ Lys-260), γ Thr-277 (γ Ile-263), β Ala-264 (β Ala-278), and β Val-265 (β Val-279), and additional non polar interaction with residues γ Ala-270 (γ Ala-256), γ Thr-273 (γ Thr-259), γ Glu-278 (γ Glu-264), α Gly-282 (α Gly-290), and α Glu-284 (α Glu-292) which are within 4Å of the bound compounds [19, 32]. Polyphenol binding pocket residues of *E. coli* ATP synthase are identical to the bovine polyphenol binding pocket residues except for two changes, namely γ Q274K and γ T277I where Gln is replaced by Lys and Thr is replaced by Ile in bovine. Bovine mitochondrial numbers are shown in parentheses. We studied the inhibitory effect of seventeen polyphenols with structural modifications through replacement, repositioning, or additions of the hydroxyl, nitro, or imino groups (Fig. 2) on the purified F_1 and membranes of *E. coli* ATP synthase to understand the enhancement in the potency of inhibition.

Figure 3 shows the inhibition of ATPase activity of purified F_1 or membrane bound enzyme in presence of varied concentrations of dihydrothymoquinone (PC01), hydroquinone (PC02), resorcinol (PC03), or catechol (PC04). While potent inhibition ~80% (IC_{50} ~20 μ M) occurs in presence of hydroquinone (PC02), the maximum amount of inhibition in presence of catechol (PC04) was ~40%, dihydrothymoquinone (PC01) was (~40%), and resorcinol (PC03) was ~20%. We consistently found that the F_1 data and the membrane data were the same for these inhibitors. This is in agreement with our previously established interpretation that inhibition of ATPase activity can be assayed using either membrane preparations or purified F_1 with equivalent results [31–32, 44–48].

Inhibitory effect of structurally modulated imino diphenolic compounds 2-[[[4-hydroxyphenyl]imino]methyl] phenol (PC05), 2-[[[3-hydroxyphenyl]imino]methyl] phenol (PC06), 3-[[[2-hydroxyphenyl]imino]methyl] phenol (PC07), , 4-[[[3-hydroxyphenyl]imino]methyl] phenol (PC08), 2-[[[2-hydroxyphenyl]imino]methyl] phenol (PC09), 3-[[[3-

²*E. coli* residue numbers are used throughout.

hydroxyphenyl)imino]methyl] phenol (PC10), or 4-[[[(4-hydroxyphenyl)imino]methyl] phenol (PC11) on the purified F₁ or membrane bound enzyme

Figure 4A and B shows the inhibitory effect of structurally modulated imino diphenolic compounds PC05, PC06, PC07, PC08, PC09, PC10, or PC11. All but PC11 beget potent inhibition with no or little left over residual activity. The IC₅₀ values are PC05 (IC₅₀~5μM), PC06 (IC₅₀~5.25μM), PC07 ((IC₅₀~9μM), PC08 (IC₅₀~9μM), PC09 (IC₅₀~7μM), or PC10 (IC₅₀~2.25μM). PC11 inhibits only ~20% with ~80% residual activity. Again the F₁ data and the membrane data were same for all the inhibitors.

Inhibitory effect of nitro diphenolic compounds 2-[[[(4-hydroxy-2-nitrophenyl)imino]methyl] phenol (PC12) or 2-[[[(2-hydroxy-4-nitrophenyl)imino]methyl] phenol (PC13) on the purified F₁ or membrane bound enzyme

Figure 5 shows the *nitro diphenolic compounds* (PC12 or PC13) induced inhibitory profiles on the purified F₁ or membrane bound *E. coli* enzyme. Introduction of nitro group in PC12 (IC₅₀~6μM) or PC13 (IC₅₀~5μM) exert potent inhibition of *E. coli* ATP synthase with no left over residual activity.

Inhibitory effect of introduction of phosphate derivatives phospho triester (PC14), or phospho diester (PC15) on the purified F₁ or membrane bound enzyme

As shown in Fig. 6 phosphate derivatives tris(4-nitrophenyl)phosphate (PC14), or bis(4-nitrophenyl)-phosphate (PC15) causes partial inhibition of ATPase activity ~45% or ~40% respectively.

Inhibitory effect of structurally modified aromatic diol (anthryl diol, PC16) or aromatic triol (anthryl triol, PC17) on the purified F₁ or membrane bound enzyme

The maximum amount of inhibition by PC16 is ~22% or by PC17 are ~ 30% with ~78 % and 70% residual activity (Fig. 7). Partial inhibition of ATP synthase is not uncommon. In previous studies [31–32, 44–52], we have noted several instances where mutant ATP synthase were incompletely inhibited by potent inhibitors like fluoroaluminate, fluoroscandium, sodium azide, NBD-Cl or by polyphenols like resveratrol, quercetrin, or quercetin-3-βglucoside. To be sure that the maximal inhibition with PC01, PC03, PC04, PC11, PC14, PC15, PC16, or PC17 had been reached, we incubated each membrane preparation or purified F₁ with 120μM (PC01), 40μM (PC03), 500μM (PC04), 70μM (PC11), 500μM (PC14), 70μM (PC16), or 140μM (PC17), the maximal inhibitory concentrations, for 1 h as in Figure 3–7 followed by supplementary pulses of the compounds and continued the incubation for an additional hour before assaying ATPase activity. As shown in Figure 8A very little or no additional inhibition occurred consistent with Figure 3–7. This shows that the inhibition by PC01, PC03, PC04, PC11, PC14, PC15, PC16, or PC17 was maximal and fully inhibited F₁ or membranes retained residual activity. Although, we used 1 hour incubation time it was observed that the maximal inhibition of purified F₁ or membrane bound enzyme was achieved within 15 minutes.

Reversal of ATPase activity of purified F₁ or membrane enzyme from the polyphenolic compounds PC01-PC17 inhibition

This experiment was carried out in two ways (i) the purified F₁ or membrane bound enzyme was inhibited with the maximum inhibitory concentrations of PC01-PC17. Then the samples were diluted to a non inhibitory concentration and ATPase activity was measured. It was found that the inhibition was totally reversible. (ii) 20 µg purified F₁ samples were inhibited with maximum inhibitory concentrations of PC01-PC17 for 1 hr. Again the inhibitory concentrations were determined based on data from figure 3–7. Then inhibited samples were passed twice through 1 ml centrifuge columns and ATPase activity was measured. It was found that in all cases activity was restored back to the near normal level as in absence of the compounds (Fig. 8B). Reversibility data indicate that the observed inhibition is not the result of protein denaturation and that the enzyme retains the ability to reactivate upon release of the compound by dilution removal through centrifuge columns.

Inhibition of growth on LB, limiting glucose, and succinate medium in presence of PC01-PC17 structurally modulated polyphenolic compounds

Inhibitory effects on ATP synthesis were studied by growing the wild-type *E. coli* strain pBWU13.4/DK8 on succinate plates, or limiting glucose, in presence or absence of PC01-PC17 compounds. Interestingly the growth was abrogated more or less in proportion to the inhibitory effect on purified F₁ or membrane bound enzyme in presence of structurally modulated compounds PC01-PC17 (see Table 1).

Discussion

The goal of this study was to examine the impact of structural modification on the inhibitory properties of a variety of polyphenolic compounds on the *E. coli* ATP synthase. Earlier we found that polyphenols resveratrol, piceatannol, quercetin, quercetrin or quercetin-3-β-D glucoside induce inhibition of *E. coli* to varying degrees [32]. Resveratrol, piceatannol or quercetin bound bovine ATP synthase X-ray structures show that polyphenols bind at the c-terminal tip of γ-subunit and interact with the binding pocket between β_{TP}-subunit and the c-terminal tip of γ-subunit [20]. The hydrophobic interaction between the above polyphenol inhibitor compounds and mitochondrial enzyme was shown to involve γK260, γI263, β_{TP}V279 and β_{TP}A278 [20]. The equivalent residues in the *E. coli* enzyme are γQ274 (γK260), γT277 (γI263), βV265 (βV279) and βA264 (βA278) [31–32]. Parentheses show the bovine numbers. Based on resveratrol, piceatannol or quercetin binding to the polyphenol binding pocket we imitated the binding of structurally modulated polyphenols in the polyphenol binding pocket (Fig. 1). Although these binding are supported by the biochemical inhibitory profiles shown in figures 3–7 the schematic orientation for all the six polyphenols compounds shown in figure 1 is purely speculative.

Hydroquinone causes ~80 inhibition. Dihydrothymoquinone, resorcinol, or catechol cause partial inhibition with large leftover residual activity (Fig. 3). All four compounds have single phenolic rings with variation in the positioning of –OH groups (Fig. 2). The relative positions of –OH groups appear to be critical for the degree of inhibition as a result of better and tighter binding of the compounds in the polyphenol binding pocket. The strong

inhibition by hydroquinone (~80%) may be due to the extended locations of –OH groups that allows stronger interaction with the polyphenol binding site residues. The isopropyl group in dihydrothymoquinone may have caused steric hindrance resulting in abrogated inhibition (~60% residual activity). In resorcinol and catechol meta and ortho positioned –OH groups may not be able to form H-bonding with the polyphenol binding pocket residues resulting in ~80% and ~60% residual activity respectively.

The significance of –OH group positioning is also apparent from the seven imino diphenolic compounds induced inhibitory profiles represented in fig. 4A and 4B. While six compounds, 2-[[[(4-hydroxyphenyl)imino]methyl] phenol (PC05), 2-[[[(3-hydroxyphenyl)imino]methyl] phenol (PC06), 3-[[[(2-hydroxyphenyl)imino]methyl] phenol (PC07), , 4-[[[(3-hydroxyphenyl)imino]methyl] phenol (PC08), 2-[[[(2-hydroxyphenyl)imino]methyl] phenol (PC09), 3-[[[(3-hydroxyphenyl)imino]methyl] phenol (PC10), cause complete or near to complete inhibition of *E. coli* ATP synthase, the seventh compound 4-[[[(4-hydroxyphenyl)imino]methyl] phenol (PC11) results in only twenty percent inhibition or ~80% residual activity. This suggests that two ringed structure along with properly positioned –OH groups are important for binding and inhibition. Extended positioning of –OH groups seems to diminish the inhibitory effect of imino diphenolic compound PC11. This is contrary inhibitory effect observed in presence of simple single ringed phenolic compound PC02 where positioning of extended –OH groups augmented the inhibitory effect.

Earlier we found that resveratrol is not a potent inhibitor of *E. coli* ATP synthase (IC₅₀ ~94μM) leading to only ~40% inhibition [32]. Structural modification of resveratrol by removing one –OH group, introduction of imino group, and repositioning of the two –OH groups, makes them potent inhibitors with IC₅₀ values ranging between 2.25μM to 9.0μM (Fig. 4A and B). Likewise, introduction of NO₂ group at varied positions (PC12 or13) also enhances inhibition on molar scale. The presence of NO₂ group results in the IC₅₀ ~6μM for 2-[[[(4-hydroxy-2-nitrophenyl)imino]methyl]phenol (PC12) and IC₅₀ ~5μM for 2-[[[(2-hydroxy-4-nitrophenyl)imino]methyl] phenol (PC13) in comparison to IC₅₀ ~94μM for resveratrol (Fig. 5). The drastic decrease in the IC₅₀ values can be accounted for by the fact that NO₂ group having electron withdrawing power may result in stronger hydrogen bonding between the phenolic –OH groups and the polyphenol binding pocket residues.

The inhibitory effect of two phosphate derivatives Tris(4-nitrophenyl)phosphate (phospho triester, PC14) or Bis(4-nitrophenyl)-phosphate (phospho diester, PC15) resulted in ~55% and 60% residual activity (Fig. 6). The partial inhibition of ATPase activity in presence of such compounds may be ascribed to (i) structural incompatibility between polyphenol binding site and phosphate derivative. The bulkiness of phospho triester may generate steric hindrances against H-bonding between NO₂ groups and residues from the polyphenols binding pocket, and (ii) the repulsion between negatively charged phosphate of phospho diester (PC15) and the negatively charged residues αE284 and or γE278 of the polyphenol binding pocket.

The inhibitory effects of anthradiol (1, 8-dihydroxy-9, 10-anthraquinone, PC16) or anthratriol (1, 8, 9-anthracetriol, PC17) shown in figure 7 appear consistent with their

structures. Both compounds are flat and rigid. Lack of structural flexibility may obstruct the H-bonding between the –OH groups of the compounds and residues of the polyphenol binding pocket.

Our results show that the extent of *E. coli* enzyme inhibition depends on the nature of modulation/modification of polyphenol compounds (Fig. 3–7). While some compounds caused maximal inhibition with nearly zero residual activity others resulted in partial inhibition with residual activity ranging between 5 to 80% (Table 1). For those compounds which resulted in partial inhibition addition of extra shot of compounds to the previously inhibited purified F₁ or membrane did not change the degree of inhibition significantly (Fig. 8A). This suggests that the purified F₁ or membrane were fully inhibited with the compounds and the extent of observed inhibition was the true inhibition. In addition, partial inhibition is not due to uninhibited enzyme or degradation of the compounds with time. The process of inhibition was also found to be completely reversible. A fully reacted F₁ regained activity once they were passed through the centrifuge columns to remove the compounds. Similarly purified F₁ or membrane regained activity once they were brought back to lower non inhibitory concentrations of compound after exposing them to higher concentrations by dilution with buffer (Figure 8B). Readily reversible process also confirms the non covalent binding of the compounds with no further inhibition on addition of second shot of the compounds.

Growth pattern was observed in presence of all the seventeen compounds (Table 1). It is noteworthy that the loss of growth was nearly in proportion to the extent of ATPase inhibition. Thus, abrogation of oxidative phosphorylation or loss of ATP synthesis corroborates the inhibitory effects of structurally modulated compound used in this study. Our null strain (pUC118/DK8) usually will grow 40–50% of the wild-type (pBWU13.4/DK) (Table 1). This is because the null strain uses only glycolysis to generate ATP whereas the wild-type uses both glycolysis and oxidative phosphorylation. Apparently compounds are inhibiting only oxidative phosphorylation and not the glycolysis. In earlier studies polyphenol compounds like quercetin, quercitrin, quercetin-3- β -glucoside, or antibiotic venturicidin were shown to inhibit the F₁-ATPase activity but not the growth [31–32, 53]. Thus, the structural modulation of compounds is important tool for generating potent inhibitors of both ATPase and ATP synthesis.

The physiological importance of inhibitory effects of polyphenols on ATP synthase is ascribed to the vital roles that dietary polyphenols play in human health. Many degenerating diseases such as cancer, aging and neurological disorders are attributed to the mitochondrial dysfunction [54–55]. Thus, it is imaginable that ATP synthase being a major player in mitochondria the inhibition of ATP synthase by polyphenols might play a considerable role in the physiology of such conditions [20]. The uncontrolled growth of tumor cells requires additional energy in the form of ATP generated by ATP synthase. Thus the inhibition of ATP synthase will deprive the tumor cells of required energy leading to cell death or apoptosis [20, 32]. Resveratrol was shown to induce apoptosis via a mitochondrial pathway [27, 56]. Interestingly enough it was shown that oligomycin a highly specific ATP synthase inhibitor induces an apoptotic suicide response in cultured human lymphoblastoid and other mammalian cells within 12–18 hrs but not in ρ^0 cells that are depleted of a functional

mitochondrial respiratory chain [57]. Inhibition of the components of mitochondrial pathways may lead to marking of some cells, via CD14, for cell death, while allowing commitment to differentiation to occur in the surviving population [58]. Cell death can also be prompted through alteration of cellular bioenergetics. 1, 4-benzodiazepine (Bz-423) was shown to bind to the oligomycin sensitivity –conferring protein subunit of F_1F_0 and inhibiting the ATP synthase. This causes a significant decrease in ATP synthesis and increase in the production of free radicals which in turn activates redox regulated apoptosis [59]. Apparently, ATP synthase inhibitors in general and polyphenol inhibitors in particular may be used to target tumor cells without affecting normal cells [20, 31–32].

The inhibition of biofilm formation and acid production by *S. mutans* through the inhibition of proton-translocating F_1 -ATPase activity in presence of a variety of polyphenols [29–30] and the involvement of mycobacterium ATP synthase in conferring resistance against the anti-tuberculosis drug diarylquinoline due to two C-subunit mutations D32V and A63P [60–61] suggests a need for potent inhibitors of bacterial ATP synthase enzymes. It is evident from the results of this study that structural modulation/modification of polyphenol compounds could provide starting point to develop potent inhibitors against bacterial pathogens such as *S. mutans* and *M. tuberculosis*. Nevertheless, mutagenic analysis of polyphenol binding site residues should be the next step to advance the current understanding of dietary benefits of polyphenols in combating disease conditions [63].

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Figure 1. X-ray structures of mitochondrial ATP synthase using PDB file 2jj1 [19]

Rasmol software was used to generate these figures. Polyphenolic compounds PC02 (A), PC05 (B), PC10 (C), PC12 (D), PC15 (E), or PC16 (F) in ball and stick display were generated by ACD/ChemSketch 12.0 program [<http://www.freechemsketch.com/>]. The schematic orientation of these compounds in polyphenol binding pocket is purely speculative. Residues involved in the interaction with compounds are identified. γ Q274K, γ T277I in red is showing the difference between bovine and *E. coli* ATP synthase. In place of Q and T bovine has K and I residues. *E. coli* residue numbering is shown.

Figure 2. Structures of polyphenolic inhibitors of *E. coli* ATP synthase are shown in five groups (I) simple phenolic compounds, PC01-PC04; (II) imino diphenolic compounds, PC05-PC11; (III) nitro diphenolic compounds, PC12 and PC13; (IV) phosphate derivatives, PC14 and PC15; and (V) aromatic diol or triol, PC16, PC17.

Figure 3. Inhibition of ATPase activity in purified F_1 or membrane-bound ATP synthase by dihydrothymoquinone (PC01), hydroquinone (PC02), resorcinol (PC03), or catechol (PC04) Membranes or Purified F_1 were preincubated for 60 min at 23°C with varied concentration of dihydrothymoquinone (PC01), hydroquinone (PC02), resorcinol (PC03), or catechol (PC04), and then aliquots added to 1 ml of assay buffer and ATPase activity determined. Details are given in Materials and Methods. Each data point represents average of at least four experiments done in duplicate tubes, using two independent membrane or F_1 preparations. Results agreed within $\pm 10\%$.

Fig 4A **Fig 4B**

Figure 4. A and B. Inhibition of ATPase activity in purified F₁ or membrane-bound ATP synthase by imino diphenolic compounds (4A) PC05-PC08; (4B) (PC09-PC11) Membranes or Purified F₁ were preincubated for 60 min at 23°C with varied concentration of (4A) 2-[[[(4-hydroxyphenyl)imino]methyl]phenol] (PC05), 2-[[[(3-hydroxyphenyl)imino]methyl]phenol] (PC06), 3-[[[(2-hydroxyphenyl)imino]methyl]phenol] (PC07), or 4-[[[(3-hydroxyphenyl)imino]methyl]phenol] (PC08); (4B) 2-[[[(2-hydroxyphenyl)imino]methyl]phenol] (PC09), 3-[[[(3-hydroxyphenyl)imino]methyl]phenol] (PC10),

or 4-[[[4-hydroxyphenyl)imino)methyl] phenol (PC11) and then aliquots added to 1 ml of assay buffer and ATPase activity determined. Details can be found Materials and Methods section. Each data point represents average of at least four experiments done in duplicate tubes, using two independent membrane or F_1 preparations. Results agreed within $\pm 10\%$.

Figure 5. Inhibitory effect of introduction of nitro diphenolic compounds PC12 or PC13 on the purified F_1 or membrane bound enzyme

Membranes or Purified F_1 were preincubated for 60 min at 23°C with varied concentration of 2-[[4-hydroxy-2-nitrophenyl imino]methyl] phenol, (PC12) or 2-[[2-hydroxy-4-nitrophenyl]imino]methyl] phenol, (PC13), and then aliquots added to 1 ml of assay buffer and ATPase activity determined. Each data point represents average of at least four experiments done in duplicate tubes, using two independent membrane or F_1 preparations. Results agreed within $\pm 10\%$. Figure inhibition by resveratrol was taken from [32].

Figure 6. Inhibitory effect of phosphate derivatives PC14 or PC15 on the purified F_1 or membrane bound enzyme

Membranes or Purified F_1 were preincubated for 60 min at 23°C with varied concentration of [Tris(4-nitrophenyl) phosphate] (PC14), or [Bis (4-nitrophenyl)phosphate] (PC15)), and then aliquots added to 1 ml of assay buffer and ATPase activity determined. Each data point represents average of at least four experiments done in duplicate tubes, using two independent membrane or F_1 preparations. Results agreed within $\pm 10\%$.

Figure 7. Inhibition of ATPase activity in purified F_1 or membrane-bound ATP synthase by aromatic diol (PC16) or triol (PC17)

Purified F_1 or Membranes were preincubated for 60 min at 23°C with varied concentration of 1,8-dihydroxy-9,10-anthraquinone (PC16), or 1,8,9-anthracetriol/dithranol (PC17) and then aliquots added to 1 ml of assay buffer and ATPase activity determined. Each data point represents average of at least four experiments done in duplicate tubes, using two independent membrane or F_1 preparations. Results agreed within $\pm 10\%$.

Figure 8. Results of Extra pulse of polyphenolic compounds and reversal of inhibition by passing through centrifuge columns

(A), Membrane bound ATP synthase (Mbr) or purified F₁ (F₁) was inhibited with inhibitory concentrations of the structurally modulated polyphenol compounds shown in the figure for 60 min under conditions as described in Fig 3–7. Then a further pulse of identical inhibitory concentrations was added and incubation continued for 1 h before assay. The last digits represent the compound concentrations in [μM]. (B) Purified F₁ was incubated with inhibitory concentrations of compounds PC01–αPC17 for 60 min under conditions as described in Fig 3–7. Then the inhibited samples were passed twice through 1 ml centrifuge columns and ATPase activity was measured. The first bar is for purified F₁ with no compound (F₁), followed by bars in presence of compounds. Although reversibility of inhibition was followed in presence of all seventeen compounds (PC01–PC17) for clarity reversal data for only six compounds is shown in this figure.

Table 1

Growth of *Escherichia coli* cells and residual ATPase activity in presence of structurally modulated polyphenolic compounds

Polyphenolic compounds	^a Growth on succinate plates	^b Growth yield in limiting glucose (%)	^c Residual ATPase Activity (%)
^d Control	++++	100	N/A
^e Null	–	45	N/A
Dihydrothymoquinone (PC01)	++	66	60
Hydroquinone (PC02)	+	55	20
Resorcinol (PC03)	++	79	80
Catechol (PC04)	+	57	60
2-[[[(4-hydroxyphenyl)imino]methyl]phenol (PC05)	–	42	0
2-[[[(3-Hydroxyphenyl)imino]methyl]phenol (PC06)	–	51	15
3-[[[(2-Hydroxyphenyl)imino]methyl]phenol (PC07)	–	53	15
4-[[[(3-Hydroxyphenyl)imino]methyl]phenol (PC08)	–	46	0
2-[[[(2-Hydroxyphenyl)imino]methyl]phenol (PC09)	–	43	5
3-[[[(3-Hydroxyphenyl)imino]methyl]phenol (PC10)	–	44	0
4-[[[(4-Hydroxyphenyl)imino]methyl]phenol (PC11)	+++	84	80
2-[[[(4-Hydroxy-2-nitrophenyl)imino]methyl]phenol (PC12)	–	44	0
2-[[[(2-Hydroxy-4-nitrophenyl)imino]methyl]phenol (PC13)	–	47	0
Tris(4-nitrophenyl)phosphate (PC14)	+	57	55
Bis(4-nitrophenyl)phosphate (PC15)	++	63	60
1,8-Dihydroxy-9,10-anthraquinone (PC16)	+++	80	78
1,8,9-Anthracetriol (PC17)	+++	69	70

^a Growth on succinate plates after 3 days was determined by visual inspection. +++++, high growth; +++, moderate growth; ++, low growth; +, minimal growth; or –, no growth.

^b Growth yield on limiting glucose was measured as OD₅₉₅ after ~20 hours growth at 37 °C.

^c Residual activity is the left over ATPase activity

^{d, e} Control, pBWU13.4/DK8 which contains UNC⁺ gene encoding ATP synthase; Null, pUC118/DK8 with UNC[–] gene. Growth of positive and negative controls in absence of polyphenol compounds. Data are means of four to six experiments each at 37 °C. Each individual experimental point is itself the mean of duplicate assays.