

Review Article

Natural Compounds as Therapeutic Agents to Treat Cystic Fibrosis

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Abstract

Despite being identified as a unique disease almost 100 years ago, drugs aimed at treating the basic defect in cystic fibrosis (CF) have only recently been approved for clinical use. Marketed as Kalydeco and Orkambi (Vertex Pharmaceuticals), these medications have improved the lives of many CF patients; yet the goal of treating all CF patients remains to be achieved. Although effective, concern has been raised regarding the annual cost of treating patients with these drugs, especially since patients will be prescribed such medications for life. The current move to a more holistic approach to medical care has prompted many people to try various herbal or “natural” remedies. In this review, we assess three such natural treatments; genistein, curcumin and resveratrol, and evaluate their potential as adjunct therapies for patients with CF.

INTRODUCTION

Cystic Fibrosis (CF) is a common lethal genetic disease of Caucasians, and results from mutations in a cyclic AMP regulated anion channel [1,2]. This ion channel, CFTR (cystic fibrosis transmembrane conductance regulator), is present in many epithelia where it regulates the movement of ions such as chloride [3,4], thiocyanate and glutathione [5-7], and bicarbonate [8,9]. The absence of CFTR protein and/or function in patients with CF results in defective exocrine pancreatic function, intestinal blockage, and in males, azoospermia due to absent vas deferens. The organ most responsible for morbidity and mortality in CF patients is the lungs. Inappropriate salt and water transport across airway epithelia leads to the accumulation of thick sticky mucus in the lumen of the airways, which traps bacteria, causing a persistent airway infection and associated inflammation; such chronic inflammation eventually leading to tissue fibrosis and destruction. Prior to the incorporation of pancreatic enzyme supplements in the therapy of patients with cystic fibrosis, CF was regarded primarily as a gastrointestinal disease due to the failure to thrive and early death from malnutrition in infants [10]. Chronic lung infections are now the primary cause of morbidity and mortality in patients with CF [1,11].

Gene Therapy

Following the cloning of the CFTR gene in 1989 [12], the early hope for a therapy to treat patients with CF was founded firmly in the realm of gene therapy, with both viral and non-viral vectors being proposed. Indeed, several high profile gene therapy trials were initiated, yet none lived up to expectations. Early studies

focused primarily on proof-of-concept in human nasal tissues, using an adenoviral construct [13]. Although CFTR mRNA and protein were undetectable, electrophysiological studies hinted at some improvement. Subsequent administration of gene therapy to the lungs of CF patients also suggested a partial correction [14], even though the amount of correction diminished with each subsequent treatment. Given current improvements in molecular biology, it is likely that future gene therapies may involve gene editing of the patient's chromosomal DNA rather than introduction of a transgene [15,16].

Drugs

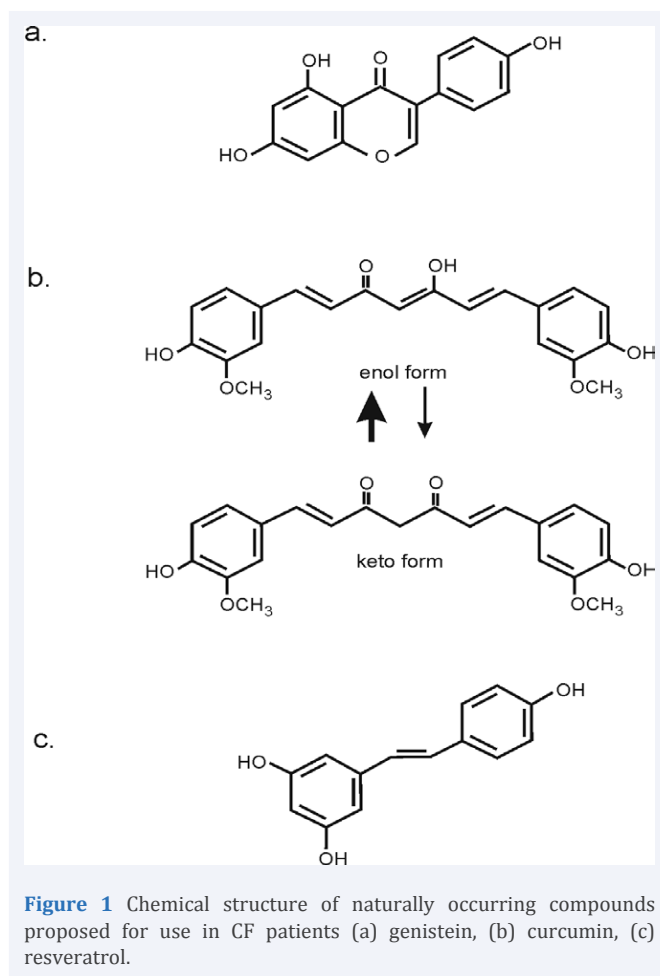
Pharmacological treatments directed towards the basic defect in CF are designed to restore normal salt and water transport across affected epithelia [17]. Even moderate increases in the function of mutant CFTR are of benefit, since studies on individuals with splice variants of CFTR who exhibit only ~10% of wild-type CFTR levels appear to have normal lung function and normal life expectancy [18]. Although >2,000 different mutations have been described in the *cftr* gene, giving rise to clinical CF, they nonetheless fall into two broad categories; those that affect protein production, and those that affect protein function [2,17]. Some mutations do appear in both categories, as is the case for the most prevalent mutation, $\Delta F508$, which constitutes about 70% of the mutant CFTR alleles in North America [19]. Given the two broad classes of CFTR mutation, it has become apparent that two categories of drug are likely to be required to treat patients with CF, based upon their unique genetic makeup. Thus, compounds that increase the protein expression of mutant CFTR are referred to as “correctors”, whilst those that

increase the functional activity of mutant CFTR are referred to as “potentiators”. High throughput screening (HTS) strategies by Vertex Pharmaceuticals (Cambridge, MA) resulted in the identification of the “potentiator” ivacaftor (VX-770) [20] and the “corrector” lumacaftor (VX-809) [21]. In 2012, the Food and Drug Administration (FDA) of the US Government approved the first drug to treat the basic defect in CF. Marketed as Kalydeco, VX-770 targeted one of the more common mutations in CF patients of Scandinavian descent, G551D. The G551D protein is characterized as a protein which is able to exit the ER and inserts into the plasma membrane but has markedly reduced ion channel function. In 2015, Vertex Pharmaceuticals received further FDA approval for a drug that combined the potentiator ivacaftor with the CFTR corrector lumacaftor, and marketed as Orkambi. This combination is primarily aimed at the common $\Delta F508$ mutation, which displays both protein production challenges and functional problems.

Whilst clinically of enormous benefit [22,23], a criticism of the Vertex drugs has been the pricing structure, with Orkambi and Kalydeco priced at more than \$300,000 a year. Paul Quinton, a Professor of Biomedical Science at the University of California at San Diego, a pioneer in CF research and himself a CF patient has called this pricing “egregious” [24], a sentiment echoed by many CF clinicians, including Dr. David Orenstein, co-director of the Palumbo Cystic Fibrosis Center at the University of Pittsburgh. With this in mind, and the current trend in natural therapies, it is not surprising that many patients and their families have sought alternatives to “big pharma” solutions. Indeed, a growing trend amongst patients with chronic diseases, such as CF, diabetes or coeliac disease, is the pursuit of alternative or “natural” remedies. This trend is reflected in the growing number of health food stores with advertising for a myriad “herbal cures”. Perhaps one of the classical examples of this approach is in the treatment of chronic pain or headache, where extracts from Willow bark have proven to be beneficial. The active ingredient in such extracts is the compound salicylin, a forerunner of the modern pharmaceutical acetylsalicylic acid, or aspirin [25]. Interestingly, several “natural” compounds have received attention as drugs reported to increase CFTR activity, including isoflavones, flavones, capsaicin, curcumin and resveratrol [26]. In this review, we highlight three of the proposed therapies for CF arising from natural sources, and evaluate their scientific merit.

Genistein

One of the first compounds found to impact mutant CFTR was genistein [27,28] (Figure 1a). Genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)4*H*-1-benzopyran-4-one) is part of a family of compounds referred to as isoflavones; heterocyclic polyphenols found naturally in many legumes [29,30]. Perhaps one of the richest sources of genistein is soya (although in soya, genistein occurs as the glycoside, genistin). Numerous health benefits have been ascribed to genistein, including its actions as a phytoestrogen, an antioxidant and a tyrosine kinase inhibitor [30,31], and genistein has been proposed to be effective in various disorders such as cancer, cardiovascular disease and menopausal problems [30]. Although the effects of genistein can be somewhat weak, its low toxicity has encouraged researchers to evaluate genistein as a potential therapeutic agent. The discovery that genistein could



act as a CFTR “potentiator” drug [27,32-34] raised the possibility that genistein could be used in patients with CF. Indeed, studies suggested that not only could genistein augment the ion channel activity of G551D CFTR [27,35], a function/gating class of mutant, but also the common $\Delta F508$ mutation a mixed function/amount mutant [27,36], and intriguingly, wt CFTR [27]. The notion that genistein might be effective against G551D CFTR is attractive, since the G551D mutation results in a protein that reaches the plasma membrane as a mature protein, therefore in the correct cellular location, but with severely impaired function [36]. i.e., a single molecular defect. Excised patch clamp studies showed that direct application of genistein could increase CFTR currents, implying that CFTR itself was the target for genistein [27,37-40]. Indeed, a recent molecular modeling study has identified five possible binding sites for genistein in the nucleotide binding domains (NBDs) of CFTR [41], although further work is required to verify functionally each of these sites. One possible explanation for the beneficial effects of genistein on CFTR, is that genistein may stabilize the NBD dimer in CFTR by binding at the interface or by inducing conformational changes [26]. In addition to its actions as a potentiator, there are indications that genistein may also have corrector activity, since long term treatment of cells with genistein has been shown to increase the level of protein expression for mutant CFTR [42]; however 3-fold higher concentrations were found to be inhibitory.

Since CFTR is an ion channel, its activity can be measured electrically. One such method, applicable in patients with CF, is a nasal potential difference (NPD) measurement [43], which measures the voltage across the nasal epithelium. This voltage arises from transepithelial ion transport, and in part reflects CFTR function. Differences between NPD of control and CF patients was identified over 30 years ago [44], thus changes in NPD can be reflective of the efficacy of therapeutic treatments. In one study, application of genistein (50 μM) to the nasal epithelium of CF patients bearing the G551D mutation restored 15% of wild type CFTR function [45]. Given its low toxicity, genistein appears to be a good candidate for the treatment of patients with CF. Importantly, the effective concentration for channel modulation ($\sim 2\text{-}3\ \mu\text{M}$), is within the range of achievable plasma levels of genistein ($\sim 1\text{-}2\ \mu\text{M}$) [46]. With regards to CFTR protein production, the observation that 100 μM genistein is inhibitory is somewhat irrelevant given achievable plasma concentrations. However, it also means that "corrector" concentrations of $\sim 30\ \mu\text{M}$ are also unlikely to be achievable. Although a clinical trial using a combination therapy of 4-phenylbutyrate and genistein has been planned, it was cancelled before the trial was initiated. What the long term exposure of patients to genistein would be, particularly exposure since infancy remains to be determined [47].

Curcumin

Turmeric, a root belonging to the ginger family, is a spice widely used in Asian cuisine, and has also been used for centuries as a part of the herbal therapies in Siddha medicine [48]. Discovered in the 1800's the principal active ingredient in turmeric is the diarylhepanoid curcumin (Figure 1b), a compound which give turmeric its characteristic yellow colouring. Scientific interest in curcumin arose with a paper published in 1949 describing the antibacterial actions of curcumin [49], specifically against *Staphylococcus aureus*. This is of particular interest since *S. aureus* is one of the main contributors to airway infection in patients with CF. Curcumin is now widely available as a nutritional supplement, and is reported to have anti-inflammatory, anti-tumour and antioxidant effects [50-52]. *In vitro*, curcumin has been shown to inhibit a number of enzymes, including HDAC1,3,8 [53,54], cyclooxygenase [55], and importantly for ΔF508 CFTR, the sarcoplasmic - endoplasmic reticulum calcium ATPase (SERCA) [56-58]. Inhibition of SERCA by curcumin presumably blocks ATP-dependent uptake of calcium into the endoplasmic reticulum, thus interfering with calcium-dependent processes within the ER, including a number of calcium-dependent chaperones. In fact, earlier studies had shown that the SERCA inhibitor thapsigargin, could facilitate ER exit of ΔF508 CFTR, with subsequent appearance of the mutant protein in the plasma membrane [59], where it could be available for activation. Similarly, exposure of baby hamster kidney cells, expressing human ΔF508 CFTR, to curcumin was reported to improve the processing of ΔF508 CFTR allowing mutant CFTR to exit the ER and insert into the plasma membrane [60]. Thus, there is some evidence that curcumin can facilitate exit of ΔF508 CFTR, and that this might be due to low ER calcium levels [61,62], however a mechanism based on SERCA inhibition has been challenged by other groups. Grubb *et al* measured ability of the calcium dependent chaperone calnexin to interact with ΔF508 CFTR in the presence of curcumin; an interaction

that may be expected to change if ER calcium balance is upset. However, these workers found no evidence of alteration in the interaction between calnexin and ΔF508 CFTR in the presence of curcumin [63]. In contrast, recent studies from other groups have argued that the effects of curcumin may be due to changes in the interaction of ΔF508 CFTR, not with calcium-dependent chaperones, but rather with cytokeratins [64,65].

Although the *in vitro* studies of Egan *et al* suggested a modest improvement in ΔF508 CFTR ER export (by whatever mechanism), *in vivo* studies were particularly exciting, since administration of oral curcumin to ΔF508 CF mice, resulted in sufficient correction of ΔF508 CFTR trafficking that normalized nasal potential-difference measurements could be attained [61]. This also included a reduction in the level of epithelial sodium transport (a process which is thought to be a significant contributor to CF lung pathology [60]). Intestinal obstruction, a hallmark of CF disease in many mouse models (presumably due to congestion of the gut by reduced water transport into the gut lumen) and a major cause of death in CF mice, was also corrected in CF mice exposed to curcumin. The intriguing consequence of these studies was the notion that a single, simple, agent was capable of correcting ΔF508 CFTR in a clinically beneficial manner. In addition to its ability to facilitate the exit of ΔF508 CFTR from the ER (i.e., a corrector), curcumin has also been shown to have potentiator activity directed at wt and ΔF508 CFTR [66] and G551D CFTR [67]. A third reported effect of curcumin is the oligomerization of CFTR molecules [68]. The slow oligomerization of CFTR may, in part, account for the actions of curcumin on CFTR. Thus, short exposure of G551D CFTR to curcumin induces a reversible activation, whereas prolonged activation produces an irreversible robust activation [67]. Studies by Kirk *et al*, however, have shown that the cross-linking characteristics of curcumin are also separable from the potentiating aspects of curcumin. Thus, cyclic derivatives of curcumin, synthesized *de novo* lacked the ability to dimerize CFTR polypeptides, yet retained the ability to activate both wt and G551D CFTR [68]. Therefore, there appears to be intriguing evidence that curcumin is efficacious against mutant CFTR. Moreover, since curcumin is found in various foods, and is sold as a herbal remedy, the idea of clinical trials based on a compound with hundreds of years of biosafety was very appealing.

Despite the enthusiasm with which the initial report of the efficacy of curcumin towards ΔF508 CFTR in both cell lines and mouse models [61] was greeted, numerous subsequent studies failed to replicate the data. Dragomir and colleagues reported that curcumin was unable to induce a forskolin stimulated chloride current in either human airway epithelial cells (CFBE) or CF nasal epithelial cells [69]. Interestingly, in ΔF508 CFTR expressing BHK cells, curcumin caused a modest increase in ΔF508 CFTR activity [69]. Studies by Berger *et al* using well differentiated airway epithelial cells were unable to detect any correction of ΔF508 CFTR by curcumin [66]. Finally, studies using Fischer Rat Thyroid (FRT) cells expressing ΔF508 CFTR showed no evidence of enhanced iodide influx (a surrogate assay for chloride efflux) in the presence of curcumin, compared to its absence [70]. It is difficult to reconcile the positive data from Egan's group with the overwhelmingly negative data obtained from other investigators. One might argue that the cell line data reflects an over expression

artefact, as initial studies used $\Delta F508$ expression in BHK cells [61]. However other groups also using BHK cells expressing $\Delta F508$ were unable to document any beneficial curcumin effects [71]. With regards to the *in vivo* mouse studies, the positive studies by Egan *et al* were performed on a mixed background (129/sv and C57BL/6) [61]; whereas the negative studies of Song *et al* were on a CD-1 background [70]. In contrast, negative data from Grubb *et al* was obtained using mice of the same genetic background (although a slightly different strain) as that for Egan and colleagues (Grubb; UNC/CWRU: congenic C57BL/6, Egan; C57BL/6), suggesting that murine genetic background is not a significant contributor to the variance between the positive and negative data surrounding curcumin. An important clinical problem regarding the use of curcumin is the achievable plasma concentrations, an important issue in human trials. In Phase 1 clinical trials, dietary curcumin has been shown to exhibit very poor bioavailability, which coupled with rapid metabolism and excretion [70,72-75] means a very low serum concentration. Thus, high concentrations of curcumin cannot be achieved and maintained in plasma and tissues following oral ingestion. In human studies with healthy volunteers ingesting 10 or 12 g curcumin, only one subject had plasma curcumin levels above the detection limit of 5 ng/mL [76]. Similarly, Song *et al* reported a peak plasma concentration of 60 nM for curcumin [70] in CF mouse studies. This raised concerns of mechanism of action, as the initial proposal that curcumin was inhibiting SERCA seemed unlikely given the known IC₅₀ for curcumin of 5-15 μ M, orders of magnitude greater than physiologically achievable in plasma. This, of course, does not rule out the possibility that curcumin may be doing something to $\Delta F508$ CFTR other than impacting its interaction with calcium-dependent chaperones, but clearly raises the issue as to whether SERCA could be a target for curcumin at physiologically relevant plasma concentrations.

Genistein and Curcumin in Combination

Despite the apparent lack of strong data in support of curcumin being a corrector, several groups have reported that curcumin has potentiator activity [66,77]. Thus, recent studies have investigated the use of combined genistein and curcumin as drugs to treat G551D CFTR. In whole-cell patch studies by Yu *et al*, genistein caused a peak increase in G551D CFTR currents of almost 25-fold at a concentration of 80 μ M, compared to curcumin with a peak increase in G551D CFTR currents of 10-fold at a concentration of \sim 40 μ M [77]. In excised inside-out patch clamp studies, Berger reported a 150-fold increase in current above baseline, at 10 μ M curcumin [66]. Using G551D-CFTR expressing CHO cells, Yu *et al* observed that curcumin was able to further increase G551D-CFTR channel activity stimulated by genistein [77]. Despite both curcumin and genistein being CFTR potentiators, the observation that curcumin and genistein had additive effects suggests that they work through different mechanisms. Curiously, genistein stimulated channel activity in cells expressing wt CFTR was inhibited by the further application of curcumin [66]. One of the clinical advantages of synergism between compounds is the notion that each drug can be used at lower concentrations than either compound would require if used alone, in fact this seems to be the case for combined genistein and curcumin [26]. An ongoing clinical trial in the Netherlands is focused on "Comparing the effect of curcumin

with genistein to treatment with ivacaftor in CF patients with a class III mutation"; class III mutations being gating mutations, as exemplified by G551D-CFTR. It will be of interest to evaluate the results of this study, as the financial implications of such data are clearly significant.

Resveratrol

Resveratrol (Figure 1c) has recently received attention as the primary ingredient contributing to the health benefits associated with red wine. Resveratrol (3,4',5-trihydroxystilbene) is a naturally occurring polyphenolic compound found in vegetables and fruits, and abundant in grapes and peanuts [78]. Similarly to curcumin, resveratrol is widely available in health food stores, and is reported to be effective due to its anti-mutagenic, anti-inflammatory, anti-oxidant and chemo-protective properties [79,80]. The mechanism(s) by which resveratrol achieves the effects are not well documented, however it is known that resveratrol can increase cellular cAMP levels through direct activation of adenylate cyclase [81] and by inhibiting cAMP phosphodiesterases [82]. Several reports using cell lines, primary mouse tissues, and *in vivo* mouse NP, have shown that resveratrol can increase the ability of $\Delta F508$ CFTR to exit the ER and traffic to the cell surface and be functional [83-86]. Such studies reported an increase in conversion from immature core glycosylated band b (ER form) $\Delta F508$ CFTR to mature fully glycosylated band c (post Golgi form) $\Delta F508$ CFTR, and salutary effects including increased airway fluid secretion and mucociliary clearance. One interesting observation was that resveratrol appeared to increase the activity of ENaC, enhancing absorptive sodium transport [85], potentially further exacerbating the enhanced sodium hyper-absorption seen in CF airways [87]. In the hands of other researchers, resveratrol was able to increase wt CFTR expression, but was unable to increase $\Delta F508$ CFTR expression in expression systems [88]. Using primary human airway epithelial cells from patients homozygous for the $\Delta F508$ CFTR mutation, our studies were unable to demonstrate any benefit from resveratrol exposure, even though known "correctors" were effective [88]. Moreover we were also unable to see any effects on amiloride sensitive sodium currents, suggesting that ENaC was not a target for resveratrol. Interestingly, resveratrol by itself could stimulate chloride secretion across a human colonic monolayer, a stimulation that was markedly enhanced by the addition of a small amount of forskolin. Such observations are at least consistent with the hypothesis that resveratrol can enhance CFTR activity by acting as a phosphodiesterase inhibitor [88,89]. It is possible that resveratrol works directly on CFTR by acting as a potentiator, indeed, resveratrol has been reported to increase the open probability (Po) of murine CFTR [85], although it should be noted that murine CFTR has different electrophysiological properties than human CFTR [90]. Intriguingly, although monomeric resveratrol can increase CFTR activity, oligomeric resveratrol is a CFTR inhibitor [91].

What accounts for the differences in these studies using resveratrol? At present it is not entirely clear, however there are certainly differences in cell models used. Another issue is the concentration of resveratrol used in the studies. The majority of studies seeing efficaciousness of resveratrol do so at concentrations >50 μ M. Indeed the studies of Jai *et al* also

see an effect of resveratrol on wt CFTR at concentrations above 50 μM [88]. However, as with curcumin, the issue of effective *in vitro* concentration versus achievable plasma concentration is an issue that has to be addressed. Although beneficial effects for resveratrol are reported at concentrations about 50 μM , the maximal achievable plasma concentration is $\sim 2 \mu\text{M}$ [80,92,93], even with high dose oral administration. When physiologically relevant levels of resveratrol were applied to primary human CF tissue, no beneficial effects on chloride transport were observed [88]. Thus, although resveratrol may be useful in cell models, its current use in humans seems premature.

DISCUSSION AND CONCLUSION

At the same time as pharmaceutical companies are developing new synthetic drugs to treat CFTR mutations, compounds from natural sources are also being evaluated. Such compounds range from exotic extracts of South Pacific sponges [94], to plants that can be found in any neighborhood grocery store. What should be the response of CF patients and their families to these natural compounds discussed above? Should patients be placed on a steady diet of curries and red wine? It is an unfortunate truth that many preparations of natural remedies are not standardized, nor do they always contain the level of active ingredient that they are purported to have. Furthermore such remedies are not subject to regulatory oversight, as are drugs from pharmaceutical companies. However, it is also true that while the current pricing for FDA approved CF drugs from Vertex Pharmaceuticals is $\sim \$300,000$ per year, supplements such as genistein, curcumin and resveratrol can be obtained for a few hundred dollars per year. Certainly for curcumin and resveratrol, the achievable plasma concentrations are significantly lower than those that are reported to be efficacious in mutant CFTR correction. From an achievable plasma concentration standpoint, genistein likely holds the most potential. Current clinical trials employing genistein should help provide a clear answer as to the utility of genistein in treating patients with CF. Given the wide availability of the naturally occurring compounds discussed, it is not surprising that CF patients are willing to test such compounds on themselves.

The fact that compounds such as curcumin, genistein and resveratrol are common dietary ingredients does not prove they have a strong safety profile, since other common dietary constituents have shown toxicity when used as dietary supplements [95]. For example, 7 μM curcumin has been shown to induce both mitochondrial and nuclear DNA damage [96]. Oleoresin, an organic extract of turmeric containing levels of curcumin similar to those found in commercial grade curcumin [97], when fed to rats over two years, was associated with increased incidence of ulcers, hyperplasia and intestinal inflammation [97]. Even in humans, ingestion of 0.8 to 3.6 g/day curcumin for 1 – 4 months led to nausea, diarrhea and increases in serum lactate dehydrogenase and alkaline phosphatase [98]. Moreover, the cytotoxic properties of curcumin appear to be enhanced in the presence of many over the counter (OTC) medications, including ibuprofen, aspirin and acetaminophen [99]. In contrast to curcumin, genistein and resveratrol appear relatively benign. In a multiple dose study in which health volunteers received one dose of resveratrol (25 – 150 mg, or

placebo) every 4 h for 48 h, no significant adverse effects were reported [100]. Longer term animal studies (750 mg/kg/day for 3 months) in rabbits and rats, also failed to note any overt toxicity [101]. Although soy and its constituents (e.g. genistein) have been consumed at high levels in Asian populations for millennia without apparent adverse effects, the fact that genistein is a phytoestrogen has raised concerns about the potential endocrine effects of genistein. High soy consumers have serum genistein in the range of 1 – 5 μM [102], and such levels have not been associated with any negative effects. Similarly, animal studies have shown that high soy diets have no adverse effect on the reproductive system of prepubertal rhesus monkeys [103]. Although long term studies with high genistein consumption remain to be performed, genistein appears to have a very good safety profile.

We are beginning to tease out the mechanisms whereby natural compounds impact upon CFTR biology, whether directly on CFTR as appears to be the case with genistein, or with CFTR gene promoters as may be the case with resveratrol. At present it still remains to be determined what mechanistic effects, if any, are associated with curcumin on CFTR. Whether natural compounds will ever be a truly viable therapy for patients with CF remains unclear. What previous studies have shown however, is that it is important to understand the exact mechanistic actions by which such compounds impinge on mutant CFTR to cause it to traffic and/or function better. Such knowledge has the potential to impact on a rational design of synthetic drugs for CFTR, such that ultimately a safe, effective and inexpensive drug is available to treat patients with CF.

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