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Bioconjugates of curcumin display improved protection against glutathione depletion mediated oxidative stress in a dopaminergic neuronal cell line: Implications for Parkinson's disease

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ABSTRACT

Oxidative stress is implicated in mitochondrial dysfunction associated with neurodegeneration in Parkinson's disease (PD). Depletion of the cellular antioxidant glutathione (GSH) resulting in oxidative stress is considered as an early event in neurodegeneration. We previously showed that curcumin, a dietary polyphenol from turmeric induced GSH synthesis in experimental models and protected against oxidative stress. Here we tested the effect of three bioconjugates of curcumin (involving diesters of demethylenated piperic acid, valine and glutamic acid) against GSH depletion mediated oxidative stress in dopaminergic neuronal cells and found that the glutamic acid derivative displayed improved neuroprotection compared to curcumin.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by loss of dopaminergic neurons of the substantia nigra (SN) associated with dopamine loss in the basal ganglia. Among the early events causing neurodegeneration are oxidative stress and mitochondrial dysfunction.[1–4] Oxidative stress during early PD is elicited by a significant depletion of the cellular antioxidant glutathione (GSH+GSSG) in the SN.[5] GSH depletion precedes both mitochondrial dysfunction and dopamine loss and is therefore considered as the earliest triggering factor of neurodegeneration.[6,7] GSH depletion in dopaminergic cells in culture increased oxidative stress and decreased mitochondrial function.[8] These results suggest that the early GSH loss in the SN of PD patients could be linked to subsequent mitochondrial dysfunction ultimately leading to neurodegeneration.[1,8] We recently demonstrated the dynamic relationship between GSH metabolism and mitochondrial dysfunction associated with PD.[9] It could be surmised that therapeutic restoration of intracellular GSH levels might prevent triggering of the early events of pre-symptomatic PD. Further, such therapeutic molecules are efficacious only if they are non-toxic with the ability to cross the blood brain barrier (BBB).

Turmeric (*Curcuma longa*) is a dietary spice used in Indian cuisine and traditional medicine. Curcumin (diferuloylmethane), the most active and non-toxic component of turmeric is a polyphenol,[10,11] with antioxidant, anti-cancer properties and therapeutic potential in neurological disorders and ability to cross the blood brain barrier (BBB).[12–15] Curcumin is neuroprotective against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mediated neurotoxicity in vivo.[16,17] We earlier showed that curcumin protects mitochondria against nitrosative stress and oxidative stress both in vitro and in vivo.[18,19] Through systems biology, we have also explained the antioxidant mechanism of curcumin action and its effect on GSH metabolism.[19]

However, the bioavailability of curcumin is very limited due to poor absorption, rapid metabolism and quick systemic elimination.

Abbreviations: PD, Parkinson's disease; SN, substantia nigra; GSH, glutathione (reduced); GSSG, glutathione (oxidized); DTNB, 5,5-dithio-bis-2-nitro benzoic acid; ROS, reactive oxygen species; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; γ-GCL, gamma-glutamyl cysteine ligase; BSO, buthionine sulfoximine; H_2O_2, hydrogen peroxide; glu, glutamate; cys, cysteine; D1, di-demethylenated piperoyl curcumin; D2, di-valinoyl curcumin; D3, di-glutamoyl curcumin.

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To improve its bioavailability, we have reported the synthesis of three curcumin derivatives involving diesters at the phenolic groups: curcumin D1 (di-piperoyl), D2 (di-valinoyl), and D3 (di-glutamoyl). These bioconjugates have improved bioavailability than curcumin, due to improved solubility, enhanced adsorption and slow metabolism.[20–22] Unpublished data from our laboratory indicated that D3 exhibited increased detoxification of PN in vitro thus protecting brain mitochondria and preventing protein nitration and against 1-methyl-4-phenylpyridinium (MPP+) neurotoxicity in cell culture. In the current study, we have tested the neuroprotective ability of these derivatives against GSH depletion mediated oxidative stress in dopaminergic neurons in culture. Our overall aim is to obtain superior curcumin derivatives possessing both improved antioxidant capacity and increased bioavailability.

2. Materials and methods

All the chemicals and solvents were of analytical grade. Routine chemicals and bulk solvents were obtained from Sisco research Laboratories (Mumbai, Maharashtra, India) and Merck (Whitehouse Station, NJ, USA). Fine chemicals for biochemical assays were obtained from Sigma (St. Louis, MO, USA). Curcumin, valine, glutamic acid and piperine for synthesis were purchased from Merck (Schuchardt, Germany).

2.1. Preparation of curcumin derivatives

The bioconjugates of curcumin were synthesized by covalent linking of different moieties through the two phenolic groups of curcumin resulting in diesters. The moieties selected were demethylenated piperic acid, valine (val) and glutamic acid (glu) and the corresponding derivatives were denoted as **D1**, **D2**, and **D3**, respectively. The ester bonds in these compounds are biodegradable, that is, hydrolysable with common cellular esterases making these derivatives potent pro-drugs in the target cells. The following is the design, synthesis and analysis of these curcumin derivatives as reported earlier (Fig. 1):[20]

2.1.1. 1,7-Bis-[4-O-demethylenated piperoyl-3-methoxyphenyl]-1,6 heptadiene-3,5-dione (D1)

Curcumin (0.736 g, 2 mmol) was taken in dry pyridine and mixed with demethylenated piperoyl chloride (1.008 g, 4.5 mmol) and stirred at room temperature (rt) for 6 h. After the completion of reaction as indicated by thin layer chromatography (TLC), the reaction mixture was poured onto crushed ice and repeatedly extracted with EtOAc. The organic layer was concentrated and purified on a silica gel column using a DCM/methanol (MeOH) gradient. Yield: 45% (0.667 g); R_f: 0.74 (DCM/MeOH 9.5:0.5); UV λ_{max} (MeOH): 395 and 295 nm. Anal. Calcd for $C_{43}H_{36}O_{12}$: C, 69.29; H, 4.87; O, 25.78. Found: C, 69.34; H, 4.83; O, 25.80. ^{1}H NMR ($CDCl_3$) δ ppm = 3.65 (s, 6H, $-OCH_3$), 4.15 (s, 2H, C_4–H), 4.65 (d, 2H, O=C–CH_2), 5.03 (s, 4H, aromatic C–OH), 5.65 (d, 2H, –CH=CH–C=O), 6.38 (d, 2H, –CH=C), 6.72–7.80 (m, 8H, olefinic and aromatic), 6.90 (d, 1H, Ar–H), 7.18–7.48 (d, 2H, CH=CH=C=O), 7.48 (d, 2H, C_1–H and C_7–H).

2.1.2. 1,7-Bis-(4-*O*-valinoyl-3-methoxy phenyl) 1,6-heptadiene-3,5-dione (D2)

Curcumin (0.368 g, 1 mmol) was taken in dry pyridine and mixed with *N*-phthaloyl-valinoyl chloride (0.53 g, 2 mmol) and stirred at rt for 8 h. After completion of the reaction as indicated by TLC, the reaction mixture was poured into crushed ice and

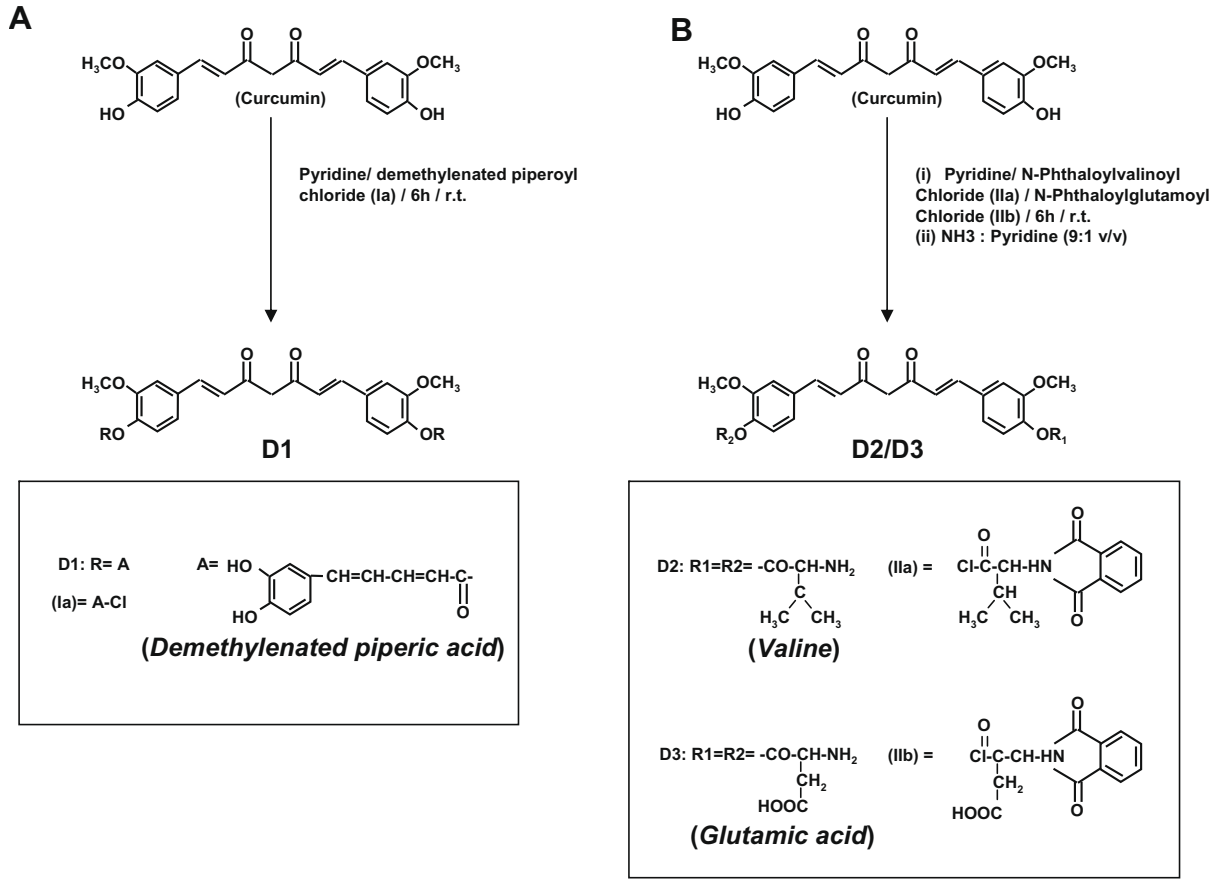

Figure 1. Chemical structure and scheme of synthesis of the curcumin derivatives used in this study. Scheme of synthesis of D1 (di-demethylenated piperoyl curcumin) (A), D2 (di-valinoyl curcumin), and D3 (di-glutamoyl curcumin) (B) (see Section 2).

repeatedly extracted with ethyl acetate. The organic layer was concentrated and treated with ammonia/pyridine (9:1 v/v) to remove phthaloyl group. The organic layer was concentrated and purified by silica gel column chromatography using DCM/methanol gradient. Yield: 45% (0.254 g); R_f: 0.9 (DCM/MeOH 9:1); UV λ_{max} (MeOH): 295 nm. Anal. Calcd for $C_{31}H_{38}O_8N_2$: C, 65.71; H, 6.76; O, 22.59; N, 4.94. Found: C, 65.74; H, 6.71; O, 22.60; N, 4.91. ^{1}H NMR ($CDCl_3$) δ ppm = 1.03 (d, 12H, CH–$(CH_3)_2$), 2.65 (m, 2H, 3′-C of valine), 3.70 (s, 6H, –OCH_3), 4.09 (s, 2H, C_4–H), 4.19 (m, 2H, CH–NH_2), 6.53 (d, 2H, C_2–H and C_6–H), 6.82–7.13 (m, 6H, Ar–H), 7.56 (d, 2H, C_1–H and C_7–H).

2.1.3. 1,7-Bis-(4-*O*-glutamoyl-3-methoxy phenyl) 1,6-heptadiene-3,5-dione (D3)

Curcumin (0.368 g, 1 mmol) was taken in dry pyridine and mixed with *N*-phthaloyl-glutamoyl chloride (0.55 g, 2 mmol) and stirred at rt for 8 h. After completion of the reaction as indicated by TLC, the reaction mixture was poured into crushed ice and repeatedly extracted with ethyl acetate. The organic layer was concentrated and treated with ammonia/pyridine (9:1 v/v) to remove phthaloyl group. The organic layer was concentrated and purified by silica gel column chromatography using DCM/methanol gradient. Yield: 40% (0.250 g); R_f: 0.9 (DCM/MeOH 9:1); UV λ_{max} (MeOH): 290 nm. Anal. Calcd for $C_{31}H_{34}O_{12}N_2$: C, 59.42; H, 5.47; O, 30.64; N, 4.47. Found: C, 59.39; H, 5.40; O, 30.67; N, 4.40. ^{1}H NMR ($CDCl_3$) δ ppm = 2.06–2.18 (m, 8H, 3′,4′-C of glutamate), 3.45 (m, 2H, CH–NH), 3.70 (s, 6H, –OCH_3), 4.46 (s, 2H, C_4–H), 4.09 (m, 2H, CH–NH_2), 6.53 (d, 2H, C_2–H and C_6–H), 6.83–7.11 (m, 6H, Ar–H), 7.54 (d, 2H, C_1–H and C_7–H), 11.2 (s, 2H, HO–C–O).

2.2. Cell culture

$1RB_3AN_{27}$ (N27) dopaminergic neuronal cell line has been used throughout this study. N27 cell line was generated by SV40 LT-antigen immortalization of rat mesencephalic cultures.[23] N27 cells possess all the physiological and biochemical properties of dopaminergic neurons and emulate those cells that are lost during PD.[24] N27 cells were grown as described earlier.[9,18]

2.3. Total glutathione (GSH + GSSG) estimation

Following different treatments, soluble N27 cell extracts were subjected to total glutathione estimations by the 5,5′-dithio-bis-2-nitro benzoic acid (DTNB) recycling method as described earlier.[18] The cell pellet was resuspended in PE buffer (100 mM potassium phosphate buffer, pH 7.4 containing 1 mM EDTA) and homogenized by sonication (5 s × 6 on ice) and total protein was estimated. 80 μl of the homogenate was acid precipitated with an equal volume of 2% sulfosalicylic acid (w/v). The mixture was centrifuged at 15,000*g* (15 min) and the resultant supernatant was used for the assay. 20 μl aliquot of the supernatant was incubated with assay buffer [PE buffer containing 0.8 mM DTNB and 0.32 U/ml glutathione reductase] in a final reaction volume of 450 μl. The reaction was initiated by addition of 0.6 mM NADPH. The reaction kinetics of DTNB recycling which was dependent on total glutathione levels was monitored at 412 nm for 3 min. The absolute GSH level in each sample was calculated based on the maximum reaction rate compared with GSSG standards (0–250 ng). All estimations were conducted in triplicate and GSH concentrations were normalized per mg protein.

2.4. Estimation of H_2O_2

Water soluble hydroperoxide levels in N27 cells were determined following ferrous iron oxidation with xylenol orange (FOX1) as previously described.[25] Briefly, N27 cells in 100–150 μl suspension were sonicated (5 s × 4 on ice; pulsed setting) and centrifuged at 13,500*g* (10 min) at 4 °C. Thirty microliters of the supernatant (corresponding to ~0.2 mg protein) was added to 0.95 ml of FOX1 reagent and incubated at room temperature for 30 min. The reaction mixture was centrifuged at 800*g* (10 min) and the absorbance of the supernatant was measured at 560 nm. The amount of H_2O_2 was calculated using the molar extinction coefficient of H_2O_2 (1.5×10^4 $mol^{-1}cm^{-1}$) and the amount of H_2O_2 was expressed as nM/mg of protein.

2.5. Estimation of lipid peroxidation

Lipid peroxidation was measured by estimation of malondialdehyde (MDA) by thiobarbituric acid reaction method modified from Ohkawa et al.[26] Briefly, N27 cells were sonicated and centrifuged at 16,000*g* (10 min) at 4 °C. One hundred microliters of the supernatant was added to a mixture containing 0.75 ml of acetic acid (pH 3.5, 20% v/v), 0.1 ml SDS (8% w/v) and 0.75 ml of thiobarbituric acid (0.8%, w/v). The reaction mixture was heated in boiling water bath for 45 min. The adducts formed were extracted into 1.5 ml of 1-butanol by vortexing and centrifugation at 2500 rpm (10 min) and their absorbance was measured at 532 nm. The amount of MDA formed was calculated using the molar extinction coefficient (241 $mol^{-1}cm^{-1}$).

2.6. Measurement of reactive oxygen species (ROS)

Total ROS in N27 cells were measured by a modified method of Ohashi et al.[27] Briefly, the medium from treated or untreated N27 cells was replaced with 1 ml Locke's solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 5 mM HEPES, 2 mM $CaCl_2$ and 10 mM Glucose, pH 7.4). Ten micromoles of dihydrodichlorofluorescein diacetate (DCFDA) was then added and the cells were incubated at 37 °C (10 min) in a CO_2 incubator. The Locke's solution was then removed and the cells were harvested. The cell pellet was washed with 1X phosphate buffered saline (PBS), pH 7.4, twice and reconstituted in lysis buffer (10 mM Tris–HCl containing 0.5% Tween-20). The lysate was centrifuged at 1000*g* (10 min) and the fluorescence of the supernatant was measured (Excitation—480 nm; Emission—530 nm).

2.7. Glutathione-S-transferase (GST) assay

GST was assayed by measuring the rate of enzyme catalyzed conjugation of GSH with 1-chloro 2-4-dinitro benzene (CDNB) according to the method of Guthenberg et al.[28] Briefly, to 1 ml reaction mixture containing phosphate buffer (0.1 M, pH 6.5; 0.5 mM EDTA), CDNB (1.5 mM) and 50 μl GSH (1 mM) and 50 μl sample was added and the increase in absorbance at 340 nm was monitored for 5 min. The enzyme activity was expressed as nmoles of S-2,4, dinitrophenyl glutathione formed /min/mg protein (M EC-9.6 mM^{-1} cm^{-1}).

2.8. Statistical analyses

All quantitative data were accumulated from at least three independent experiments. The final data are expressed as mean ± SEM. Differences between mean values were analyzed by one-way analysis of variance (ANOVA) and p value <0.05 was considered to be significant.

2.9. Predictive data from virtual experimental system

2.9.1. Model description

We recently reported predictive studies linking GSH metabolism and mitochondrial dysfunction associated with PD.[9] We also described the effect of curcumin administration on GSH

metabolism.[19] The current experiment includes the comparison of curcumin and its derivatives in terms of their effect on GSH metabolism and oxidative stress in a module of the dopaminergic neuronal platform. This module evaluates the induction of gamma-glutamyl cysteine ligase (γ-GCL) and generation of GSH, ATP, ROS, and reactive nitrogen species (RNS). The nodes included in each of the individual modules are qualitatively described here with the equations and kinetic parameters for each node described previously.[9,19]

Oxidative stress due to GSH depletion: The homeostasis of ROS and RNS in the neuron under normal physiological conditions is maintained mainly by GSH and associated enzymes.[9] The decline in the protective antioxidant system with lowered GSH levels impacts by oxidative damage to the mitochondria.[29]

2.9.1.2. GSH synthesis and metabolism

This module incorporating GSH synthesis, its cellular distribution and antioxidant function have been described earlier.[9,30] Curcumin (and its derivatives) administered into the system induces GSH synthesis via up regulation of γ-GCL.[31]

2.9.2. Model construction

The model is a dynamic representation of GSH metabolism modulated by curcumin. The model construction and its assumptions have been described earlier.[9] The reaction kinetics for all nodes in the dynamic integrated model is summarized in the supplementary data of previously reports.[9,19]

2.9.3. Simulation protocol

Initial conditions: The initial conditions of the neuronal platform represent normal mitochondrial energetics with the free radicals generated being scavenged by GSH antioxidant system. In the neuron, the ATP utilization is ~80 μM/s[32] and the PMF is ~90–200 mV. The normal cytosolic and mitochondrial concentrations of GSH are 1–3 mM[33] and 10–50 μM, respectively. The steady-state concentrations of H_2O_2 and ROS in the mitochondria are ~10 nM and 0.1 nM, respectively.[34] The normal physiological concentrations of NO and calcium in matrix are ~0.02–0.1 μM and 0.2 μM, respectively.[35,36]

Administration of curcumin and its derivatives: In this experiment, the dopaminergic neuronal platform was exposed to the conditions indicated below followed by estimation of cellular GSH and ROS:

(a) Curcumin alone (0.5 μM).
(b) Improved bioavailability of curcumin representing the derivatives D1 and D2 and indicated by doubled curcumin levels (1 μM).
(c) Improved bioavailability of curcumin (1 μM) in addition to mild increase in glutamate levels (from 1 μM to 3 μM) representing D3.

Curcumin was modeled as having the following dual characteristics as described in Jagatha et al.:[19]

(a) *GSH up regulation*: Curcumin administration (1 μM) to a normal cell increases γ-GCL levels from 0.26 μM to 0.33 μM resulting in a ~1.5-fold increase of GSH level.
(b) *Direct scavenging of ROS and RNS*: Curcumin shows SOD activity and PN scavenging activity.[37] The cumulative effects of both reactions (i) and (ii) have been modeled.

3. Results

Oxidative stress implicated in PD pathology involves GSH depletion as the earliest event.[7] Hence, therapeutic intervention to decelerate neurodegeneration requires molecules that restore neuronal GSH levels and prevent oxidative damage. We showed that curcumin protects against oxidative and nitrosative stress in vivo either by direct detoxification of toxic species or via induction of GSH synthesis.[18,19] However, the bioavailability of curcumin poses a major obstacle for its therapeutic efficacy. The synthesis and improved cellular uptake of curcumin bioconjugates have been recently reported.[20–22] In the current study, we have tested the neuroprotective ability of three derivatives of curcumin (diester with demethylenated piperic acid or D1; diester with val or D2; diester with glu or D3) compared to curcumin against GSH depletion mediated oxidative stress in dopaminergic neurons (see Fig. 1 for structures of the compounds).

We utilized N27 cell line as a model for GSH depletion associated with PD. N27 cell line, generated by immortalization of dopaminergic neurons from rat fetal mesencephalon are comparable to the neurons selectively lost in PD.[23,24] Hence N27 cell line represents an excellent cell model to understand GSH depletion mediated oxidative stress associated with PD. N27 neuronal cells treated with BSO (0–25 μM; 24 h) showed a dose dependent decrease in the levels of total cellular GSH (Fig. 2A) with 50% depletion obtained at 1.5 μM BSO and maximum decrease at 25 μM. For subsequent experiments, cells were exposed to 1.5 μM BSO for 24 h to maintain ~50% depletion of cellular GSH, similar to the decreases observed in the PD SN.[7]

We confirmed the neuroprotective ability of curcumin and its derivatives against GSH depletion using the BSO-N27 model.[19] Treatment of N27 cells with curcumin or the derivatives alone (0.5 μM, 24 h) significantly increased total GSH as shown earlier[18,19] with D3 showing maximum increase (~64% compared with untreated control) compared to curcumin (30%), D1 (~25%), and D2 (~25%) (Fig. 2B). However, higher concentrations of curcumin and the derivatives did not cause further increase in GSH levels (data not shown) indicating that under these conditions, 0.5 μM curcumin or its derivatives is sufficient to induce maximum GSH levels in these neurons.

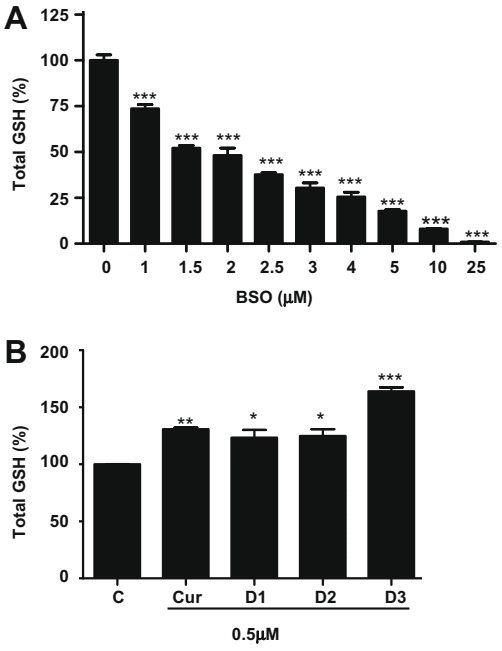

Figure 2. Effect of BSO and curcumin on total GSH levels in N27 dopaminergic neurons. (A) Estimation of total GSH in N27 cells incubated with 0–25 μM BSO for 24 h ($n = 5$). Values shown as percentage of GSH compared with untreated control (100% GSH = 23.3 ± 3.0 nmol/mg protein). (B) Estimation of total GSH in N27 cells incubated with 0.5 μM of curcumin, D1, D2, and D3 for 24 h ($n = 3$). $^{*}p$ <0.05, $^{**}p$ <0.01 and $^{***}p$ <0.001 compared to untreated control (in A and B).

When N27 cells were treated with BSO (1.5 μM for 24 h) followed by BSO withdrawal for 24 h, it completely restored the cellular GSH levels indicating that BSO mediated GSH depletion was specific and reversible (Fig. 3). This regimen involving 24 h BSO treatment followed by 24 h withdrawal was applied in all the subsequent experiments. Pre treatment of BSO treated N27 cells with curcumin and its derivatives (0.5 μM, 24 h) significantly restored the GSH levels compared to BSO alone (1.5 μM, 24 h) (curcumin = 78%, D1 = 78%, D2 = 72% and D3 = 83% compared to BSO = ~50% and control = 100%) (Fig. 3). However, post treatment (0.5 μM, 24 h) caused tremendous increase in GSH compared with the BSO alone and much higher compared to untreated control (curcumin = 127%, D1 = 124%, D2 = 122% and D3 = 136% compared to control = 100% and BSO alone = 50%) (Fig. 3). We observed that D3 showed higher GSH levels both in the pre and post treatment experiments compared to curcumin.

It is known that GSH depletion causes increased ROS production.[7] Since curcumin and its derivatives can restore GSH levels in the BSO-N27 model, we tested whether these derivatives could detoxify ROS more efficiently compared to curcumin. Accordingly, we measured the cellular levels of MDA (marker of lipid peroxidation), H_2O_2 and total ROS in N27 cells treated either with BSO (0.5 μM) alone or in combination with curcumin or its derivatives (both pre and post-treatment) compared to untreated N27 cells. Figure 4A shows that BSO treatment resulted in ~25% increase in total MDA levels compared to untreated extracts, which was significantly decreased following BSO withdrawal for 24 h (104%). While curcumin pre treatment resulted in ~40% reduction in lipid peroxidation compared to BSO alone, pretreatment with D1 and D3 resulted in ~50% reduction and D2 caused ~30% reduction in lipid peroxidation. On the other hand, post treatment with these compounds caused higher reduction in MDA levels as shown in Figure 4A (65% reduction with curcumin, 75% with D1, 80% with D2, 90% with D3 compared with BSO alone). These levels were much lower than the MDA levels observed in untreated cell extracts. Such drastic detoxification of lipid peroxidation might be possible due to significantly higher GSH levels during pretreatment (as shown previously in Fig. 3). We observed that the exposure to D3 resulted in maximum reduction in lipid peroxidation compared with curcumin and other derivatives.

We surmised that the decrease in MDA levels in cells treated with curcumin and derivatives might be due to the direct involvement of cellular GST activity. Accordingly, GST assay in BSO treated N27 extracts showed ~20% reduction in activity compared with untreated controls (Fig. 4B). Further, withdrawal of BSO or incubation with curcumin and its derivatives (both pre and post treatments) restored GST activity in BSO treated cells comparable to untreated controls. However, a direct correlation between the magnitude of restoration of GST activity and the corresponding MDA values in respective treatment groups was not observed.

Estimation of H_2O_2 indicated ~40% increase in BSO treated N27 cells which decreased by ~25% following BSO withdrawal (Fig. 4C). The H_2O_2 levels were further reduced (~50% reduction compared to BSO alone) in cells either pre or post-treated with curcumin or its derivatives. Except for D2 (post-treatment) which showed ~80% decrease in H_2O_2, the other derivatives showed similar reduction compared with curcumin. Nevertheless, these levels were much lower than the H_2O_2 levels observed in untreated cell extracts.

Similarly, ROS estimation indicated that compared to untreated controls, there was ~65% increase in BSO treated N27 cells which was decreased by ~30% following BSO withdrawal (Fig. 4D). The ROS levels were further decreased in cells pre-treated with curcumin or its derivatives (50% reduction in ROS with curcumin, 55% with D1, 70% with D2, and 80% with D3 compared with BSO alone). Post treatment was also able to abrogate BSO mediated ROS generation (45% reduction in ROS with curcumin, 65% with D1, 60% with D2 and 90% with D3 compared with BSO alone). As seen in the MDA experiment, exposure to D3 caused maximum scavenging of ROS compared with curcumin and other derivatives. However, cells treated with either curcumin or the derivatives alone did not show significant increase in ROS levels (data not shown).

Based on the data, we conclude that among the derivatives, D3 displayed significantly enhanced antioxidant potential against oxidative stress in dopaminergic neurons compared with curcumin. To explain the mechanism underlying this, we carried out a systems biology based predictive study of curcumin and its derivatives in a dopaminergic neuronal virtual platform described earlier.[9,19] Figure 5 shows the dynamics of GSH metabolism in the brain which represents a steady-state GSH concentration of 2.5 mM. Curcumin (0.5 μM) administration into the system increased the expression of the enzyme γ-GCL by Nrf2-dependent pathway as described earlier,[19] resulting in elevation of GSH levels up to ~3 mM (Fig. 5A). Increase in curcumin concentration up to 1 μM mimicking improved bioavailability in derivatives did not elevate the GSH levels further thus corroborating our cell culture data. However, when curcumin at 0.5 μM along with increased glutamate levels mimicking the glutamoyl diester of curcumin (D3) was administered, the GSH levels almost doubled to 4.75 mM. This trend is similar to the increase in GSH levels in D3 treated N27 cells compared to curcumin. Consequently, D3 showed improved detoxification of ROS compared with curcumin and other derivatives (Fig. 5B). Figure 6 is a schematic representation of the effect of curcumin and its derivatives on GSH metabolism in the dynamic model.

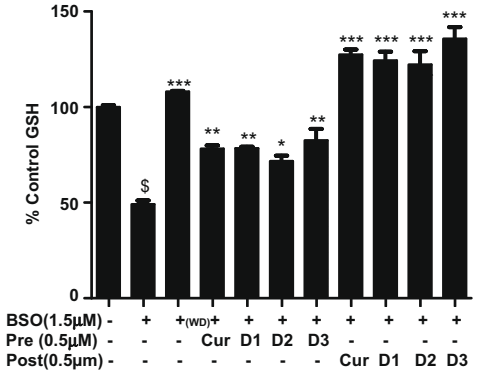

Figure 3. Neuroprotective effects of curcumin on total GSH levels GSH-depleted dopaminergic neurons. Estimation of total GSH in BSO (1.5 μM) treated N27 cells incubated with or without 0.5 μM curcumin or its derivatives (D1, D2, and D3) (pre or post-treatment) ($n = 6$). WD = BSO withdrawal for 24 h. $^{\$}p$ <0.001 compared to untreated control; $^{*}p$ <0.05, $^{**}p$ <0.01, and $^{***}p$ <0.001 compared to BSO alone.

4. Discussion

There have been significant advances in PD therapy including surgical and pharmacological interventions. Currently, there are several options for pharmacological treatment of PD with Levodopa (L-dopa) being the most popular drug. However, the occurrence of severe drug-induced side effects in chronic L-dopa therapy has allowed utilization of alternative molecules including dopamine receptor agonists, anti-cholinergic drugs, monoamine oxidase-B inhibitors, catechol-*O*-methyl transferase inhibitors etc. These therapeutic strategies are mainly symptomatic and strive to replenish striatal dopamine. But their ability to prevent or slowdown neurodegeneration of SN neurons has not been completely validated in humans. Therefore, there is scope for exploration of novel therapeutic molecules that prevent neurodegeneration and

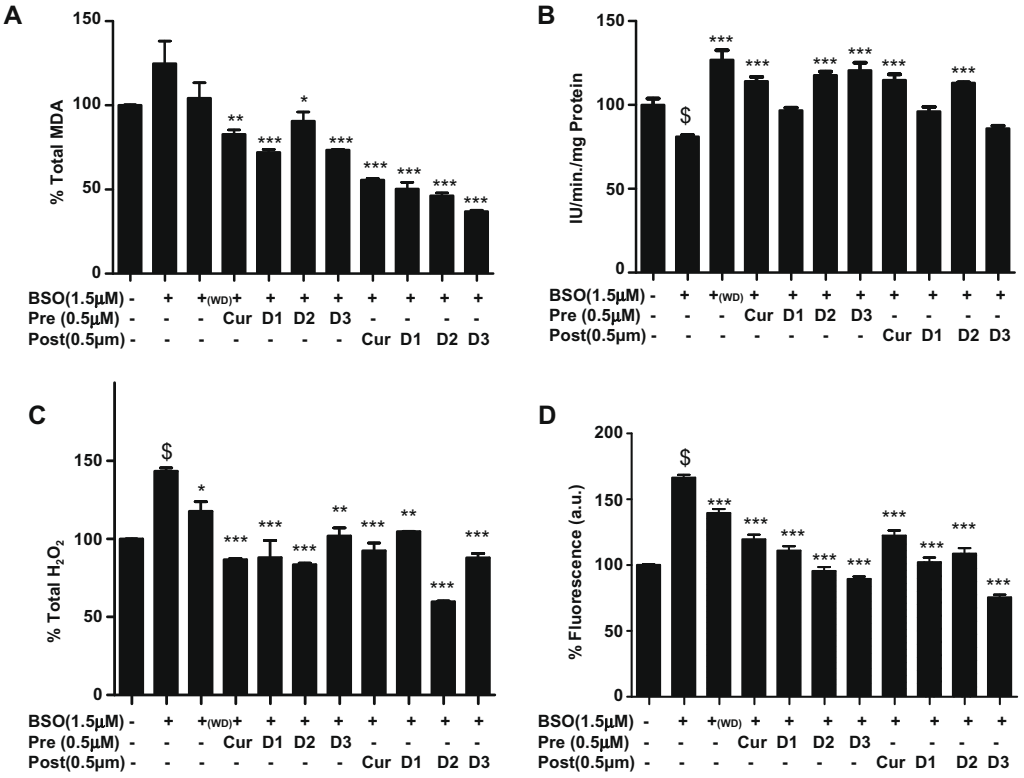

Figure 4. Neuroprotective effects of curcumin on lipid peroxidation, GST activity, hydrogen peroxide production, and total ROS in GSH-depleted dopaminergic neurons. Total cell extracts from untreated and BSO (1.5 μM) treated N27 cells incubated with 0.5 μM curcumin or its derivatives (D1, D2, and D3) (pre or post-treatment) were used to estimate total cellular concentrations of (A) MDA ($n = 6$); values shown as percentage of MDA compared with untreated control (100% MDA = 115 ± 5 nmol/mg protein); $^{*}p$ <0.05, $^{**}p$ <0.01, and $^{***}p$ <0.001 compared to BSO alone. (B) GST activity ($n = 3$); values shown as percentage of GST activity compared with untreated control (100% GST activity = 0.407 ± 0.02 IU/mg protein/min); $^{\$}p$ <0.05 compared to untreated control; $^{***}p$ <0.001 compared to BSO only. (C) H_2O_2 ($n = 3$); values shown as percentage of H_2O_2 compared with untreated control (100% H_2O_2 = 13 ± 2 nmol/mg protein); $^{\$}p$ <0.001 compared with untreated control; $^{*}p$ <0.05, $^{**}p$ <0.01, and $^{***}p$ <0.001 compared to BSO alone; and (D) Total ROS content ($n = 6$); values indicated by percentage arbitrary units (au) of DCF fluorescence; (100% Fluorescence = 264.4 ± 1.2 au/mg protein); $^{\$}p$ <0.001 compared with untreated control; $^{***}p$ <0.001 compared with BSO alone. WD = BSO withdrawal for 24 h in all the figures.

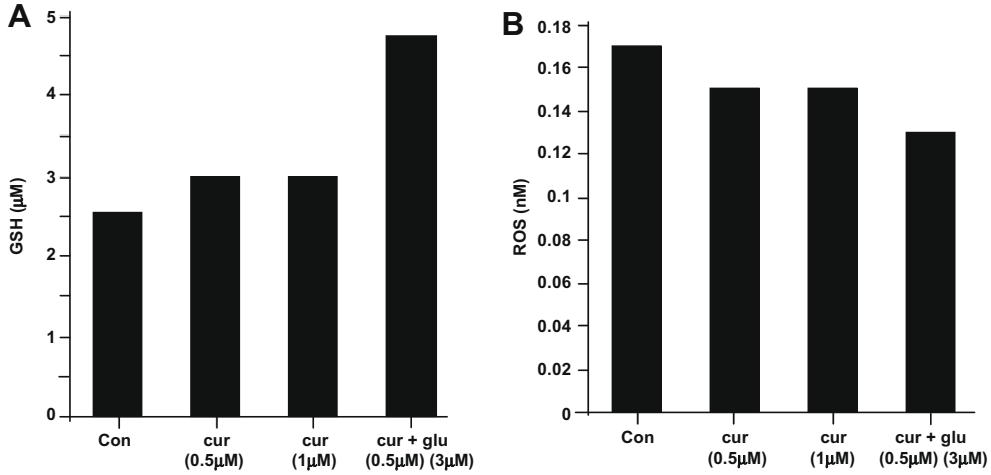

Figure 5. In silico predictive data from the virtual experimental system on the effect of curcumin and its derivatives on cellular GSH synthesis and scavenging of ROS. Curcumin (either at 0.5 and 1 μM) and glutamate (at 3 μM) were introduced into the dopaminergic neuronal platform followed by (A) estimation of GSH synthesis and (B) estimation of ROS.

could be used as adjunctive therapies along with dopamine replacement.[38]

Curcumin exhibits neuroprotective effect against MPTP[16,17] and 6-hydroxy dopamine (6-OHDA) in vivo.[39] We have recently reported that curcumin protects mitochondria against PN and GSH depletion mediated toxicity in vitro and in vivo.[18,19] Curcumin also binds to the redox-active metals, iron and copper thereby exerting neuroprotection against oxidative damage.[40] Interestingly, curcumin induces GSH synthesis in cells by activation of GCL activity in vivo thereby enhancing the antioxidant potential.[31] Dickinson et al.[31] demonstrated that curcumin-induced elevation of GCL activity is probably by enhanced transcription of GCL genes via

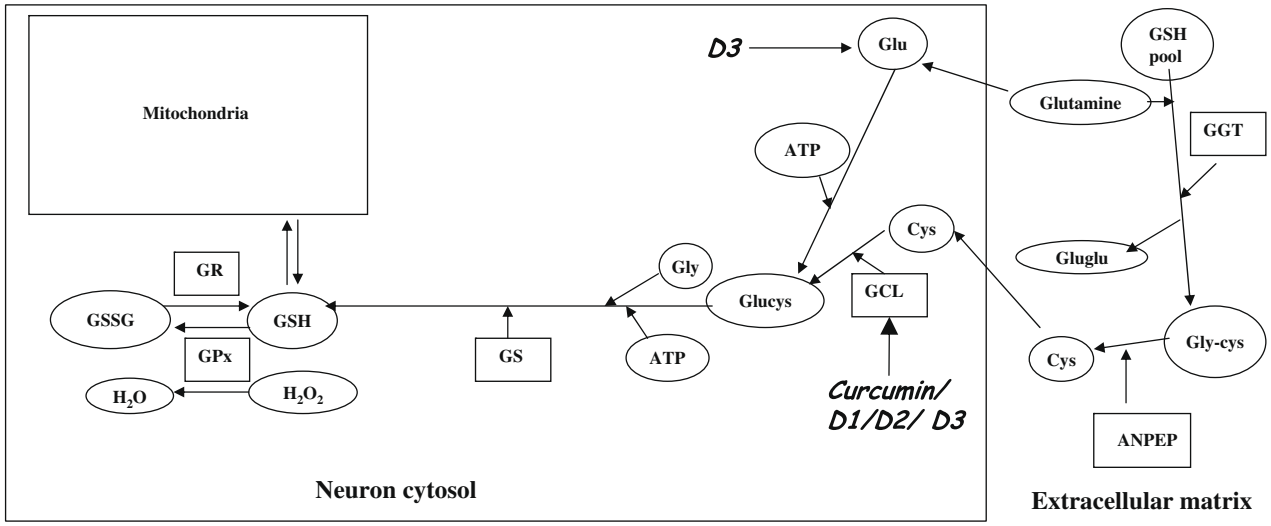

Figure 6. Schematic overview of the effect of curcumin or its derivatives on GSH metabolism. De novo synthesis of GSH from its constituent amino acids in the neuronal cytoplasm occurs as a two-step reaction. Whereas curcumin and its derivatives D1, D2, and D3 contribute to GSH synthesis at the rate limiting step by increasing the gene expression of γ-GCL, D3 also contributes via a minor elevation of glu levels in the cytosol. Conversion of the oxidized form of GSH, GSSG also contributes to the total cellular GSH pool. GSH released from the cell is hydrolyzed by the activity of GGT in the extracellular space to its constituent amino acids, which could later be taken up by the cells for de novo GSH synthesis. The mitochondrial GSH pool is maintained by uptake of cytosolic GSH via an energy-dependent mechanism. GSH detoxifies ROS and RNS generated in the mitochondria during different metabolic reactions. List of abbreviations: GSSG, GSH disulfide; GR, GSH reductase; ANPEP, alanyl (membrane) aminopeptidase; Gly, glycine; gluCys, glutamate-cysteine; Cys, cysteine; Glu, glutamate; GGT, gamma-glutamyl transpeptidase; GS, GSH synthase; GPx, Glutathione peroxidase; H_2O_2, hydrogen peroxide.

binding of specific transcription factor complexes to TRE and EpRE elements. Further, the authors showed that curcumin modulates the components of transcription factors that compose EpRE and AP-1 complexes which bind to these cis elements, thereby impacting the GCL expression. Lavoie et al.[41] demonstrated both in astrocytes and neuronal cells that curcumin caused a specific and significant increase in the expression of the gene coding for the modifier subunit of γ-GCL (GCLM) which is required for the up regulation of γ-GCL activity. Based on these data and our previous reports,[18,19] we surmise that curcumin could be a potential candidate for adjunctive pharmacotherapy in PD.

However, there are several reports indicating poor bioavailability of curcumin due to the rapid systemic derivatization in the GI tract suggesting that small doses of curcumin are necessary for its neuroprotective effect.[42] Limited bioavailability might be due to poor absorption, rapid metabolism and quick systemic elimination. In order to improve its bioavailability, the use of adjuvants like piperine to prevent glucuronidation, use of curcumin nanoparticles, curcumin structural analogues, liposomal curcumin or curcumin phospholipid complexes have been attempted.[43]

We have utilized diester derivatives of curcumin (di-piperoyl, di-valinoyl and di-glutamoyl) that improve its bioavailability.[20] These conjugates could be dissociated at the target site by cellular esterases releasing curcumin thereby functioning on as pro-drugs. Studies in animal models and human volunteers showed that piperine, an active natural compound from black pepper enhances the activity and bioavailability of curcumin several fold.[44] Similarly, valinoyl ester pro-drugs exhibit improved bioavailability.[45] The diesters of curcumin used in our study have: (i) enhanced metabolic stability due to protection of phenolic hydroxyl groups thus delaying their glucuronidation (ii) improved cellular uptake due to transportation via amino acid carriers and (iii) better solubility due to increased hydrophilicity.[20]

We observed that all the three derivatives tested were either equal to or more potent compared to curcumin in countering the GSH depletion mediated oxidative stress. Moreover, the derivatives showed neuroprotection both during pre and post treatment suggesting that they could reverse the oxidative damage caused during GSH depletion. Among the three bioconjugates, D3 exhibited significant increase in GSH levels and showed maximum detoxification of ROS. The predictive study carried out to delineate the beneficial effects of D3 indicated the following: During GSH synthesis, the rate limiting step is the formation of the dipeptide gamma-glutamyl cysteine.[46] This requires influx of glu (as glutamine) and cys (as cystine) from the extracellular matrix as shown in the schematic representation in Figure 6.[9] Since it was not possible to introduce the glutamoyl diester of curcumin in our neuronal platform, we chose to increase the extracellular glu levels (thus increasing its influx into the neuronal cytosol) from 1 μM (control condition) to 3 μM along with increased levels of cytosolic curcumin. Consequently, the initial increase in GSH was basically due to increased neuronal glu without any significant changes in cystine uptake and intracellular cys levels. With increased glu in the μM range in the system, we observed initial elevation in cellular GSH. However, when the glutamate levels increased to 1–5 mM, cystine uptake in astrocytes was inhibited leading to reduction in neuronal cys thereby decreasing GSH levels (data not shown). Despite abundant glutamate levels, GSH levels dropped since cys here became rate limiting for synthesis.

Our study indicated that administration of D3 probably enhanced cellular GSH levels not only via increased accumulation of curcumin in the cell, but also by improved intracellular concentrations of glu in the non-toxic range. D3 not only exhibits improved bioavailability but also independently enhances GSH levels making it a specific pro-drug for diseases involving oxidative stress. In conclusion, compounds such as D3 with improved uptake, ROS-scavenging capacity and neuroprotective efficiency in vivo compared with curcumin with a capacity to cross blood brain barrier could serve as potential neuroprotective strategies in disorders such as PD.

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