

Pharmacokinetic-Pharmacodynamic (PK-PD) Modeling of Effect of Naringenin and Its Surface Modified Nanocarriers on Associated and Core Behaviors of Autism Spectrum Disorders (ASD)



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ABSTRACT

The pharmacokinetic and pharmacodynamic (PK-PD) model was developed to describe the relationship between plasma/brain concentration of naringenin and its nanocarriers with behavioral and biochemical alterations in a rat model of autism spectrum disorders (ASD). Behavioral parameters like sensorimotor dysfunction, hyperlocomotion, anxiety-like behavior, social interaction, and repetitive behavior were investigated by rotarod, actophotometer, open-field, reciprocal social interaction, and repetitive self-grooming test respectively. Naringenin was administered in doses (25, 50, and 100 mg/kg) and in the form of its uncoated and glutathione as well as tween 80-coated PLGA nanocarriers (25 mg/kg) thrice daily (8 hourly). Sigmoid E_{max} model was applied to study the relationship between the concentration of naringenin in plasma/brain and behavioral effects (in terms of sensorimotor dysfunction, locomotor activity, anxiety-like behavior, social interaction ability, repetitive behavior) as well as biochemical changes (plasma levels of TNF- α , MMP-9, and HSP-70, and Pgp at BBB). Model parameters such as E_0 , E_{max} , and EC_{50} indicate that maximum effect occurred after administration of GSH-coated naringenin nanoparticles and the minimum effect occurred with the 25 mg/kg dose of unencapsulated naringenin. The R^2 value of 0.99 and small Akaike information criterion indicate the goodness of fit of the model. The PK-PD modeling done by sigmoid E_{max} model showed a positive correlation between plasma/brain drug concentration and neuroinflammatory markers as well as behaviors consistent with the ASD phenotype.

LIST OF ABBREVIATIONS

PK-PD	Pharmacokinetic and Pharmacodynamic
ASD	Autism Spectrum Disorders
PLGA	Poly(lactic-co-glycolic acid)
TNF-alpha	Tumour necrosis factor-alpha
MMP-9	matrix metalloproteinases 9
HSP-70	Heat Shock Protein 70
Pgp	P-glycoprotein
BBB	Blood brain barrier
IL-6	Interleukin-6
NP	nanoparticle
PPA	Propanoic acid
GSH	Glutathione
NGN	Naringenin
IL- β	Interleukin-Beta

Introduction

Autism spectrum disorders (ASD) include complex neurodevelopmental disorders associated with characteristic symptoms that manifest in children at 3 y of age. These characteristic symptoms include impairments in social interaction and communicative skills as well as the presence of restrictive, repetitive, pervasive, and stereotypic behavior. In addition to the above, some co-morbidities like irritable nature, anxiety, aggression, cognitive deficits, hampered adaptive skills, and accompanied disorders like attention deficit hyperactivity disorder, epilepsy, and sensory processing disorder are also associated with ASD [1, 2]. According to the National Institute of Mental Health, 2.41% of children in the US have ASD. Prevalence of ASD has been reported by the U.S. Center for Disease Control and Prevention to be 1 in 59 children in 2018 in comparison to 2010 reports of 1 in 88. The World Health Organization reports of 2017 show that worldwide 1 in 160 children is suffering from ASD [3]. ASDs show significant skewness in occurrence in boys with a sex ratio of 4:1 [4–6]. ASD presents a complex integration of genetic, epigenetic, and environmental factors. It is a complex interaction between preexisting genetic factors and environmental factors that alter the functional capacity of the brain [6–10].

Apart from several behavioral and cognitive complications arising as a result of central nervous system dysfunction, there are various physiological co-morbidities associated with ASD that can also worsen the behavioral complications. Research and clinical studies indicated many physiological co-morbidities such as immune system deregulation, neuroinflammation, oxidative stress, mitochondrial dysfunction, and gastrointestinal complications [6, 11, 12]. The release of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF- α), IL-6, IL- β , and other triggers of neuroinflammation such as heat shock protein-70 (HSP-70) and matrix metalloproteinases-9 (MMP-9) as a result of the generation of oxidative stress and immune system activation is one of the components of the pathogenesis of ASD [13, 14].

Behavioral and gastrointestinal symptoms worsen in ASD children after intake of high carbohydrate containing propanoic acid (PPA) as a preservative [15, 16]. PPA, a weak organic acid, can cause

the release of inflammatory cytokines as well as depletion of endogenous antioxidants like glutathione (GSH) and superoxide dismutase as well as the elevation of lipid peroxidase leading to increased oxidative stress. Intracerebroventricular administration of PPA in adolescent rats induced ASD phenotype [17, 18]. We have also validated this model in our lab with some modifications and evaluated the therapeutic potential of curcumin resveratrol and naringenin (NGN) [19–21]. We have observed the increase in markers of neuroinflammation such as TNF- α , MMP-9, and HSP-70 in our previous studies as well as depletion of endogenous antioxidants as a result of oxidative stress that results in the development of ASD like core as well as associated behaviors.

(\pm)-Naringenin (5,7-dihydroxy-2-(4-hydroxyphenyl) chroman-4-one) is a flavanone that is abundantly found in grapefruit as well as in oranges and tomato skin [22]. NGN has been found also to exert its antioxidant, antihyperlipidemic, antidepressant, antiproliferative, and anti-inflammatory effects [23–27]. Despite its therapeutic potential NGN's role clinically has been hampered as a result of its poor bioavailability, instability, and extensive first-pass metabolism before reaching the systemic circulation [28, 29]. In order to improve the bioavailability and enhance the brain uptake of NGN, we developed NGN-loaded PLGA nanoparticles in our laboratory and also coated these with reduced GSH and tween 80 in order to inhibit the P-glycoprotein (P-gp) efflux transporter. We evaluated their brain uptake, studied *in vivo* pharmacokinetics, and observed that there is an improvement in bioavailability of NGN and its enhanced uptake in the brain after surface coating of nanoparticles with GSH and tween 80 (our unpublished study).

We have explored the therapeutic potential of NGN as well as its coated and uncoated nanoparticles in the experimental paradigm of ASD [21].

The pharmacokinetic-pharmacodynamic (PK-PD) model is a prospective aid for efficient drug development as its usage can avoid high attrition rates of drugs from the market as a result of lack of efficacy and safety issues raised during clinical trials or post-marketing surveillance phase [30]. It is a promising tool in translational drug research that can predict about safety and efficacy of drugs on the basis of *in vitro* and *in vivo* studies. It can prove to be of help in optimization of dosage regimen especially for controlled release formulations in clinical trials, simulating, and optimizing of phase 3 clinical trial design and in early drug development [31]. PK-PD modeling is a useful tool that can connect PK and PD so that effect and time relationship occurring after the administration of a particular dose of a drug can be predicted from the dose-response relationship [32]. When pharmacodynamic parameters are not dependent on time and concentration of the drug is constant at the active site (i. e., steady-state has been achieved), then concentration and effect can be predicted utilizing the models such as the linear effect concentration model, log-linear effect concentration model, fixed-effect model, and E_{max} and sigmoid E_{max} model. Time-dependent study of data shows the presence of hysteresis loop (i. e., there is some delay between the plasma concentration of drug at the site of action and its effect). The hysteresis loop is either clockwise or counterclockwise. The 2 non-steady-state models are the effect compartment model and physiological indirect response models. Modeling criteria depends on either the mode of administration of the drug or the dependence of pharmacodynamic pa-

rameters on time. Hence, when a steady state is achieved after multiple dosing or long-term infusion, then steady-state models are used, but when a single dose of the drug is administered and pharmacodynamic parameters show a time-dependent relationship, then more complicated models are used such as those meant for the non-steady state. Simple and empirical models have limitation in their capability to predict the relationship between effect and concentration as they do not take into account the mechanism behind the delay in response. Thus, more mechanism-based models for PK-PD correlation are required that are based on a mechanism that a drug undergoes at the site of action and presents forth actual relationship between the concentration of drug and effect [31–35].

With this background, we have designed this PK-PD study to establish a relationship between various concentrations of NGN in plasma and brain achieved via administration of various doses of unencapsulated as well as its uncoated and coated nanocarriers and their effect on various behavioral/biochemical alterations.

Results

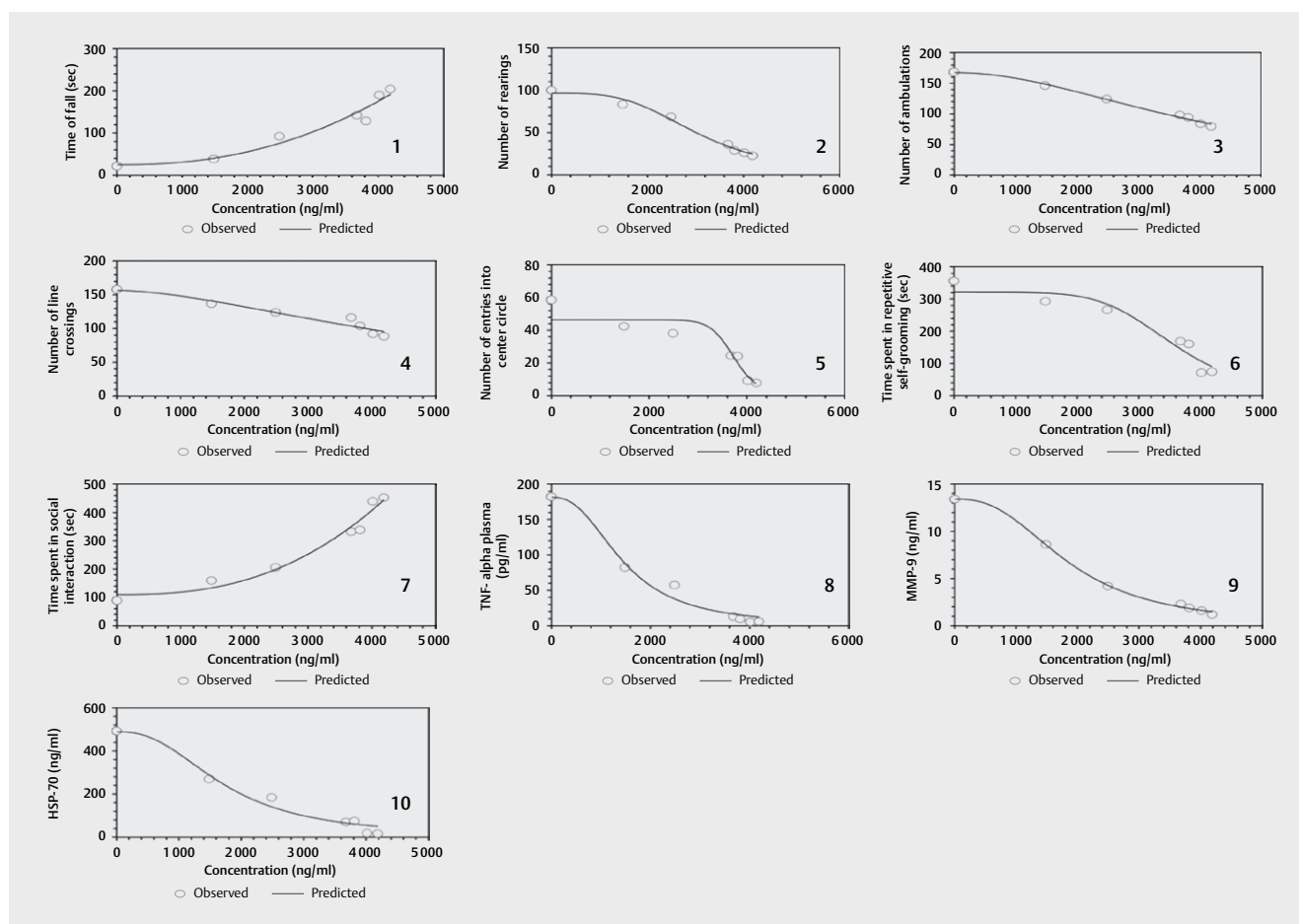
Six groups of animals out of 7 groups, which were administered 1M PPA, were treated with NGN (25, 50, and 100 mg/kg) and with uncoated as well as GSH or tween 80-coated NGN-loaded PLGA nanoparticles (25 mg/kg). Different plasma concentrations (approximately equal to the minimum residual dose) of NGN were achieved on the 22nd day, 8 h after the administration of the last dose on the 21st day. The minimum plasma concentration of (1482.8 ± 0.22 ng/mL) was for NGN (25 mg/kg) and highest was for GSH-coated nanoparticles (25 mg/kg) (4185.3 ± 0.62 ng/mL). The plasma concentration of NGN for the group receiving NGN-PLGA nanoparticles (3674.6 ± 0.78 ng/mL) was comparable to that of the unencapsulated drug (100 mg/kg) (3811.2 ± 0.45 ng/mL). Similarly, the concentration of NGN in the brain was minimum for the group receiving NGN (25 mg/kg) (314.62 ± 0.18 ng/mL) and highest was for the group receiving GSH-coated nanoparticles (1296.28 ± 0.45 ng/mL). The concentration of NGN in brain for group receiving NGN-PLGA nanoparticles (25 mg/kg) (989.42 ± 0.88 ng/mL) was comparable to that of free NGN (100 mg/kg) (926.2 ± 0.96 ng/mL). Various pharmacological responses were observed such as time of fall (sensorimotor dysfunction), ambulations, rearing (locomotor activity), number of line crossings and entries into a center circle (anxiety-like behavior), time spent in reciprocal social interaction, and repetitive self-grooming. PPA administration resulted in a decrease in time of fall (21.6 ± 0.8 s) that was increased to 204.6 ± 0.96 s after administration of GSH-coated NGN-loaded nanoparticles. Anxiety results in hyperlocomotion, as seen from a large number of rearings (100.2 ± 2.5), ambulations (168.4 ± 0.9), number of line crossings (158.2 ± 1.8), and number of entries in center circle (58.6 ± 1.6). PPA administration (i. e., induction of ASD-like phenotype) also results in a decrease in time spent in social interaction (89.3 ± 0.45 s) and increase in time spent in repetitive self-grooming (356.2 ± 0.75 s). Maximum reduction was observed after administration of GSH-coated NGN-loaded nanoparticles and the minimum reduction was after administration of NGN (25 mg/kg). GSH NGN-NPs showed significant reduction in neuroinflammatory markers like TNF-α (182.1 ± 1.2 pg/mL to 6.2 ± 2.0 pg/mL), MMP-9 (13.4 ± 0.8 ng/mL to

1.2 ± 0.94 ng/mL), and HSP-70 (492.8 ± 1.7 ng/mL to 17.2 ± 2.8 ng/mL) in plasma. TNF-α levels in the brain also showed reduction after administration of GSH NGN-PLGA nanoparticles (1122.2 ± 1.5 pg/mL to 28.4 ± 1.5 pg/mL). Efflux transporter (P-gp) at BBB was upregulated in PPA administered rats as observed from P-gp concentration (58.3 ± 0.8 ng/mL versus control reading of 43.98 ± 1.75 ng/mL) in the brain homogenate. Maximum reduction in P-gp concentration was reported in GSH-coated nanoparticles (2.92 ± 0.8 ng/mL) treated group.

To find out the relationship between PK and PD parameters, the results were analyzed using the sigmoid E_{max} model with the help of PK Solver 2.0 (Microsoft Excel Add-Ins program). The graphs showing predicted and observed responses at various concentrations for plasma (► Fig. 1) as well as the brain (► Fig. 2). Various PD parameters like E_0 (baseline effect when no concentration is present), E_{max} (maximum effect), EC_{50} (concentration at which 50% effect occurs), γ (sigmoidicity factor), R^2 (regression coefficient), and Akaike information criterion (AIC) (measure of goodness of fit for the model) for various behavioral tests, neuroinflammatory markers, and efflux transporter described above have been given in ► Table 1.

Discussion

The main aim for the development of PK-PD model of NGN and its nanocarriers was to understand the relationship between plasma and brain concentration of NGN and behavioral alterations such as social behavior, repetitive behavior, sensorimotor dysfunction, hyperlocomotion, and anxiety as well as neuroinflammatory markers such as TNF-α, MMP-9, and HSP-70 and P-gp. Sigmoid E_{max} model was used to establish a PK-PD correlation. PPA administration resulted in a decrease in time of fall (21.6 s), increase in number of rearings and ambulations (100.2 and 168.4), increase in number of line crossings and entries into center circle (158.2 and 58.6) indicating anxiety, increase in time spent in self-grooming (356.2 s), and decrease in social interaction time (89.3 s) as well as increase in levels of neuroinflammatory markers such as TNF-α, MMP-9, and HSP-70 and upregulation of efflux transporter, P-gp. E_0 values indicated the parameters after administration of PPA and before the beginning of treatment with NGN and its nanocarriers. These results are consistent with the scientific literature indicating about the effect of short-chain fatty acids such as PPA in generating neuroinflammatory cascade and causing alterations in behavior, biochemical, and molecular alterations in autistic patients [36–38]. Moreover, clinical findings also suggest an increase in the levels of neuroinflammatory markers such as TNF-α, MMP-9, and HSP-70 and oxidative stress in autistic patients [39, 40]. The results of sigmoid E_{max} model showed that after administration of unencapsulated NGN (25, 50, and 100 mg/kg) as well as its coated and uncoated nanocarriers (25 mg/kg), maximum effect occurred after administration of GSH-coated NGN-loaded nanoparticles as indicated by E_{max} values and minimum after administration of NGN (25 mg/kg) as maximum plasma and brain concentrations of NGN were achieved after administration of GSH-coated nanoparticles and minimum with NGN (25 mg/kg). Hence, after administration of different doses of NGN and its nanocarriers, there was an improvement in behavior and reduction in the levels of neuroinflam-



► **Fig. 1** Sigmoid E_{\max} model correlating various pharmacological responses and neuroinflammatory markers with plasma concentration of NGN after administration of NGN (25, 50, and 100 mg/kg), NGN-PLGA-NPs (25 mg/kg), and GSH NGN-PLGA-NPs and Tween 80-NGN-PLGA-NPs. 1 Time of fall (s). 2 Number of rearings. 3 Number of ambulations. 4 Number of line crossings. 5 Number of entries in center circle. 6 Time spent in repetitive self-grooming. 7 Time spent in social interaction. 8 Levels of TNF- α . 9 Levels of MMP-9. 10 Levels of circulating antibodies to HSP-70.

matory markers such as TNF- α , MMP-9, and HSP-70 and P-gp by virtue of its neuroprotective effect as a result of its antioxidant action [41]. However, the effect was maximum as a result of brain-targeted action of GSH-coated nanocarriers. The sigmoid E_{\max} model showed good fit as indicated from values of R^2 , which was 0.99 and showed the closeness of observed and predicted value (observed value of E_{\max} for number of line crossings was 88.6 s but the predicted value was 95.74). A similar pattern was observed for other effects. A small AIC value indicates the appropriateness of the model and goodness of fit of the data. Sigmoidity factor (γ) is the curve-fitting parameter in the sigmoid E_{\max} model indicating the steepness of the concentration-effect relationship. Its values ranged from 1.31–21.4 (brain) and 1.68–14.95 (plasma). The maximum value of γ was for TNF- α levels in the brain indicating a very steep sigmoid curve. The P-gp concentration in the Sigmoid E_{\max} model also indicated enhanced inhibition of these efflux transporters due to the presence of the coating. Hence, encapsulation of NGN in the nanoparticles as well as coating with GSH and tween 80 not only resulted in improved bioavailability but also resulted in brain targeting as a result of inhibition P-gp. The increase in plasma as well as brain concentration of NGN correlated with improve-

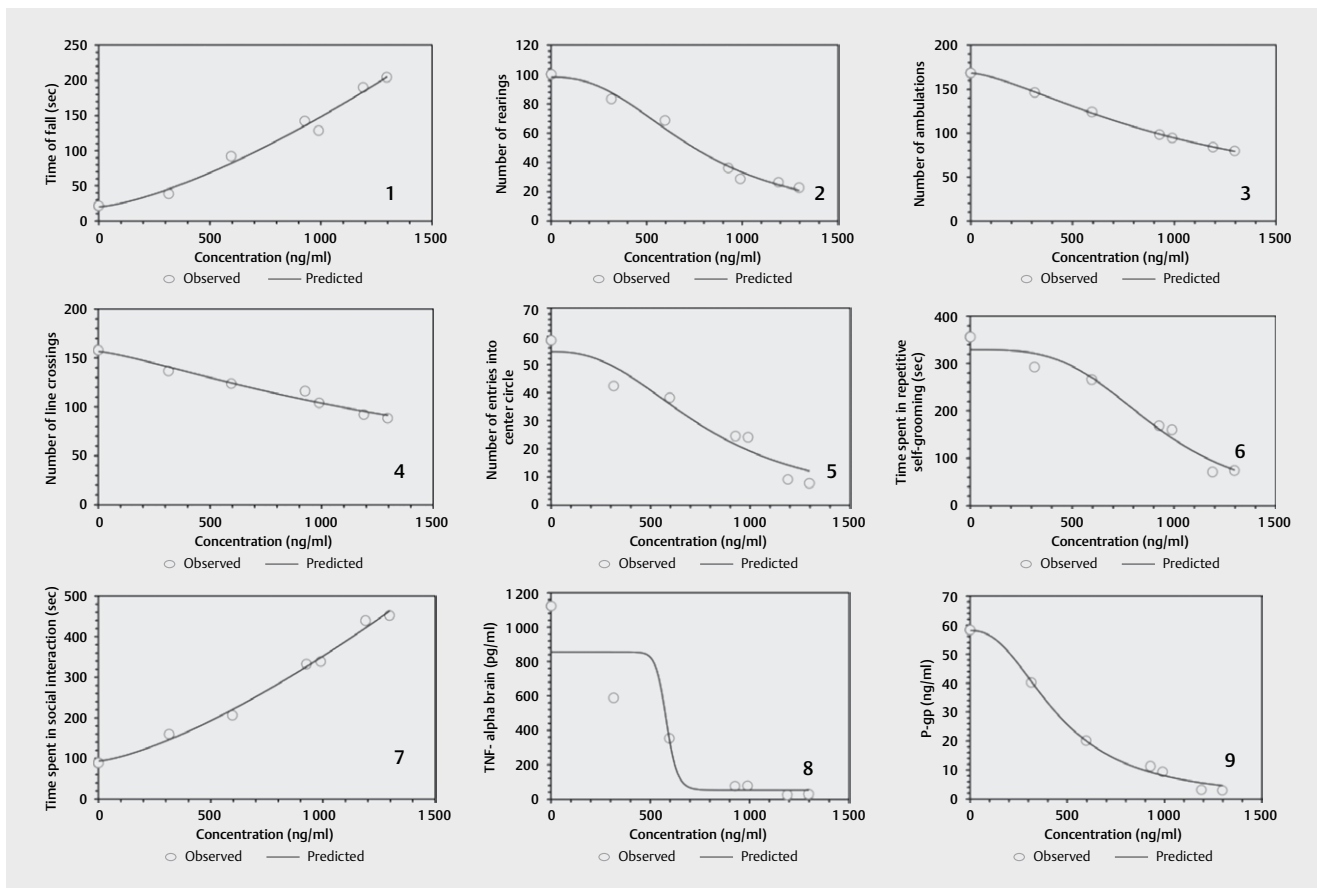
ment in social interaction ability, repetitive behavior, sensorimotor dysfunction, and anxiety-like behavior after administration of coated nanoparticles (25 mg/kg) as compared to uncoated and even with the highest dose of NGN (i. e., 100 mg/kg). Our results are in lines with those documented in the literature that indicated improvement in bioavailability after encapsulation of drug in nanoparticles and inhibition of P-gp efflux and enhanced brain uptake after coating with GSH and tween 80 [42–45].

Hence, the PK-PD modeling done by sigmoid E_{\max} model showed a positive correlation between plasma/brain drug concentration and neuroinflammatory markers as well as behaviors consistent with the ASD phenotype.

Material and Methods

Animals and drugs

Male Sprague-Dawley rats (250–280 g), 3–4 m old and bred in Central Animal House Facility of Panjab University, Chandigarh (India), were used. The rats were housed individually in cages and given free access to standard laboratory food (Ashirwad Industries) and



► **Fig. 2** Sigmoid E_{max} model correlating various pharmacological responses and P-gp concentration with a concentration of NGN in the brain after administration of NGN (25, 50, 100 mg/kg), NGN-PLGA-NPs (25 mg/kg), and GSH NGN-PLGA-NPs and tween 80-NGN-PLGA-NPs. 1 Time of fall (s). 2 Number of rearings. 3 Number of ambulations. 4 Number of line crossings. 5 Number of entries in center circle. 6 Time spent in repetitive self-grooming. 7 Time spent in social interaction. 8 Levels of TNF- α (brain). 9 Concentration of P-gp.

water. The experimental protocol was approved by Institutional Animal Ethics Committee of Panjab University, Chandigarh (PU/45/99/CPCSEA/IAEC/2018/115) and was conducted according to Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) guidelines for the use and care of experimental animals.

PPA, NGN (MW = 272.25, purity \geq 98% HPLC), Resomer RG 502 H (Poly(DL-lactide-co-glycolide) (PLGA)), 50:50, MW = 7 000–17 000), Kolliphor P 188 (poloxamer 188), polysorbate 80 (tween 80), and reduced GSH were purchased from Sigma Chemical Co. TNF- α ELISA and MMP-9 assay kits were purchased from R&D Systems. Assay kits for HSP-70 and P-gp were purchased from Qayee Biotechnology-life sciences and Wuhan Sciences, respectively. All other chemicals used for biochemical estimations were of analytical grade.

Preparation of NGN-PLGA uncoated as well as coated nanoparticles

The NGN-PLGA nanoparticles were prepared by nanoprecipitation method reported by Fessi et al. [46] with minor modifications. NGN-PLGA nanoparticles were further coated with reduced GSH and polysorbate 80 in order to enhance brain delivery. One percent poly-

sorbate 80 coating over NGN nanoparticles was done as reported by Wilson et al. (2008) with minor modifications. While GSH-coated nanoparticles were prepared with minor modifications in the method reported by Geldenhuys et al. [45]. The nanoparticles were characterized for size, morphology, entrapment efficiency, and in vitro drug release. The average particle size of uncoated NGN nanoparticles was found to be 143.93 ± 2.68 nm while GSH and tween 80-coated nanoparticles were found to be 223.86 ± 6.09 nm and 152.4 ± 2.1 nm, respectively (to-be published data).

Study design

ASD was induced in rats by administering an intracerebroventricular injection of 1M PPA according to the procedure of MacFabe et al. with some modifications and as in our published studies [19–21].

For the current study, rats were randomly selected and divided into 7 groups of 5 animals each. The first group was ASD induced group that received 1M PPA. The second, third, and fourth groups consisted of ASD-induced animals (administered with 1M PPA) treated with 25, 50, and 100 mg/kg (peroral) NGN administered thrice daily (8 hourly). The fifth, sixth, and seventh groups were administered with NGN-loaded PLGA nanoparticles (NGN-PLGA-NP), reduced GSH-coated NGN-loaded PLGA nanoparticles (GSH NGN-

► **Table 1** Various PD parameters derived from sigmoid E_{max} model (different concentrations achieved using different doses of naringenin (25, 50, and 100 mg/kg) and its nanocarriers (uncoated and coated).

S.NO	Time of fall (s)	Rearing (no.)	Ambulations (no.)	No. of line crossings	No. of entries into the circle	Time spent in social interaction (s)	Time spent in self-grooming (s)	TNF- α (pg/ml)	HSP-70 (ng/mL)	MMP-9 (ng/mL)	P-gp (ng/mL)
Plasma											
E_{max}	204.6	22.8	79.8	88.6	7.8	452.4	74.3	6.2	17.2	1.2	
E_0	21.6	100.2	168.4	158.2	58.6	89.3	356.2	182.1	492.8	13.4	
EC_{50} (ng/ml)	3170.7	2578.8	2451.9	2515.4	3508.9	3149.5	3128.4	1395.1	1648.7	1733.3	
γ	2.3	3.5	1.9	1.7	14.9	2.6	5.7	2.5	2.5	2.6	
R^2	0.98	0.996	0.999	0.997	0.967	0.994	0.984	0.986	0.986	0.99	
AIC	59.8	38.5	36.1	47.4	46.9	65.4	68.0	52.5	67.2	-3.4	
Brain											
E_{max}	204.6	22.8	79.8	88.6	7.8	452.4	74.3	28.4			2.9
E_0	21.6	100.2	168.4	158.2	58.6	89.3	356.2	1122.2			58.3
EC_{50}	798.7	615.4	576.1	615.4	641.6	759.43	765.9	552.6			432.6
γ	1.4	2.4	1.5	1.3	2.5	1.4	3.5	21.4			2.3
R^2	0.995	0.996	0.999	0.998	0.982	0.998	0.992	0.92			0.997
AIC	52.4	39.4	16	41.6	42.8	56.1	62.6	91.2			26.9

PLGA-NP), and tween 80-coated NGN-loaded PLGA nanoparticles (tween 80-NGN-PLGA-NP) after induction with ASD at the dose of 25 mg/kg, administered thrice daily (8 hourly). Starting from the second day of experiment till 21st day, NGN, as well as its lyophilized nanoparticles, were administered after suspension in 0.5 % w/v sodium carboxymethylcellulose, thrice daily.

On the 22nd day, blood samples were taken, 8 h after the last dose on the 21st day, and plasma was separated for estimating the concentration of NGN as well as levels of TNF- α , MMP-9, and HSP-70. The behavioral tests such as rotarod (sensorimotor dysfunction), actophotometer (locomotion), open-field (anxiety), reciprocal social interaction, and repetitive self-grooming were performed, and the animals were sacrificed under deep anesthesia. The brains were excised and analyzed for concentration of NGN as well as TNF- α and P-gp concentrations.

Behavioral tests and neuroinflammatory biomarkers

ASD is associated with core symptoms such as the inability to socially interact and repetitive behavior, whereas sensorimotor dysfunction changes in locomotor activity and anxiety are some of the associated behaviors occurring in ASD. Hence, in order to establish a PK-PD model for understanding the effect of treatment with various doses of NGN and its coated and uncoated nanocarriers, the following behavioral tests were conducted and biomarkers were evaluated. Details of the behavioral tests are given in the Supporting Information (section S1).

Behavioral tests

Briefly, associated behaviors like sensorimotor dysfunction, locomotor activity, and anxiety were assessed by rotarod test [47], actophotometer [48], and open-field test [49], respectively. Neurobehavioral tests for core autistic behaviors such as social interaction ability and repetitive behavior were assessed by reciprocal social interaction test [50] and repetitive self-grooming [51], respectively.

These tests were conducted 8 h after the administration of the last dose.

Neuroinflammatory biomarkers and concentration of P-gp at BBB as pharmacodynamic parameters

ELISA assay was conducted to analyze neuroinflammatory biomarkers such as TNF- α , MMP-9, and HSP-70 using the rat plasma; however, levels of TNF- α were also assessed in brain homogenate. These were assessed using a rat TNF- α kit (R&D Systems) and quantikine MMP-9 (R&D Systems). HSP-70 was estimated to assay the level of HSP-70 in plasma (Qayee Biotechnology) and concentration of P-gp in the brain samples were determined by rat P-gp kit (Wuhan Sciences). P-gp concentration was assessed in brain homogenate as neuroinflammation leads to upregulation of P-gp.

Blood sampling and brain tissue collection

Thirty-five Sprague-Dawley rats were randomly divided into 7 groups with 5 animals in each group. Five-hundred-microliter blood samples were collected 8 h after the last dose on the 21st day by retro-orbital venous plexus puncture under mild ether anesthesia. The samples were collected in heparinized tubes and plasma was separated by centrifugation at 10,000 rpm for 10 min. Brain samples were collected by cervical dislocation after the behavioral

tests. Both plasma and brain samples were stored at -80°C for further analysis.

Estimation of NGN in plasma and brain using RP-HPLC method

A simple, sensitive, robust, and effective reversed phase (RP)-HPLC method was developed with some modifications and validated in our laboratory for detection and quantification of NGN in plasma and brain [52].

PK-PD modeling

The onset, intensity, and duration of pharmacological effect of the drug depend on the dose and pharmacokinetics of the drug, which determines drug concentration in plasma as well as a receptor site. We applied sigmoid E_{max} model [35] to study the relationship between the concentration of NGN in plasma and pharmacological effects like sensorimotor dysfunction, locomotor activity, anxiety-like behavior, reciprocal social interaction, repetitive self-grooming, and TNF- α , MMP-9, and HSP-70 levels. The same model was applied to study the relationship between the concentration of NGN in the brain and above-mentioned pharmacological effects except for MMP-9 and HSP-70. Also, the concentration of P-gp at the blood-brain barrier was considered.

Sigmoid E_{max} model

This model, a generalization of the E_{max} model, is based on the observation that an increase in drug concentration near maximum pharmacologic response produces a disproportionately smaller increase in pharmacologic response. This model has been derived from the theory of drug-receptor interaction. The sigmoid E_{max} model, like the E_{max} model, describes drug action in terms of maximum effect (E_{max}) and EC_{50} (the drug concentration that produces 50% of E_{max}). The equation for the sigmoid E_{max} model is

$$E = \frac{E_{\text{max}} \times C^{\gamma}}{EC_{50} + C^{\gamma}} + E_0$$

where E_0 represents the value of E when no drug is present. The exponent γ is the sigmoid factor or steepness of the curve; $\gamma = 1$ for the hyperbolic curve, $\gamma > 1$ for steeper curve, and $\gamma < 1$ for a smoother curve. A very large γ value may indicate allosteric or cooperative effect in the interaction of the drug molecules with the receptor.

In order to study PK-PD correlation, 3 doses (25, 50, and 100 mg/kg) of unencapsulated NGN and 3 doses consisting of different formulations, namely uncoated NGN nanoparticles (25 mg/kg) as well as GSH and tween 80-coated NGN nanoparticles, (25 mg/kg) were administered, which resulted in 6 different concentrations of NGN in plasma and brain. Our pharmacokinetic study (unpublished data) indicated that the peak plasma concentration of NGN after the administration of free NGN (25 mg/kg) was 4227.08 ± 0.52 ng/mL. The peak plasma concentrations of NGN were significantly enhanced after administration of NGN-PLGA nanoparticles (5325.057 ± 0.57 ng/mL) and were further increased as a result of their surface modification with GSH (6323.09 ± 0.415 ng/mL) and tween 80 (6189.05 ± 0.305 ng/mL). Brain concentration was also

1.63 times and 1.39 times enhanced after administration of GSH and tween 80-coated nanocarriers as compared to uncoated nanocarriers. Also, from our previous pharmacokinetic studies (unpublished data), it was observed that half-life for coated as well as uncoated NGN-loaded PLGA nanoparticles is approximately 4 h, whereas for free NGN, the half-life is approximately 2.6 h. Therefore, in case of nanoparticles, the minimum residual dose on the 22nd day (8 h after the last dose on 21st day) would be a higher proportion of the C_{max} of the corresponding dose as compared to unencapsulated NGN. Thus, plasma and brain concentrations of NGN vary with different doses of NGN (unencapsulated) and coated as well as uncoated nanocarriers resulting in 6 different concentrations. The pharmacodynamic parameters were measured for all the above 6 dosage forms.

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Conflicts of Interest

The authors declare no conflicts of interest.

References

- [1] American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders. American Psychiatric Association; 2013 Accessed at <http://www.psychiatryonline.org/doi/book/10.1176/appi.books.9780890425596>
- [2] Estabillo JA, Matson JL, Cervantes PE. Autism symptoms and problem behaviors in children with and without developmental regression. *J Dev Phys Disabil* 2018; 30: 17-26
- [3] World Health Organization. Autism spectrum disorders. 2017; Accessed at, <http://www.who.int/mediacentre/factsheets/fs2017/05/autism-spectrum-disorders/en/>
- [4] Holt R, Monaco AP. Links between genetics and pathophysiology in the autism spectrum disorders. *EMBO Mol Med* 2011; 3: 438-450
- [5] Santangelo SL, Tsatsanis K. What is known about autism: genes, brain, and behavior. *Am J Pharmacogenomics* 2005; 5: 71-92
- [6] Newschaffer CJ, Croen LA, Daniels J, Giarelli E, Grether JK, Levy SE, Mandell DS, Miller LA, Pinto-Martin J, Reaven J, Reynolds AM, Rice CE, Schendel D, Windham GC. The epidemiology of autism spectrum disorders. *Annu Rev Public Health* 2007; 28: 235-258
- [7] EJM Jones, Gliga T, Bedford R, Charman T, Johnson MH. Developmental pathways to autism: a review of prospective studies of infants at risk. *Neurosci Biobehav Rev* 2014; 39: 1-33
- [8] LaSalle JM. Epigenomic strategies at the interface of genetic and environmental risk factors for autism. *J Hum Genet* 2013; 58: 396-401

- [9] Modabbernia A, Velthorst E, Reichenberg A. Environmental risk factors for autism: an evidence-based review of systematic reviews and meta-analyses. *Mol Autism* 2017; 8: 13
- [10] Ciernia AV, Laufer BI, Dunaway KW, Mordaunt CE, Coulson RL, Yasui DH, LaSalle JM. Epigenomic convergence of genetic and immune risk factors in autism brain. *bioRxiv* 270827; doi: <https://doi.org/10.1101/270827>; Accessed at: <https://www.biorxiv.org/content/10.1101/270827v2>
- [11] Rossignol DA, Frye RE. A review of research trends in physiological abnormalities in autism spectrum disorders: Immune dysregulation, inflammation, oxidative stress, mitochondrial dysfunction and environmental toxicant exposures. *Mol Psychiatry* 2012; 17: 389–401
- [12] Nadeem A, Ahmad SF, Attia SM, Bakheet SA, Al-Harbi NO, AL-Ayadhi LY. Activation of IL-17 receptor leads to increased oxidative inflammation in peripheral monocytes of autistic children. *Brain Behav Immun* 2018; 67: 335–344
- [13] Vargas DL, Nascimbene C, Krishnan C, Zimmerman AW, Pardo CA. Neuroglial activation and neuroinflammation in the brain of patients with autism. *Ann Neurol* 2005; 57: 67–81
- [14] Bjorkland G, Saad K, Chirumbolo S, Kern JK, Geier DA, Geier MR, Urbina MA. Immune dysfunction and neuroinflammation in autism spectrum disorder. *Acta Neurobiol Exp* 2016; 76: 257–268
- [15] Jyonouchi H, Sun S, Itokazu N. Innate immunity associated with inflammatory responses and cytokine production against common dietary proteins in patients with autism spectrum disorder. *Neuropsychobiology* 2002; 46: 76–84
- [16] Horvath K, Papadimitriou JC, Rabsztyan A, Drachenberg C, Tildon JT. Gastrointestinal abnormalities in children with autistic disorder. *J Pediatr* 1999; 135: 559–563
- [17] MacFabe DF, Cain DP, Rodriguez-Capote K, Franklin AE, Hoffman JE, Boon F, Taylor AR, Kavaliers M, Ossenkopp KP. Neurobiological effects of intraventricular propionic acid in rats: Possible role of short chain fatty acids on the pathogenesis and characteristics of autism spectrum disorders. *Behav Brain Res* 2007; 176: 149–169
- [18] Choi J, Lee S, Won J, Jin Y, Hong Y, Hur TY, Kim JH, Lee SR, Hong Y. Pathophysiological and neurobehavioral characteristics of a propionic acid-mediated autism-like rat model. *PLoS One* 2018; 13: e0192925
- [19] Bhandari R, Kuhad A. Neuropsychopharmacotherapeutic efficacy of curcumin in experimental paradigm of autism spectrum disorders. *Life Sci* 2015; 141: 156–169
- [20] Bhandari R, Kuhad A. Resveratrol suppresses neuroinflammation in the experimental paradigm of autism spectrum disorders. *Neurochem Int* 2017; 103: 8–23
- [21] Bhandari R, Paliwal JK, Kuhad A. Naringenin and its nanocarriers as potential phytotherapy for autism spectrum disorders. *J Funct Foods* 2018; 47: 361–375
- [22] Felgines C, Texier O, Morand C, Manach C, Scalbert A, Régerat F, Rémésy C. Bioavailability of the flavanone naringenin and its glycosides in rats. *Am J Physiol Gastrointest Liver Physiol* 2000; 279: G1148–G1154
- [23] Kumar S, Tiku AB. Biochemical and molecular mechanisms of radioprotective effects of naringenin, a phytochemical from citrus fruits. *J Agric Food Chem* 2016; 64: 1676–1685
- [24] Nahmias Y, Goldwasser J, Casali M, Van Poll D, Wakita T, Chung RT, Yarmush ML. Apolipoprotein B-dependent hepatitis C virus secretion is inhibited by the grapefruit flavonoid naringenin. *Hepatology* 2008; 47: 1437–1445
- [25] Yi LT, Liu B Bin, Li J, Luo L, Liu Q, Geng D, Tang Y, Xia Y, Wu D. BDNF signaling is necessary for the antidepressant-like effect of naringenin. *Prog Neuro-Psychopharmacology. Biol Psychiatry* 2014; 48: 135–141
- [26] Wu LH, Lin C, Lin HY, Liu YS, Wu CY, Tsai CF, Chang PC, Yeh WL, Lu DY. Naringenin suppresses neuroinflammatory responses through inducing suppressor of cytokine signaling 3 expression. *Mol Neurobiol* 2016; 53: 1080–1091
- [27] Birt DF, Hendrich S, Wang W. Dietary agents in cancer prevention: flavonoids and isoflavonoids. *Pharmacol Ther* 2001; 90: 157–177
- [28] Ratnam DV, Ankola DD, Bhardwaj V, Sahana DK, Kumar MNVR. Role of antioxidants in prophylaxis and therapy: A pharmaceutical perspective. *J Control Release* 2006; 113: 189–207
- [29] Yen FL, Wu TH, Lin LT, Cham TM, Lin CC. Naringenin-loaded nanoparticles improve the physicochemical properties and the hepatoprotective effects of naringenin in orally-administered rats with CCl₄-induced acute liver failure. *Pharm Res* 2009; 26: 893–902
- [30] Chien JY, Friedrich S, Heathman MA, de Alwis DP, Sinha V. Pharmacokinetics/pharmacodynamics and the stages of drug development: role of modeling and simulation. *AAPS J* 2005; 7: E544–E559
- [31] Danhof M, de Lange ECM, Della Pasqua OE, Ploeger BA, Voskuyl RA. Mechanism-based pharmacokinetic-pharmacodynamic (PK-PD) modeling in translational drug research. *Trends Pharmacol Sci* 2008; 29: 186–191
- [32] Meibohm B, Derendorf H. Basic concepts of pharmacokinetic/ pharmacodynamic (PK/PD) modelling. *Int J Clin Pharmacol Ther* 1997; 35: 401–413
- [33] Wright DFB, Winter HR, Duffull SB. Understanding the time course of pharmacological effect: a PKPD approach. *Br J Clin Pharmacol* 2011; 71: 815–823
- [34] Khan DD, Friberg LE, Nielsen EI. A pharmacokinetic-pharmacodynamic (PKPD) model based on in vitro time-kill data predicts the in vivo PK/PD index of colistin. *J Antimicrob Chemother* 2016; 71: 1881–1884
- [35] Shargel L, Wu-Pong S, Yu ABC. *Applied Biopharmaceutics & Pharmacokinetics*. 5th ed New York: McGraw-Hill Medical; 2005: 527–563
- [36] MacFabe DF, Rodríguez-Capote K, Hoffman JE, Franklin AE, Mohammad-Asef Y, Taylor AR, Boon F, Cain DP, Kavaliers M, Possmayer F, Ossenkopp KP. A novel rodent model of autism: intraventricular infusions of propionic acid increase locomotor activity and induce neuroinflammation and oxidative stress in discrete regions of adult rat brain. *Am J Biochem Biotechnol* 2008; 4: 146–166
- [37] El-Ansary AK, Bacha A Ben, Kotb M. Etiology of autistic features: the persisting neurotoxic effects of propionic acid. *J Neuroinflammation* 2012; 9: 661
- [38] Shultz SR, Macfabe DF, Martin S, Jackson J, Taylor R, Boon F, Ossenkopp KP, Cain DP. Intracerebroventricular injections of the enteric bacterial metabolic product propionic acid impair cognition and sensorimotor ability in the Long-Evans rat: Further development of a rodent model of autism. *Behav Brain Res* 2009; 200: 33–41
- [39] Abdallah MW, Michel TM. Matrix metalloproteinases in autism spectrum disorders. *J Mol Psychiatry* 2013; 1: 16
- [40] González-Fraguela ME, Hung MD, Vera H, Maragoto C, Noris E, Blanco L, Galvizo R, Robinson M. Oxidative stress markers in children with autism spectrum disorders. *British J Med Res* 2013; 3: 307–317
- [41] Wu LH, Lin C, Lin HY, Liu YS, Wu CY, Tsai CF, Chang PC, Yeh WL, Lu DY. Naringenin suppresses neuroinflammatory responses through inducing suppressor of cytokine signaling 3 expression. *Mol Neurobiol* 2016; 53: 1080–1091
- [42] Wilson B, Samanta MK, Santhi K, Kumar KPS, Paramakrishnan N, Suresh B. Targeted delivery of tacrine into the brain with polysorbate 80-coated poly(n-butylcyanoacrylate) nanoparticles. *Eur J Pharm Biopharm* 2008; 70: 75–84
- [43] Jain A, Jain A, Garg NK, Tyagi RK, Singh B, Katare OP, Webster TJ, Soni V. Surface engineered polymeric nanocarriers mediate the delivery of transferrin-methotrexate conjugates for an improved understanding of brain cancer. *Acta Biomater* 2015; 24: 140–151
- [44] Grover A, Hirani A, Pathak Y, Sutariya V. Brain-targeted delivery of docetaxel by glutathione-coated nanoparticles for brain cancer. *AAPS PharmSciTech* 2014; 15: 1562–1568

- [45] Geldenhuys W, Mbimba T, Bui T, Harrison K, Sutariya V. Brain-targeted delivery of paclitaxel using glutathione-coated nanoparticles for brain cancers. *J Drug Target* 2011; 19: 837–845
- [46] Fessi H, Puisieux F, Devissaguet JP, Ammoury N, Benita S. Nanocapsule Formation by interfacial polymer deposition following solvent displacement. *Int J Pharm* 1989; 55: R1–R4
- [47] Dunham NW, Myua TS. A note on a simple apparatus for detecting neurological deficit in rats and mice. *J Am Pharm Assoc Am Pharm Assoc (Baltim)* 1957; 46: 208–209
- [48] Sachdeva AK, Kuhad A, Chopra K. Epigallocatechin gallate ameliorates behavioral and biochemical deficits in rat model of load-induced chronic fatigue syndrome. *Brain Res Bull* 2011; 86: 165–172
- [49] Raghavendra V, Chopra K, Kulkarni SK. Brain renin angiotensin system (RAS) in stress-induced analgesia and impaired retention. *Peptides* 1999; 20: 335–342
- [50] Silverman JL, Yang M, Lord C, Crawley JN. Behavioural phenotyping assays for mouse models of autism. *Nat Rev Neurosci* 2010; 11: 490–502
- [51] Moretti P, Bouwknecht JA, Teague R, Paylor R, Zoghbi HY. Abnormalities of social interactions and home-cage behavior in a mouse model of Rett syndrome. *Hum Mol Genet* 2005; 14: 205–220
- [52] Bhandari R, Kuhad A, Paliwal JK, Kuhad A. Development of a new, sensitive, and robust analytical and bio-analytical RP- HPLC method for in vitro and in vivo quantification of naringenin in polymeric nanocarriers. *J Anal Sci Technol* 2019; 10: 11