Developing Integrated PBPK/PD Coupled mechanistic pathway model (miRNA-BDNF): an approach towards System toxicology

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Abstract:

Integration of a dynamic signal transduction pathway into the tissue dosimetry model is a major advancement in the area of computational toxicology. This paper illustrates the ways to incorporate the use of existing system biological model in the field of toxicology via its coupling to the Physiological based Pharmacokinetics and Pharmacodynamics (PBPK/PD) model.This expansion framework of integrated PBPK/PD coupled mechanistic system pathway model can be called as system toxicology that describes the kinetics of both -the chemicals and –biomolecules, help us to understand the dynamic and steady-state behaviors of molecular pathways under perturbed condition. The objective of this article is to illustrate a system toxicology based approach by developing an integrated PBPK/PD coupled miRNA-BDNF pathway model and to demonstrate its application by taking a case study of PFOS mediated neurotoxicity. System dynamic involves miRNA-mediated BDNF regulation, which plays an important role in the control of neuronal cell proliferation, differentiation, and survivability.

Key words: PBPK/PD, miRNA, BDNF, Neuroendocrine, System biology, PFOS

1. Introduction

In the field of quantitative risk assessment, a journey of classical dose-response models is categorized into different classes for the better quantification and estimation of early possible risk (Andersen et al., 2005). These include –a) Physiological based pharmacokinetic and pharmacodynamic modeling (PBPK) for the quantification of internal biophase concentrations in different tissues, b) pharmacodynamics (PD) model quantifies the interactions of chemicals with target biomolecules c) System Biology describes the dynamic relationship of biological components for a robust physiological response. Perturbation of these biological components can be quantified through the integration of PBPK/PD model into the system biological models providing a predictive tool for measuring toxicological impact at the cellular and biomolecular level (Andersen et al., 2005; Gohlke et al., 2005; Zhao and Ricci, 2010).

The PBPK model in the area of dosimetry risk assessment has been widely accepted and applied and it is among the top priority tool recommended in the vision of toxicity testing in the 21st century (Andersen and Krewski, 2009). PBPK model has been extended to develop the PBPK/PD for certain pesticides (Timchalk et al., 2002; Foxenberg et al., 2011). The integration of PD was generally done with the quantification of the response variable (biomarker) effect of an interaction of a chemical (biophase concentration estimated by PBPK) with a target biomolecule (mainly receptors). But it has a certain limitation such as lack of robust biology (biomarker relation to endpoint), and very often the endpoints are specifically remained single explanatory biomarker. Coupling of PBPK/PD model and system biology together can enlighten the effect of changes in key biomolecules considering the whole biological system. System biology comprising of genomics, metabolomics, and proteomics which rationalizes the functional interaction of biological components in a time-dependent fashion (Aderem, 2005; Kitano, 2002). Thus, it could be useful in system toxicology for understanding the altered biological pathway due to chemical induced perturbation of certain key biomolecule in a system, illustrating differences from normal pathway (Arrell and Terzic, 2010; Auffray et al., 2009; Hood et al., 2004; Kell, 2006). Understanding the biomolecular mechanisms are of great interest to identify the toxicological effects at the very early stages of the disease (toxicological response). However, often we lack sufficient information to link chemically perturbed biological components (molecular biomarker) to an altered biological system. This lead to the use of the simplified dose-response model (simple PD) to predict the adverse outcome (disease) for a target chemical(Calabrese and Baldwin, 2003). In the field of toxicology, there is limited use of these system biology models (Waters et al., 2003). The wide use of system toxicology in human environmental risk assessment has a time lag in comparison with pharmaceuticals science as it lacks experimental data, has complex interaction pathways of environmental chemicals than the target specific drugs, and low commercial priority of applied toxicological science.

Recently use of the integrated PBPK/PD models in a field of environmental toxicology, enables development of a quantitative biologically based risk model which increases our understanding towards the relationship between tissue bio-phase concentration of chemicals and endogenous biomolecule (Timchalk et al., 2002; Foxenberg et al., 2011). Furthermore, signaling pathways could be used as an extension of PBPK/PD, given dynamic interactions of chemicals with biological components are known, the first step towards system toxicology (Bhattacharya et al., 2012; Gim et al., 2010). It has benefits such as: easy to implement if the signaling pathway already developed, often data from

the dose-response experiments for known biomolecules can be used, a good step to use Adverse Outcome Pathways (AOPs) knowledge to develop the generic PBPK/PD model for multi-species and multi-chemicals.

Neuroendocrine or neurotrophins such as nerve growth factors, BDNF and neurotrophin-3 are proteins, basically processed and secreted in constitutive and regulatory fashion in non-neuron, neurons and neuroendocrine cells (Lu, 2003; Mowla et al., 1999). Among them, BDNF is immensely expressed and extensively scattered than other neurotrophins, and play an important role in neuronal survival and differentiation (Boulle et al., 2012; Michael et al., 1997; Murer et al., 2001). BDNF binds with a Tropomyosin receptor kinase B (TrkB) presents on the neuronal cell surface causing sequential activation of following pathways such as Mitogen-activated protein kinases (MAPKs), Extracellular-signal-regulated kinase (ERK), and Protein kinase B (AKT) that are mainly involved in differentiation and survivability of neurons (Michael et al., 1997; Murer et al., 2001 Bursac et al., 2010; Boulle et al., 2012). It has been seen that reduced BDNF protein and mRNA expression is linked with several neurological disorders such as Alzheimer's and Parkinson's (Bursac et al., 2010). Moreover, dopaminergic, GABAergic, cholinergic, and serotonergic neurons are known to require BDNF for their proper development and survival (Lipsky and Marini, 2007; Murer et al., 2001), signifies BDNF as an important biomarker for neurodevelopmental function.

It has been reported that miRNA regulates the synthesis of BDNF via posttranscriptional modification of BDNFmRNA (Caputo et al., 2011; You et al., 2016). Muiños-Gimeno et al., (2011) reported the involvement of miRNA-22 associated panic disorders in the Spanish and North European population. Later, the transcriptomic analysis studied by Li et al., (2015) in SH-SY5Y cell line also found the involvement of miRNA-22 dependent decrease in the BDNF level and neuronal cell survivability. The miRNAs are turning out to be significant regulators of mRNAs and the related proteins. In this proposed study, miRNA (micro-RNA) regulated BDNF (Brain- derived neurotropic factor) and its effect on neuronal survivability mechanisms was selected for the development of the mechanistic base model. Perfluorooctanesulfonic acid (PFOS) was selected as a case study to illustrate the ways to incorporate the use of system biological model in the field of toxicology via Pharmacodynamic coupled tissue dosimetry model(PBPK/PD).

Case studies on PFOS

PFOS is well recognized among industrial chemicals that can easily cross the BBB (blood brain barrier) (Sato et al., 2009) and its exposure was related to several developmental neurotoxicity effects (Johansson et al., 2008; Yang et al., 2015; Goudarzi et al., 2016; Vuong et al., 2016) . For instance, it was found that PFOS exposure to zebrafish causing an alteration in the expression of more than 40 different type of miRNAs allied with the developmental toxicities (Zhang et al., 2011). The several mechanisms were hypothesized for the PFOS causing development neurotoxicity disorders such as oxidative stress, altering neurotransmitters level and upregulation and downregulation of apoptotic and pro-survival factors from various animals and cell line studies (Long et al., 2013; Chen et al., 2014; Yu et al., 2016). In a recent study, it was found that PFOS can decrease the neuronal cell survivability by altering the level of miRNA in human neuroblastoma cell line(Li et al., 2015). This could be an important mechanism of PFOS as it has been seen that miRNAs regulate the proteins level by

regulating their mRNAs expression level. The purpose of our model is to test the hypothesis that PFOS perturbed the miRNA affecting neuronal survivability via regulating BDNF at mRNA level. The human dosimetry study has shown the longer residence time of PFOS inside the body and relatively higher concentration in the brain tissue than comparing to other perfluoroalkyl substances (PFASs) (Fabrega et al., 2014). Furthermore, its continuous exposure and potential to cross the BBB could put the humans at high risk of neurodevelopmental disorders which is in consonance with recently published paper related to neurotoxicity of PFOS (Yang et al., 2015; Vuong et al., 2016). The PFOS PBPK model has been well developed previously by Fabrega et al., (2014) that predicts internal tissue dose. However, for a better understanding of toxicological mechanisms in the context of risk assessment, we would need one more step towards the system toxicology. This gap could fill by coupling integrated PBPK/PD model into a mechanistic system model.

The objective of this study was the development of a mechanistic pathway system (miRNA-BDNF mRNA- BDNF- cell survivability) model and coupling of above model with a PBPK/PD taking a case study of the PFOS induced neurotoxicity.

2. Materials and Methods

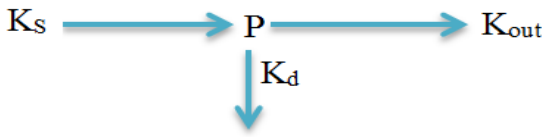
2.1. **miRNA-mRNA-BDNF-cell survival mechanistic pathway (figure 1)**

Generally, miRNA post-transcriptionally regulates the protein molecule via binding at 3´UTR of mRNA (Perruisseau-Carrier et al., 2011). It has been found that miRNA decreases the level of BDNF either via degradation of mRNA or facilitating ribosome induced silencing complex formation with mRNA (RISCm) (Bartel, 2004; Djuranovic et al., 2011). The other mechanism involves miRNA inhibits the BDNF regulation by downregulating the expression ofcyclic response element-binding protein (CREB) (Caputo et al., 2011; You et al., 2016). Nonetheless, the numbers of the regulatory pathways have been proposed (Zeng et al., 2011; Sandhya et al., 2013; York, 2015). Moreover, a study on population affected with neuronal disorders showed an inverse relationship between miRNA and BDNF level (Muiños-Gimeno et al., 2011) strengthens the evidence of regulation of BDNF via miRNA. BDNF dependent cell survival pathways can be extremely important from a regulatory perspective. The relationship between BDNF concentration and cell survival are quite well known via the dose-response curve obtained from the in-vitro cell line study (O'Leary and Hughes, 1998). Nevertheless, intermediate molecular signaling pathways are prevailed in-between the binding of BDNF with TrkB receptors to the effects on the neuronal cell. This involves activation of MAPK/ERK and AKT-PI3K pathways that increase the neuronal survival and differentiation process via increasing expression of CREB (Michael et al., 1997; Murer et al., 2001 Bursac et al., 2010; Boulle et al., 2012). The conceptual diagram is provided in figure 1.

2.1.1. miRNA regulatory BDNF pathway model

The regulatory pathway of BDNF involves different intermediate biomolecules. However, in this study, the generic miRNA-BDNF pathway was adapted from the previously published work of Wang et al., (2010) to developed exclusively miRNA regulatory BDNF model. The whole pathways are modeled by applying mass balance equation based on reaction kinetics applying ordinary differential equations. This allows the estimation of a biomolecule given the model parameter corresponds to the

reaction rates. BDNF is the output of the miRNA-BDNF model, which was then used as an input for the estimation of neuronal survival. The generic form of the system dynamic model is as follow:



Where Ks is synthesis rate constant for the endogenous molecules, P is the concentration of an endogenous molecule, Kd is the degradation rate constant, Kout is the dissipation rate constant of P available for the synthesis of the subsequent endogenous molecule. Following this schematic, concentration of endogenous biomolecules is estimated by the following differential equation;

$$\frac{d}{dt}(P) = Ks - Kd * P - Kout * P \qquad \textbf{Eq. (1)}$$

2.1.2. BDNF - cell survival Emax model-

To simplify the model, we have applied hills sigmoid equations to get the output of the neuronal survival by applying Emax and EC50 value of BDNF for neuronal cell survival from experimental data (O'Leary and Hughes, 1998). The percentage of cell survivability with respect to BDNF concentration was estimated by the use of sigmoid Emax model applying the following equations;

$$Cell\ survivablity = E0 + ¿ \quad (Emax * C^n)/¿ \quad EC50 + C^n \quad)) \qquad \textbf{Eq. (2)}$$

Where, $Cell\ survivability$ = percentage of cell survivability as function of BDNF conc., Eo = baseline response, Emax = maximum response, C= BDNF concentration, EC50= concentration at which BDNF shows 50% response of Emax, n= hill coefficient

This developed Emax model was integrated into indirect response model eq. (3) that provides the neuronal cell survivability as a function of time. More details on indirect response models can be found in Bonate, (2011).

$$\frac{d}{dt} Cell\ survivablity = kout_{BDNF} * cell\ survivablity - kd * cell\ survivability(t) \qquad \textbf{Eq. (3)}$$

Where $\frac{d}{dt} Cell\ survivability = percentage\ of\ cell\ survivability \in the\ time\ domain$, $kout_{BDNF}$ is BDNF conc. assumed to be responsible for neuronal cell survivability, kd is the degradation rate of the neuronal cell.

2.2. PFOS PBPK (a case study)

The PBPK model of PFOS was adapted from the previously published model (Fabrega et al., 2014). The concentration of PFOS in a brain considered as the effective target dose (target tissue dosimetry), considering the brain as a target organ in relation to potential neurodevelopment deficit disorders. PBPK model generates time course of PFOS concentration in the brain, which is used as input for the mechanistic pathway model. At the end, integration of the PBPK model of PFOS into the mechanistic BDNF –cell survivability model analyzes the perturbation of PFOS on the whole pathway results in decreased in neuronal cell survival rate. The conceptual model for this integration is provided in figure 2.

Concentrations in the respective compartment (muscle, richly perfused, fat, kidney, Brain and liver) are estimated by applying the following equation:

$$\frac{dCi}{dt} = \frac{Qi \times \left(Ca - \frac{Ci}{Ki:p} \right)}{Vi} \qquad \textbf{Eq. (4)}$$

Where, Ci is the concentration in the tissue i (ng/L), Qi is the blood flow in the tissue i (L/h), Ca is the arterial concentration (ng/L), Ki:p is the partition coefficient of tissue i, and Vi is the volume of the tissue i (L). Detail description of PBPK model can be found in our other publications (Fabrega et al., 2014; Fàbrega et al., 2016).

All the physiological, Physicochemical parameters and model equations for the PBPK are provided in the Annex-I

2.3. IVIVE for dose Equivalency

In-vitro in-vivo extrapolation (IVIVE) method was used in order to estimate the oral equivalent dose from the given in-vitro dose. It has an assumption that the in-vitro area under the curve (AUC), calculated by multiplying dose with the total duration of exposure, would be similar with the AUC of target in-vivo organ (in this case Brain).

Li et al., (2015) in-vitro studies on SH-SY5Y cell line was selected, where a decrease in neuronal cell survivability found to depend on miRNA and BDNF. In Li et al., an experiment they used 12 in-vitro doses (6 doses each for 24hr and 48 hr) for that corresponding in vivo doses was determined. The assumption was made that in-vitro doses are equivalent to internal target concentration (brain). For the reconstructing equivalent oral dose, the AUC value was calculated for each in-vitro conc., based on their duration of treatment (In this case 24hr and 48 hr). The conceptual schematic for dose reconstruction is provided in figure 3. The calculated AUC was assumed to be equivalent with in-vivo AUC brain. Dose reconstruction approach has been used, so that the given equivalent oral dose will provide the AUC in the brain that matches the AUC for the 12 different in-vitro doses (6 for 24hr and 6 for 48hr), a similar approach has been used in the previous study (Thiel et al., 2017). The oral equivalent doses were estimated to be way higher, as the PFOS concentration reaching to the brain was found to be relatively very low(Fabrega et al., 2014; Fàbrega et al., 2016). The estimated oral equivalent doses for the corresponding in-vitro doses are provided in Table 1.

2.4. Integrated PBPK/PD coupled miRNA-BDNF-cell survival pathway

Coupling of PBPK to mechanistic miRNA-BDNF pathway model has been done with the integration of brain PFOS concentration as a target input that perturbs key component miRNA of the pathway. The interaction of the PFOS with the miRNA has done based on empirical evidence but the mechanism behind the interaction is still not clear. The coupling was done by applying stimulatory Emax model that assumes PFOS increase the concentration of miRNA via increasing their synthesis rate. Finally the output we measured as a percentage of neuronal survival rate considering two scenarios; with and without PFOS exposure. The conceptual diagram is provided in figure 4.

The integration of PFOS into the BDNF pathway is done by indirect pharmacodynamic interaction model with the following equation;

$$\frac{d}{dt}(miRNA) = K_{¿miRNA} * \left(1 + \frac{Emax * C}{EC_{50} + C}\right) - K_{outmiRNA} * miRNA_0 \qquad \textbf{Eq. (5)}$$

Where, $K_{¿miRNA}$ = synthesis rate constant of miRNA, $K_{outmiRNA}$ = dissipation rate of miRNA, $miRNA_0$ = initial value of miRNA, Emax = maximum response for miRNA, C = brain concentration of PFOS, EC50 = concentration at which PFOS shows 50% response of Emax.

2.5 Model parameterization

The mi-RNA-mRNA-Protein pathway parameters were taken from the previously published model (Wang et al., 2010). Specifically, BDNF protein synthesis rate was used instead of generic protein synthesis. There was no BDNFmRNA synthesis rate data available in the literature and for that generic BDNFmRNA rate constant was used. BDNF synthesis rate was taken from the Castillo et al., (1994) and Menei et al., (1998). Furthermore, the synthesis rate was scaled accounting number of neuronal cells to the whole body per kg weight nmol/hr/kg$^{(0.75)}$. The degradation rate of BDNF was parameterized from half- life by using the following relationship: degradation rate = $Ln2/t_{1/2}$.

For the quantification of neuronal survival against BDNF exposure, the required Emax and EC50 parameters for establishing sigmoid Emax model were taken from O'Leary and Hughes,(1998). The Emax and EC50 values for the reaction are implemented as such as these parameters tend to have a similar trend across species (Gatzeva-topalova et al., 2011). PBPK parameters for the PFOS were used from the previously published article (Fabrega et al., 2014). The dynamic interaction data for the PFOS to miRNA, such as EC50 estimated from Li et al., (2015). All the parameters that were used for developing mechanistic model are provided in Table 2. All the model equations for the mechanistic and integrated PBPK/PD-mechanistic models are provided in the Annex-I

3. Results

The simulation of the model is divided into two parts; the first simulation of a PBPK and a mechanistic system pathway model individually to get the base model. Later simulation of integrated PBPK/PD coupled mechanistic model (system toxicology) was done. The integration of Pharmacodynamic interaction between PFOS and target

biomolecule was done by using indirect response model. The equivalent exposure doses for the PFOS were extrapolated from the in-vitro study of Li et al., (2015). Neuronal survivability was chosen as an end point biomarker for the model and mapping of in-vitro data (neuronal survivability) to in-vivo was done based on linear interpolation method. The PFOS PBPK model codes are provided by Fabrega et al., (2014) which was used in this paper to simulate PBPK model.

The mechanistic system model simulations were performed for the miRNA-BDNF signaling pathway and the resulting time course of BDNF was recorded as model output. The output of the BDNF time course data was used for performing the simulation to get the percentage of cell survivability by applying indirect sigmoid response model. This part of simulation results recorded as the normal baseline value for the model. The figure 6 (base model of the mechanistic pathway) showed the baseline value of important endogenous biomolecules like miRNA, BDNF, RISC(RNA-induced silencing complex), RISCm (complex form between BDNFmRNA and RISC) and percentage of neuronal cell survivability. The mechanistic system model has optimized to achieve the maximum neuronal cell survivability steady state which is in compliance with experiment data given by Gillespie et al., (2003). The model has been simulated for 20 days in order to achieve the steady state. The miRNA regulation of BDNF via forming a complex between RISC and BDNFmRNA called RISCm has been documented can be seen in the base model figure number 6 which is in compliance with Wang et al., (2010) model. This complex formation between RISC and BDNFmRNA was enhanced by the miRNA resulting in a decrease of BDNF protein synthesis. The RISC complex binds with the mRNA at the 3' UTR and inhibits its further translation to protein. The base model also able to capture the phenomena of regulating BDNF protein by miRNA considered to be one of the important biological processes. The behavior of model curve for BDNF and cell survival are in a similar trend, which was also observed in in-vivo experiments (Rodríguez-Tébar et al., 1992; O'Leary and Hughes, 1998; Fletcher et al., 2008). The model shows BDNF maintains cell survivability at the steady state level of around 95 percent. In Figure (6), a sudden drop in the cell survivability to 40 percent level could be explained considering the lag time in the attainment of BDNF steady state level. The simulation of the base model (Figure 6) shows that model able to retain the steady state for cell survivability at 95% once BDNF attained a steady state. A similar observation was reported by Gillespie et al., (2003) experimental study that survivability of neuron in presence and absence of BDNF were 90 percent and 40 percent respectively.

The PBPK model simulation was carried out for the PFOS for the estimated oral equivalent dose (12 doses) given as a single dose. Figure 5 shows the simulation of the internal target tissue (brain) concentration of PFOS with 12 different dose levels providing different Cmax in dose dependent manner over the time period. The dose was given at the 240hr as shown in figure 6 when the mechanistic base model reaches steady state.

The coupling of PBPK into the mechanistic model was done by fitting in-vitro data, estimated from Li et al., (2015) study, via applying Emax sigmoid model. The developed coupled PBPK/PD-mechanistic model quantifies the dynamic of the endogenous biomolecular concentration of different species at the different level of PFOS exposure that perturb key components of the system (in the miRNA model). The interaction of the PFOS to the given pathway was modeled by implementing indirect sigmoid response model **Eq. (5)** for PFOS-miRNA interaction. Consequently, dynamic

changes in miRNA level as a function of PFOS concentration over time was observed (figure 7). The PFOS alter the steady state of all biological components involved in the pathway via stimulating input of miRNA disturbing whole mechanistic pathway. The integrated model was simulated for 12 different in-vitro equivalent in-vivo doses describing the whole system as one unit rendering time course of endogenous concentration after exposure to environment chemicals distinct from normal condition (Base model).

The figure 7, 8, 9 and 10 shows the effect of a chemical on the endogenous biomolecule concentration (miRNA, RISCm, BDNF) and cell survivability (in percentage) respectively over the time period. Figure 7 illustrates the dose depended effects of PFOS on miRNA level following single exposure to PFOS (dose given at 240hr). Figure 8 illustrates the increase in the formation of the RISCm complex after the PFOS exposure. The increase of RISCm complex concentration is due to increase of miRNA level which can be considered as an indirect action of PFOS. The highest level of miRNA is observed at tmax (time point of Cmax) of PFOS and, with the elimination of PFOS from the system, shifting of miRNA level to steady state concentration at the level higher than baseline concentration was observed. Consequently, a decrease in the level of BDNF (figure 9) was noted as increase miRNA level facilitates the formation of the RISCm (figure 8), posttranscriptional regulatory mechanism of miRNA (explained in 2.1). With the increase in dose level, the difference between base steady state concentration and shifted steady state concentration was higher that can be seen in figure 7, 8, 9 and 10. Figure 10, illustrates the time vs neuronal survivability that describes the effect of PFOS over time as an end point biomarker.

5. Discussion and Conclusions

In this study, an attempt was made for the development of an integrated PBPK/PD coupled mechanistic model that allows assessing or characterizing the potential impact of environmental chemicals on a biological system. An Integrated PBPK/PD PFOS model and a mechanistic (miRNA-BDNF-neuronal survival) system model were evaluated individually. The generic mi-RNA model was adapted with a modification in BDNF as a target output protein. The regulation of BDNF involves several pathways among which miRNA-dependent pathway is an important one. The endogenous level of BDNF has an important effect on the survivability of neurons. For example principal hierarchy of BDNF signaling and consequently activation of MAPK/ERK/AKT pathway is well understood (Michael et al., 1997; Murer et al., 2001 Bursac et al., 2010; Boulle et al., 2012), but how these events control cellular survival are not well understood. The reported relation between chemical exposure and significant changes in BDNF level, consequently neuronal adverse outcomes, made a plausible argument of considering BDNF as a good biomarker. To keep biological plausibility intact in our mathematical expression, we restrict our model to the miRNA-BDNF pathway, and later linking it to the cell survivability as a function of the time course of BDNF concentration by applying Emax model. The developed mechanistic model shows miRNA-dependent regulation of BDNF which is a natural phenomenon of this model retaining the regulatory mechanism of miRNA on BDNF. The mechanistic base model (figure 6) well predicted the percentage of cell survivability as a function of BDNF concentration. The PBPK model was used to estimate the internal target dose of chemicals. The output of PBPK in target organ is used as input for the mechanistic system model providing integrated coupled PBPK/PD-mechanistic system model. This will describe the whole system as one unit rendering time course of endogenous

biomolecules concentration and their steady state level with and without chemical exposure marking the difference between the normal and altered biology of the pathway.

The integrated PBPK/PD- coupled mechanistic system model well describes the observed changes in endogenous molecules level during and after discontinuation of exposure to the chemical. It can predict the adverse effect of environment chemicals considering both; the nature of changes in the system (altered biology) with respect to normal biology, and, the capability of an endogenous molecule to retain homeostasis, mimicking the real in vivo physiological scenario. Therefore, this kind of model (integrated PBPK/PD- coupled mechanistic system model) can predict risk in more quantitatively as well as mechanistically considering pharmacokinetic, pharmacodynamic and relative altered biology from normal biology pathway as a consequence of chemical exposure. The advantage of Coupled integrated PBPK/PD-mechanistic system model is; it provides more understanding towards risk not only based on the target tissue concentration but also their effect on the target molecule participating in the biological network. Integrated PBPK/PD coupled mechanistic model are able to predict endogenous molecule concentration involved in pathway over their time course as a function of chemical exposure, which was shown by current developed model as a case study for PFOS

In summary, a molecular/cellular model that presented in this article mechanistically links BDNF involved in directed neuronal growth and neuronal survival, two distinct neurodevelopmental processes that use an overlapping molecular (that is genetic) machinery. The model does not provide further insights into which of these neurodevelopmental processes would be most relevant to the etiology of neurotoxicity, or where in the brain these processes are localized to selectively impact on neural circuitry. Although epigenetically regulation of BDNF (Lubin et al., 2008) in the brain by miRNA is very important were observed from literature in the theoretical network, it is unlikely that there would just be a single explanatory model that connects to BDNF on a molecular level and corresponding neuronal adverse outcomes. Rather, several etiological cascades contributing to neuronal adverse outcome are likely to exist. However, the currently developed model considered the following pathway for a series of signaling cascade biomolecules such as chemicals-miRNA-mRNA-RISCm-BDNF-neuronal survivability, previously described in the conceptual model (figure 2). For the currently selected pathway model predicts BDNF as a very sensitive endogenous species biomolecule, which maintains the cell survivability at steady state. Although, PFOS does not directly target BDNF in our model it still remains the sensitive target which could be due to its regulation is highly dependent on miRNA level. Comparison of figure 9 and 10 allow us to see the decrease in neuronal survivability (figure 10) is highly sensitive towards BDNF level (figure 9). The model shows that BDNF regulation (miRNA based regulation) is very much important for neuronal cell survivability. This shows BDNF could be an interesting species (biomarker) which can link between both environmental exposure and neuronal adverse outcomes.

There was an assumption of the existence of an empirical relation between the in-vitro toxicity to in-vivo toxicity (Wambaugh et al., 2013). Moreover, tools have been developed to translate in-vitro toxicity dose-response to predict the in-vivo toxicity by applying reverse dosimetry concept that provides equivalent in-vivo dose required to produce in-vitro toxicity, eventually validation of model was done by comparing POD (point of departure) from predicted in vivo dose response with reported POD of

chemicals (Abdullah et al., 2016; Forsby and Blaauboer, 2007; Louisse et al., 2016; Wambaugh et al., 2013). In this case study of PFOS model (PBPK/PD coupled mechanistic model) due to lack of in-vivo data particularly for the following proposed mechanistic pathway, in worst case scenario we constrained to in-vitro data for qualitative or partial validation of the developed model. To check the performance of the developed PBPK/PD coupled mechanistic model, neuronal cell survivability was selected as an end point. Two approaches were used for this purpose; first reconstructing oral in-vivo equivalent dose for an in-vitro dose; second, response data are generated for identified in vivo doses by mapping in vitro toxicity data (in this case neuronal cell survivability). Figure 10 illustrates, the simulated response variable (% neuronal survivability), for dose equivalent to in-vitro conc., vs observed linear interpolated response variable. Although model could not able to predict all the observed data, however, most of them were within the simulated range. The simulated maximum % of neuronal cell survivability on the lower side was around 35%, which is higher than the experimental observation of around 16 to 20%. This could be possibly explained by several facts such as current model uses adaptability mechanism which lacks in the in-vitro system, only one pathway has been accounted, neglecting the possibility of several mechanisms, empirical estimation of PFOS-miRNA interaction and the inherent uncertainty in in-vitro data and model.

The purpose of this work was to develop a simple model which combines pharmacokinetic model like PBPK predicting the internal tissue dosimetry and mechanistic system model via quantifying the Pharmacodynamic interaction of chemicals with key biomolecule components involved in the mechanistic system of biology. The measurement of mi-RNA, mRNA, BDNF in the brain at different time points gives evidence in parallel changes and difference in between them; significantly improves the understanding of relation with neuronal adverse outcomes. Here in this model, the mechanistic pathway can be considered as an equivalent AOP pathway for neurotoxicity. However, this can be further extended by integrating identified new pathways responsible for neurotoxicity. There are many ways that model can be extended to increase its utility, but certainly, the mi-RNA-based post-transcription regulation of BDNF not limited to PFOS. The same concept can be further applied to other environmental chemicals altering the similar system.

In this paper, we have partially validated our model, considering our objective of this paper is to focus on the illustration of tools that use simple integrated PBPK/PD-coupled mechanistic pathway model involving three main steps 1.Development of PBPK model, 2. Development of mechanistic system model 3. Couple PBPK with the mechanistic model by integrating PD model that quantify perturbed biomolecule (a component of the mechanistic model) as a result of chemical exposure. This step developed a new framework that could utilize the existing normal mechanistic pathways model and integrated PBPK/PD model, a step towards system toxicology based models.

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Figure Labels

Figure 1. describes the miRNA-mRNA-BDNF-cell survival mechanistic pathway showing the importance of miRNA in regulating BDNF via forming a complex with RNA-induced silencing complex. Later BDNF binding to TrkB with the sequential activation of pathway **such as MAPK/ERK and PI3K/AKT causing increase in CREB expression which leads to increase in** neuronal survival, differentiation, and proliferation.

Figure 2. represents the full scheme of PBPK/PD model showing the integration of tissue dosimetry model with miRNA-BDNF-Cell survival pathway via pharmacodynamic interaction of PFOS-miRNA.

Figure 3. Schema for the estimation of in-vivo oral dose

Figure 5. Simulated brain concentrations of PFOS over the time period. The figure shows a simulation of the time course of PFOS concentration in the brain for each 12 different doses corresponding to in-vitro dose. The single oral dose was given at 240hr.

Figure 4. represents the pharmacodynamic interaction of PFOS-miRNA and the consequent effect on neuronal survivability rate.

Figure 6. Mechanistic Base model. The figure shows simulated key biomolecules such as RISC, miRNA, RISCm, BDNF and percentage neuronal cell survivability.

Figure 7. simulated time vs miRNA level The figure depicts simulated miRNA concentration after single oral dose of PFOS for 12 different dose levels.

Figure 8. Simulated time vs RISCm level. The figure shows the increase in RISCm level after single oral dose of PFOS for 12 different dose levels.

Figure 9. Simulated time vs BDNF level. The figure depicts simulated BDNF concentration after single oral dose of PFOS for 12 different dose levels.

Figure 10. Simulated vs predicted neuronal cell survivability (percentage). The figure depicts simulated vs observed neuronal cell survivability (percentage) after single oral dose of PFOS for 12 different dose levels.

741**TABLES**

742**Table 1. oral equivalent dose calculated based on AUC extrapolation method**

in-vitro dose (μM)	AUC_24 (nM*hr)	AUC_48 (nM*hr)	in-vivo dose (nM)(24hr)	in-vivo dose (nM) (48hr)
1	24000	48000	86925	130570
10	240000	480000	896550	1362850
50	1200000	2400000	4494910	6839718
100	2400000	4800000	8992868	13685810
150	3600000	7200000	13490820	20531899
200	4800000	9600000	17988780	27378025

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744**Table 2. Scaled parameters for Coupled PBPK/PD mechanistic pathway model**

Description	Parameter Symbol	Value	References
BDNF synthesis rate	Kin_BDNF	.023 nM/hr/kg 0.75	(Menei et al., 1998)
BDNF dissipation rate	**Kout_BDNF**	**0.231/hr**	**(Fukumitsu et al., 2006)**
Maximum BDNF effect on cell survival	**Emax**	**100**	**Assumed**
Half maximum concentration of BDNF for neuron survivability	**EC50_BDNF**	**5E-03 nM**	**(O'Leary and Hughes, 1998)**
Cell degradation constant	**Kd_cell**	**2.45e-5/hr**	**(Clarke et al., 2000)**
Maximum PFOS effect on miRNA	**Emax_miRNA**	**2.4**	**maximum fold change(Li et al., 2015)**
Half maximum stimulatory concentration of PFOS for miRNA	**EC50_PFOS**	**1000nM**	**(Li et al., 2015)**
Volume of cytoplasm	**V_cyt**	**4e-12/L**	(Bartlett and Davis, 2006)
Volume of nucleus	**V_nucleus**	**4e-13**	(Carlotti et al., 2000)
Pri miRNA synthesis rate	**K_primiRNA**	**3.6 nM/hr**	(Pérez-Ortín et al., 2007)
mRNA synthesis rate	**K_mRNA**	**0.36 nM/hr**	(Bartlett and Davis, 2006)
Adjusted Coefficient of R promoting pri-miRNA maturation	**R_miRNA**	**0.001 nM**	(Wang et al., 2010)

pri-miRNA to pre-miRNA(n) catalyzed by R	**K_primiRNA-premiRNA**	**360/hr**	(Wang et al., 2010)
premiRNA transport rate	**T_premiRNA**	**180/hr**	(Wang et al., 2010)
Rate of premiRNA(c) conversion to dsmRNA	**K_premiRNA-dsmRNA**	**36/hr**	(Ma et al., 2008)
miRNA formation rate	**K_miRNA**	**36/hr**	(Kohler and Schepartz, 2001)
miRNA-induced RISC formation rate	**K_RISC**	**108/hr**	(Bartlett and Davis, 2006)
mRNA-RISC complex formation rate	**K_[mRNA-RISC]**	**3.6nM/hr**	(Haley and Zamore, 2004)
mRNA cleavage rate	**Kc_mRNA**	**25.27**	(Haley and Zamore, 2004)
Dissociation rate of RISC complex	**Kd_[mRNA-RISC]**	**3.6/hr**	(Wang et al., 2010)
Rate of pri-miRNA degradation	**d_primiRNA**	**0.9/hr**	(Wang et al., 2010)
Rate of pre-miRNA(c) degradation	**d_premiRNA**	**0.9/hr**	(Wang et al., 2010)
Rate of dsRNA degradation	**d_dsRNA**	**3.96/hr**	(Wang et al., 2010)
Rate of miRNA degradation	**d_miRNA**	**0.9/hr**	(Wang et al., 2010)
Rate of RISC degradation	**d_RISC**	**0.36/hr**	(Wang et al., 2010)
Rate of mRNA-bound RISC complex degradation	**d_[mRNA-RISC]**	**0.077/hr**	(Wang et al., 2010)
Rate of mRNA degradation	**d_mRNA**	**0.36/hr**	(Wang et al., 2010)

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