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Pharmacokinetics and Pharmacodynamics in Breast Cancer Animal Models

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Abstract

The study of pharmacokinetics (PK) and pharmacodynamics (PD) in cancer drug discovery and development is often paired and described in reciprocal terms, where PK is the analysis of the change in drug concentration with time and PD is the analysis of the biological effects of the drug at various concentrations over different time courses. While PK is defined by how a compound is absorbed, distributed, metabolized, and eliminated, PD refers to the measure of a compound's ability to interact with its intended target, leading to a biologic effect. Recent advances in anti-breast cancer drug discovery have resulted in several new drugs, but there is still a high attrition rate during clinical development. One reason for this failure is attributed to inappropriate correlation between the PK and PD parameters and subsequent extrapolation to human subjects. In this chapter, we describe the protocols of PK and PD studies in breast cancer models to assess the efficacy of an anti-breast cancer compound, noting the types and endpoints employed, and explain why it is important to link PK and PD in order to establish and evaluate dose/concentration-response relationships and subsequently describe and predict the effect-time courses for a given drug dose.

Keywords

Pharmacokinetics; Pharmacodynamics; Breast cancer; Animal models; PK/PD modeling

1 Introduction

Biological response of a drug is often related to its concentration at the site of action over the course of time [1]. Thus, pharmacokinetics (PK) which studies the time course of drug's fate in the body and pharmacodynamics (PD) which studies the relationship between drug concentration and biological effect become the essential components of the modern drug discovery process and crucial towards successful clinical transition of a new drug. Breast cancer, one of the most common causes of cancer related mortalities in women worldwide, has been the focus of anticancer drug discovery over the years [2, 3]. Despite considerable diversity in molecular profile, histopathology, and treatment outcome, advances in genetics and molecular/cell biology have greatly extended our knowledge about the molecular pathogenesis of breast cancer, and subsequently unlocked new avenues for developing novel

targeted therapies through the identification of potential therapeutic targets involved in intracellular signaling processes responsible for malignant transformation and progression [4–7]. However, anti-breast cancer drug discovery and development is associated with high attrition rates, largely, due to a lack of efficacy and unexpected toxicity of new drugs in the clinical development. Generation of high-quality, predictive PK and PD information in the early phase of the drug development process could help reduce the high attrition rates in the discovery and development pipeline [8–10].

1.1 Pharmacokinetic Considerations

New drug development is highly dependent on animal studies to provide a framework for human clinical trials [11, 12]. Current cancer drug development paradigm selects starting doses for phase I clinical trials based on prior data obtained from pharmacokinetic and toxicology studies in animals using allometric scaling based on body surface area conversions [10, 11]. Though plasma PK properties have been successfully related to pharmacological effects, the heterogeneous nature of tumor tissue often impedes drug delivery from plasma to tumor, leading to complex relationships between concentrations in plasma, tumor interstitium, and tumor cells [13]. Therefore, meaningful PK data for an anticancer drug must include tumor permeability and tumor uptake data, either in vitro or in vivo [13, 14].

Prior to advancing to in vivo testing, a number of parameters are assessed that help predict the potential of the drug candidate to reach its intended target in vivo [15, 16]. These include, but are not limited to, physicochemical characterization of the drug (i.e., determinations of molecular weight, solubility, lipophilicity), extent of plasma protein binding, interactions with cytochrome P₄₅₀ (CYP) isoenzymes (both induction and inhibition), ability to migrate across a cell barrier (Caco-2, MDCK cell lines, and/or artificial lipid membranes are often used to address potential for absorption across the gut and blood–brain barrier), and stability (measured either as half-life of a compound mixed with human or rodent plasma and in solutions of varying pH simulating stomach or GI environments, or when incubated with liver microsomes or hepatocytes as an early metabolic stability readout) [15, 16]. An exhaustive in vivo PK profile that describes tissue distribution and accumulation over a time course is usually established before compounds undergo extensive efficacy testing in animal models [16–18].

1.2 Pharmacodynamic Considerations

A successful and meaningful PD analysis involves the measurement of biomarkers or clinical endpoints that can be quantitatively related to drug concentration in the body. In PD studies for anti-cancer drugs, often, four different types of outcomes are measured either, alone or in combination [19, 20]: (a) decrease in tumor size or tumor regression (this is the most frequent measure of drug efficacy); (b) modification in expression level of a biomarker which correlates with disease progression and/or survival, e.g., changes in estrogen receptor (ER) and progesterone receptor (PgR) for endocrine therapy, changes in Ki-67 levels, a cellular proliferation marker, for assessing therapeutic efficiency [21, 22]; (c) an outcome predicted based on the molecular target of the drug (e.g., an increase in apoptosis assessed by Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining with a

proapoptotic drug, or inhibition of target oncogene levels); and (d) in some cases, toxicity as a result of therapy can also be used as a clinical end-point with higher drug exposure being associated with antitumor activity and greater toxicity [10]. The concentration–response relationships that lead to the estimation of these PD parameters are both useful in preclinical drug development and in the clinic trials. Various in vitro cellular and cell free assays are often used to identify and validate molecular targets [23–31]. For further testing and confidence as a potential anti-breast cancer agent, the PD studies often include testing in animal models for target validation and therapeutic response, in relation to drug concentration in vivo.

2 Materials

Analytic grade and/or HPLC grade reagents should be used for preparation of all solutions and buffers, using ultrapure double distilled water. Prepared solutions and buffers should be filtered and stored as indicated. All cell culture equipment and biological hood and culture media should be sterile or sterilized prior to use. Gloves must be used for all procedures. Animals should be housed and treated according to regulatory guidelines and all animal study protocols must be reviewed and approved by Institutional Animal Care and Use Committee (IACUC).

2.1 Uptake and Cellular Transport Experiments

1. 60 or 100 mm dishes.
2. Cancer cell lines.
3. MDCK cell line.
4. Caco-2 cell line.
5. Cell culture medium as specified by ATCC for particular cell line.
6. FBS.
7. Penicillin–streptomycin.
8. Sterile PBS.
9. Trypsin–EDTA solution.
10. Hemocytometer for cell counting.
11. Pasteur pipettes.
12. Stop solution (200 mM KCl and 2 mM HEPES).
13. Transwell diffusion chamber.

2.2 S9 Metabolism Studies

1. 37 °C shaking water bath.
2. 25 mL round bottom glass test tube.
3. 100 mM Tris-HCl buffer (pH 7.4).

4. Methanol, UPLC grade.
5. S9 (Celsis In Vitro Technologies): CD-1 mouse S9; human S9; and Fisher 344 rat S9. All S9 fractions are in 20 mg/mL protein basis.
6. NRS-I: 2 % NaHCO₃; 1.7 mg/mL NADP, 7.8 mg/mL glucose-6-phosphate, 6 units/mL glucose-6-phosphate dehydrogenase.
7. NRS-II: 2 % NaHCO₃; 1.9 mg/mL UDPGA; 100 µg/mL PAPS.

2.3 Protein Binding Assay

1. Ultrafiltration devices (Amicon Centrifree-Amicon YM-300 filter system, Millipore Corp., Bedford, MA).
2. Water bath.
3. Centrifuge.
4. Purified mouse plasma.
5. α1-acid glycoprotein (AGP).
6. Human serum albumin (HSA).

2.4 Cytochrome P450 Enzyme Induction/Inhibition Assay

1. Fresh human hepatocytes.
2. Test compound at different concentrations.
3. CYP isoforms: CYP1A2; CYP2B6; and CYP3A4.
4. Negative control: vehicle (typically 0.1 % DMSO).
5. Positive control reagents: 3-methylchloranthrene (CYP1A2); omeprazole (CYP1A2); phenobarbital (CYP2B6); dexamethasone and rifampicin (CYP3A4).
6. Probe substrates: ethoxyresorufin (CYP1A2); bupropion (CYP2B6); midazolam (CYP3A4).

2.5 In Vitro Stability Testing

1. Mouse plasma.
2. Human plasma.
3. Water bath.

2.6 Establishment of Animal Tumor Models

1. Cell culture related materials.
2. Culture medium without FBS or antibiotics.
3. 70 % ethanol.
4. 0.9 % NaCl (physiological saline, sterile).

5. Drug preparations in appropriate vehicle.
6. 1 mL syringe with 27–30 gauge needles.
7. Sterilized animal feeding needles, 18/50 mm, curved tube style.
8. Matrigel® basement membrane matrix (Becton Dickinson Labware, Bedford, MA).
9. Xenolight RediJect d-Luciferin (850 µL of 30 mg/mL in a vial; PerkinElmer, Waltham, CA).
10. Caliper IVIS bioluminescent imaging system (Caliper, Mountain View, CA).
11. A small balance for body weight measurement.
12. Calipers for measuring tumor diameters.
13. Ear tags and ear puncher for identification of animals.
14. Disinfectant.
15. Sterile gauze.
16. Solution for sample preparation for pathology analysis: add 735 mL of absolute alcohol (100 %) into 315 mL of deionized H₂O and then add 117 mL of formalin (37 % formaldehyde).
17. Athymic nude mice, female, 4–6 weeks old, allowing 3–5 days acclimatization period.
18. Clean cages with sterile bedding, food, and water, and cage card with animal identification information.

2.7 In Vivo PK and PD Studies

1. Glass metabolic cages (for mice and rats).
2. Animal surgical instruments for blood drawing and tissue collection.
3. Diseased animal model, such as female nude mice bearing breast xenograft tumors or orthotopic tumors.
4. Normal healthy animal, such as CD-1 female mice.
5. Heparinized 1.5 mL Eppendorf tubes.
6. Tissue homogenizer.
7. Balance for tissue weight measurement.
8. Tissue storage vials and solvent.
9. Pharmacokinetic modeling program.

3 Methods

3.1 Drug Cellular Uptake Assay

1. Plate cells at a density of $\sim 5 \times 10^5$ cells/well in 6-well plates and grow under optimum conditions.
2. Prior to the experiment, wash cells twice with PBS and incubate at 37 °C for 10 min to ensure complete removal of the medium.
3. Add 2 mL of desired concentration of drug in PBS in each well and incubate at 37 °C for 30 min to 2 h (*see* Note 1).
4. Following incubation, wash the cells three times with PBS and rinse the cells three times with ice-cold stop solution to stop further uptake of drug.
5. Lyse cells by incubating with PBS at –80 °C overnight and subsequent thawing at room temperature. Aliquots from each well are transferred to 1.5 mL centrifuge tubes and extracted as per established procedure.
6. Analyze with HPLC/LC-MS (*see* Notes 2–4).
7. Measured the protein concentration of each sample by Bradford (Bio-Rad, Hercules, CA) assay.
8. All uptake data is normalized to the protein count from each well.

3.2 Cellular Transport Studies

1. Grow cell mono-layers on the Transwell™ under appropriate conditions.
2. Prior to the experiment, wash cells twice with PBS and incubate at 37 °C for 10 min to ensure complete removal of the medium.
3. Add 200 μ L of desired concentration of drug in PBS in donor chamber (*see* Note 1).
4. Fill receiver chamber with PBS. Sampling is carried out from the receiver chamber at predetermined time points and fresh PBS replaced in the receiver chamber to maintain sink conditions. All the experiments are performed at 37 °C.
5. The samples are analyzed by HPLC/LC-MS. The effective permeability (cm/s) is determined according to Eq. 1:

$$P = \frac{dC/dt}{C \times AV \times 60} \quad (1)$$

P is the permeability in cm/s, dC/dt denotes the slope of plot of concentration (nM) versus time (min), C represents the concentration of drug (nM/cm³), A is the surface area of the chamber (cm²), and V is the volume of each half-chamber (cm³).

3.3 S9 Metabolism Studies

1. Dilute the S9 to 10 mg/mL in 100 mM Tris-HCl buffer.
2. Place the test tube into the ice bath and add 200 μ L of 10 mg/mL S9.
3. Add 1280 μ L of 100 mM Tris buffer.
4. Add 20 μ L of 1000 μ M test compound.
5. Place the test tube and the NRS separately into a 37 °C shaking water bath for 5 min.
6. Add 500 μ L of 100 mM Tris-HCl or NRS I or NRS II to test tube. Start the reaction time at the addition of Tris-HCl or NRS to the samples.
7. 150 μ L (duplicate) of the mixture is moved to the Eppendorf tube at 0, 15, 30, 45, and 60 min, and twofold volume (300 μ L) of cold methanol is added. Vortex for 10 s. The mixture is centrifuged at $12,000 \times g$ for 10 min and then the whole supernatant is moved to a new 100 $\times$ 75 mm glass tube. Air-dry using compressed clean-air. The deposit is dissolved in 150 μ L of UPLC mobile phase. 100 μ L of volume is injected onto the UPLC/LC-MS column.
8. The stability of the drug is determined by analysis of intact drug post incubation with S9 fractions, in comparison with negative control (without S9 fractions) (see Notes 5 and 6).

3.4 Protein Binding Assay

1. The plasma protein binding studies are carried out on whole human plasma as also specific plasma proteins such as Human serum albumin (HSA), and α 1-acid glycoprotein (AGP).
2. α 1-acid glycoprotein (AGP) and human serum albumin (HSA) solutions are prepared in PBS (pH 7.4). The concentrations of AGP and HSA used are 0.7 g/L and 40 g/L respectively, which is equal to the levels found in normal subjects.
3. Spike mouse plasma samples, AGP, and HSA with drug at a low and high concentration and maintain at 37 °C for 1 h. Prepare control samples using PBS instead of plasma.
4. From each of these preparations, a portion is aliquoted and placed in a sample reservoir of an Amicon Centrifree® ultrafiltration system (Millipore Co., Bedford, MA). The filter systems are centrifuged at $2000 \times g$ until the reservoirs run dry. From each sample, triplicate portions are taken for analysis by HPLC or LC-MS. The Amicon Centrifree YM-300 filter system has a membrane molecular mass cutoff of 30,000 Da.
5. The amounts present in the filtrates are designated as “free drug” (*F*). The concentrations of the unfiltered solutions are also determined by triplicate analyses. This amount represents the ‘total drug’ concentration (*T*). The amount bound nonspecifically to the filter (*X*) also is determined. The percentage of test

compound bound to plasma proteins is calculated by the following formula:

$$\text{Percentage bound} = [(T - F - X)/T] \times 100 \text{ (see Note 7).}$$

3.5 Cytochrome P450 Induction/Inhibition Assay

1. Use fresh or platable cryopreserved hepatocytes, as either monolayer or sandwich culture, with a 1–2 day recovery period after plating.
2. Treat with test compound and positive controls for 2–3 days (changing medium with test compounds every 24 h) in media containing ITS, dexamethasone, and penicillin–streptomycin as media supplements (see Note 8).
3. Dissolve the test compound preferably in DMSO (v/v 0.1 %) whenever possible and incubate at three or more different concentrations (in triplicate), spanning anticipated or known therapeutic concentration range including a concentration at least an order of magnitude higher. Minimally three concentrations of the test compound should be assessed spanning the in vivo or predicted $C_{\max}$ exposure levels, such as 0.1 $\times$, 1 $\times$, and 10 $\times$ $C_{\max}$.
4. Use recommended positive controls such as 3-methylchloranthrene (2 μ M), phenobarbital (1000 μ M), and rifampicin (10 μ M) for CYP1A2, 2B6, and 3A4, respectively, (dissolved in 20 mL of incubation medium) at concentrations known to elicit maximal induction response (Table 1).
5. Prepare working concentrations of the probe substrates as follows: (a) 100 mM phenacetin in DMSO, (b) 500 mM bupropion in methanol, (c) 200 mM testosterone in methanol.
6. Prepare a negative control by adding 20 μ L DMSO into 20 mL warm incubation medium.
7. In separate conical tubes, dissolve the test compound in warm incubation medium to yield final inducing concentrations 1000-fold lower than the respective working concentrations.
8. Remove the hepatocyte plate(s) from the incubator and replace the medium in the appropriate wells with 0.5 mL of the negative control, inducers, or test compound solutions, preferably each in triplicate.
9. Return plate(s) to the incubator for 24 h.
10. After 24 h, replace the medium with freshly prepared inducing solutions, repeating **steps 4** through **8**.
11. Fresh solutions should be prepared daily and applied every 24 h during the duration of the experiment, which is typically 48–72 h prior to harvest.
12. On the day of harvest, warm the incubation medium to 37 °C, as usual and remove the induction plate(s) from the incubator.
13. Aspirate the medium from each well, wash and replace with warm incubation medium. Take precautions to avoid the hepatocytes drying out, as mentioned in the notes (see Note 9).

14. Prepare probe substrates in 20 mL warm incubation medium to yield final incubation concentrations 1000-fold lower than working concentrations.
15. Remove the wash medium from the hepatocytes and replace with 0.5 mL of the probe substrate solutions prepared in the previous step.
16. Incubations should be conducted in triplicate with the respective CYP450 treatment group.
17. Incubate on an orbital shaker in the incubator for the time specified in table. A speed of 120–150 rpm is recommended for a 24-well plate.
18. Stop the incubations at the appropriate time points by collecting and freezing the sample medium, or by removing the sample medium and replacing it with an organic stop solution. Samples can be stored at -80°C before analysis.
19. Extract and analyze samples by method of choice, typically HPLC/LC-MS by monitoring the formation of metabolite.
20. If mRNA analyses are desired, upon removal of the probe substrates, wash hepatocytes twice with Hank's Balanced Salt Solution (HBSS).
21. Remove the HBSS from the wells and replace with an appropriate volume of lysis buffer. Collect the cell lysates in RNase-free microcentrifuge tubes and extract RNA for subsequent PCR reactions.
22. CYP activity can be expressed as pmol/min/million cells where pmol is defined as the amount of metabolite formed during the reaction. The fold-induction enzyme activity is determined by the ratio: CYP activity (induced)/CYP activity (vehicle). The percent adjusted positive control is determined by: $[(\text{CYP activity (induced)} - \text{CYP activity (vehicle)}) / (\text{CYP activity (positive control)} - \text{CYP activity (vehicle)})] \times 100$. A drug that produces a change that is equal to or greater than 40 % of the positive control can be considered as an enzyme inducer in vitro and further in vivo evaluation is warranted (*see* Note 10).

3.6 In Vitro Drug Stability Assay

1. Incubate three concentrations of the test drug (spanning anticipated or known therapeutic concentration range including a concentration at least an order of magnitude higher) in purified mouse plasma.
2. Analyze triplicates of the samples that have been exposed to different conditions (time and temperature) (*see* Note 5).
3. Freeze-thaw stability: The samples are stored at -20°C for 24 h. They are then removed from the freezer and allowed to thaw unassisted at room temperature. When completely thawed, the samples are refrozen for 24 h under the same conditions. This freeze-thaw cycle is repeated twice more, for a total of three cycles. Then the samples are extracted using a validated extraction procedure and subjected to HPLC/LC-MS analysis.

4. Short-term and long-term stability: the short-term stability in plasma is assessed by analyzing samples kept at 37 °C for 2, 4, 6, 8, 12, and 24 h and samples kept at 4 °C for 4, 8, 12, and 24 h. The long-term stability is determined by assaying samples after storage at –80 °C for 2, 4, 6 and 8 weeks. Then the samples are extracted using a validated extraction procedure and subjected to HPLC/LC-MS analysis.
5. Bench-top stability: to assess the bench-top stability of test compound in a particular biological matrix at room temperature, samples are incubated in plasma or tissue homogenates and kept at ambient temperature (25 °C) for 4, 8, 12, and 24 h, which far exceeded the routine sample preparation time.
6. Homogenization stability: samples are injected into equal weights of blank control tissues (brain, heart, liver, kidneys, lungs, spleen, tumor, and muscle) in the collection tube. Care is taken to ensure the drug solution is contained inside the tissue/organs. The tissue samples are homogenized, and extracted and immediately analyzed.
7. Stock solution stability: fresh and stored at room temperature (25 °C) stock solutions of samples are tested at 6, 12, and 24 h after sample preparation. The extraction and analytical methods are identical to the above sample preparation.

3.7 Subcutaneous Xenograft Models

1. Culture the desired breast cancer cells in 10 or 15 cm dishes according to specified ATCC guidelines (*see Note 11*).
2. Harvest tumor cells in logarithmic growth phase by trypsinization and transfer to 50 mL centrifuge tubes. Centrifuge them at $500 \times g$ for 5 min (*see Note 12*).
3. Remove supernatant and wash cells twice with medium lacking serum and antibiotics.
4. Remove a small aliquot of cell suspension before the last centrifugation and count the number of cells using a hemocytometer. Calculate the total cell number by multiplying cell density (cells/mL) by the volume of cell suspension.
5. Centrifuge tubes again for 5 min at $500 \times g$ to collect cells.
6. Add necessary volume of culture medium lacking serum to the cells. Pipet thoroughly to obtain a homogeneous cell suspension (*see Note 13*).
7. Mix the cell suspension with Matrigel basement membrane matrix (2:1, v:v, final 2.5×10^7 cells/mL) (*see Note 14*).
8. Transfer the cell suspension to a 1 mL syringe with a 27 gauge needle. Inject 0.2 mL of the cell suspension (5×10^6 cells/per animal) subcutaneously into the inguinal area of the mouse (*see Notes 15–17*).
9. To establish MCF7 human breast cancer xenografts, each of the female nude mice is first subcutaneously implanted with slow release estrogen pellet (SE-121, 17- β estradiol/pellet; Innovative Research of America, Sarasota, FL).

10. Label each cage card with date and type of cell injected.
11. Observe animals for tumor growth and clinical signs every day. When tumors are easily visible, begin to measure tumor diameter with calipers and record tumor measurements (*see* Note 18).
12. Tumor mass and volume are calculated using the formula: $\text{Mass (mg)} = 0.5 (\text{short diameter}) (\text{long diameter})^2 \times 1000$. When tumors reach ~50–150 mg, initiate the treatment with test drug.
13. Divide animals randomly into treatment groups and controls (10–15 animals/group). Calculate the mean, SD, and SE in mass and volume for each group.
14. Label the cage card with the date, experiment, and treatment group numbers, drug, dose, and frequency.
15. The test compound is administered with i.p., i.v., or p.o. The injection volume is based on body weight (5 $\mu\text{L/g}$ of body weight) and the concentrations of the drug should be adjusted on the basis of the doses.
16. Tumors should be measured at least twice a week. For rapidly growing tumors, it may be necessary to measure tumors every other day.
17. Monitor animal closely for distress, lethargy, tumor morphology (presence of ulceration) and body weight. In general, when tumor reaches 10 % of the body weight or when severe toxicity due to tumor growth or treatment toxicity develops, animals should be sacrificed.
18. Final measurements and tumor size calculations should be performed prior to sacrificing animals and removing tumors.
19. At the end of the experiment, confirm that final measurements and calculations have been performed for each animal.

3.8 Orthotopic Xenograft Tumor Models

1. Establish orthotopic models using procedures described above. Only different is transfer the luciferase tagged cell suspension to a 1 mL syringe with a 30 gauge needle. Inject 30 μL of the cell suspension (5×10^6 cells/per animal) into mammary fat pad (*see* Notes 19 and 20).
2. Immediately after injection and every 3 days thereafter, monitor bioluminescence using a Caliper IVIS imaging instrument.
3. Inject Luciferin i.p. at 150 mg/kg. Wait 5–10 min after injection and then place each mouse into the anesthesia chamber, and anesthetize with isoflurane inhalant. Once the mouse is fully anesthetized (approximately 5–10 min), transfer it to the imaging chamber and keep under mild anesthesia with oxygen mixture through a nose cone until image is acquired, and then analyze optical images using the IVIS Living Image software package.
4. Begin treatment of mice with drug when all tumors have reached an average bioluminescence intensity of $\sim 1 \times 10^7$ (*see* Note 21).

3.9 In Vivo PK Study

3.9.1 Animals

1. Establish xenograft or orthotopic models using procedures described earlier.
2. Use normal model, e.g., CD-1 mice (no tumors).
3. Animals with tumor size of ~500–1000 mg are randomly divided into treatment groups for various time points (3–5 animals/group). Calculate the mean, SD, and SE of tumor mass and body weight for each group.
4. Label the cage card with the date, experiment, and treatment group numbers, and the drug, dose, and time point. Typically, the following time points are included: 5, 15, 30, and 60 min, and 2, 4, 6, 8, 24, 48, and 72 h (*see Note 22*).
5. Each animal should be identified by an ear tag.

3.9.2 Dosing for Pharmacokinetic Study

1. Measure body weight immediately prior to dosing.
2. Drug maybe given ip, i.v (via a tail vein), or p.o. (oral gavage) at one or more dose levels, e.g., 5, 10, and 20 mg/kg of body weight (*see Notes 23 and 24*).
3. The injection volume should be based on body weight (usually 5 μ L/g of body weight). The concentrations of the drug should be adjusted on the basis of the doses.
4. Each dosed animal should be placed in a glass metabolic cage and given commercial diet and water ad libitum.

3.9.3 Urine and Fecal Sample Collection

1. Urine samples should be collected using a metabolic cage at various intervals, e.g., 0–6, 6–12, 12–24, and 24–48 h. The urine volume should be measured and recorded.
2. Fecal samples should be collected using a metabolic cage at various intervals e.g., 0–12, 12–24, and 24–48 h. The weight of feces should be measured and recorded.
3. At the end of each interval, the inner wall of the metabolic cage should be rinsed twice with 50 mL of PBS. The cage wash should be collected, measured, and placed in properly labeled containers.
4. All samples and rinse water should be kept at -20°C until analysis.

3.9.4 Blood and Tissue Sample Collection

1. For each animal, check the general conditions. Weigh animal and record body weight.

2. Record time of treatment. At the designated time interval post dosing, draw blood from retro-orbital plexus and collect on ice in a heparin tube. Centrifuge at 14,000 rpm for 10 min. Transfer supernatant to a clean Eppendorf tube.
3. Collect various tissues including tumor tissue at necropsy and immediately trim of extraneous fat and connective tissue.
4. Blot on filter paper.
5. Weigh and store in labeled plastic tubes in -80°C freezer until homogenization.

3.10 PK Data Analysis

1. Plasma, urine, and tissue concentrations should be determined on the basis of quantitation of the drug via appropriate established and validated analytical method. The drug concentration is usually expressed as ng/mL for plasma and ng/g for various tissues.
2. Pharmacokinetic parameters should be estimated, including the maximal concentration (C_{max}), the time at C_{max} (T_{max}), half-life ($t_{1/2}$), the area under the curve (AUC), volume of distribution (V_d), and mean residue time (MRT) using an appropriate modeling software such as Phoenix WinNonlin (Certara, NJ).

3.11 PD Data Analysis

1. Various assay methods such as real-time reverse transcription-PCR (RT-PCR, to measure changes in the target gene/protein expression at mRNA level), Western blotting (to measure activation or inhibition of target proteins, cellular markers of processes such as metastases, inflammation), flow cytometry (to measure changes in DNA content, stem cell markers, etc.) are used to measure these outcomes [26–31].
2. A chosen end point (i.e., a biomarker or the target gene/protein is measured at different time points). Time intervals are chosen according to the aim and objective of the experiment. Therapeutic efficacy is determined by the ratio of % T/C (mean tumor mass of treated group divided by that of control).
3. The biomarkers may involve markers obtained from easily sampled fluids such as blood/urine and include enzymes (e.g., SOD, catalase, LDH, ALT, AST), blood count variables (e.g., erythrocytes, leukocytes, platelet count), hormones (e.g., estrogen) that do not require sacrifice of the animal.
4. Alternatively, the animals can be sacrificed at desired time points, tumors excised and subjected to RT-PCR or Western blotting or immunohistochemical staining (IHC) to identify effect of the drug on the target gene/protein at transcriptional and translational levels.
5. The most common PD parameters are the maximum effect (E_{max}) that can be reached and the half maximal effective concentration (EC_{50}).

$$E = E_{\max} \frac{C}{EC_{50} + C}$$

E = effort; C = concentration; $E_{\max}$ = max response; EC_{50} = concentration capable of producing 50 % of the maximum effect.

4 Notes

1. For drug uptake and cellular transport studies, ensure that drug concentrations used are lesser than those required for cytotoxic activities.
2. A reliable and sensitive analytical method must be established and validated as per regulatory guidelines—USFDA, EMEA, etc.
3. It is very important to establish a bioanalytical method where the limit of quantitation is lesser than the expected concentration of the test compound in the biological matrix. Otherwise, low concentrations in the biomatrix will not be detected and this will result in incorrect AUC determination.
4. Avoid using ultra low volumes of the biological matrix, wherever possible, (less than 5 μ L) for sample preparation as there can be errors in volume due to personnel error.
5. Use freshly prepared samples for HPLC/LC-MS, especially in stability and metabolism determination experiments.
6. If a particular metabolite (endogenous or exogenous) is to be determined, ensure appropriate sampling procedure is carried out. Variations in collection and storage of samples can lead to variability in analyte quantitation.
7. The protein binding of the drug must be determined since extensive binding to plasma proteins can impede its in vivo distribution/disposition and affect its therapeutic performance.
8. If any test compound is dissolved in a solvent other than DMSO, a negative control with an equivalent organic solvent percentage must be included in the study design for fair comparison.
9. In order to prevent hepatocytes from drying out in, each treatment group should be aspirated separately and replaced with the medium before dosing the next set.
10. If performing protein determinations to normalize data across wells, keep in mind that the protein is not solely from hepatocytes, but also includes protein in the extracellular matrix used to overlay the hepatocyte monolayers.
11. A selective subpopulation of a parental cancer cell line with migration/colonization affinity towards specific organs can be used to mimic far site metastases. For example, the MDA-MB-231BR (brain seeking) cell lines, upon injection into the bloodstream of immunocompromised mice (via intracardiac

route), travel into and colonize brain tissue; thus mimicking brain metastases [13].

12. Ensure that the cells are passaged the day before the injection and are no more than 80 % confluent on the day of the experiment.
13. Ensure cells are viable once harvested (use a trypan blue test for counting viable cells).
14. Always gently but thoroughly mix the cells before loading the syringe. Ensure there are no cell clumps in the solution that is being injected.
15. The mice used for developing the xenograft models should not be older than 6–8 weeks.
16. The use of a needle usually results in a strong, negative pressure, which can cause cell damage and death. Therefore, while withdrawing cell solution to the syringe, the needle should be removed to prevent negative pressure stress on the cells.
17. An alternative area for subcutaneous injection of the cancer cells is the upper region of the back near the neck where there is a lot of fat. This is a vascularized area for good tumor growth and the mice cannot reach the tumor as it grows.
18. The rate of tumor growth is dependent on individual cell lines and the amount of cells injected into each mouse. The optimum number of cells to be injected is determined by three variables: (a) type of tumor cell (slow growing vs. metastatic, with or without additional cells such as stromal cells); (b) location of injection (s.c., vs. orthotopic); (c) type of mouse (athymic nude vs. NOD/SCID). It is recommended using over 10^6 cells at tumor site if not sure of tumor growth activity.
19. Recently patient derived (PDX) models have been introduced that direct implantation of breast tumor tissues derived from patients into mouse mammary fat pads of immunocompromised mice and closely resemble the original tumors in the patient maintaining original histopathology as well as biomarkers, hormone dependence or independence, gene expression, etc. [32].
20. In order to achieve, successful injection of tumor cells orthotopically into organ site, ensure minimum leaking at the site after injection.
21. Bioluminescence intensity also varies from tumor to tumor. The mean photons emitted over time by a specific tumor are highly comparable with the corresponding tumor volumes. It is estimated that a bioluminescence signal at 1×10^7 is roughly equal to a tumor volume of 50–150 mm³, which is the starting point to initiate drug therapy.
22. Ensure that appropriate sampling procedures and times are chosen. For example, in studies where in vivo plasma (or tissue) drug concentrations are to be measured, ensure that sampling time is chosen according to route of

administration. Include earlier sampling points (e.g., 3, 5, and 15 min post iv dosing).

23. While doing PK/PD experiments, care should be taken to ensure uniform dispersion of drug in carrier vehicle. Improper drug distribution in vehicle will lead to improper dosing.
24. Ensure that the carrier vehicle used for drug administration is itself, nontoxic, and does not affect tumor growth. Use of a sufficiently large control group (negative control) is the solution.

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Table 1

Assay conditions for the determination of Cytochrome P450 enzyme activities in human hepatocytes

Enzyme	Prototypical inducer	Substrate	Cone. (μM)	Incubation (min)	Marker metabolite
CYP1A2	3-methylcholanthrene	Phenacetin	100	15	Acetaminophen
CYP2B6	Phenobarbital	Bupropion	500	20	Hydroxybupropion
CYP3A4	Rifampicin	Testosterone	200	15	6 β -hydroxytestosterone