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Implications of Plasma Protein Binding for Pharmacokinetics and Pharmacodynamics of the γ -Secretase Inhibitor RO4929097

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Abstract

Purpose—Understanding of plasma protein binding will provide mechanistic insights into drug interactions or unusual pharmacokinetic properties. This study investigated RO4929097 binding in plasma and its implications for the pharmacokinetics and pharmacodynamics of this compound.

Experimental Design—RO4929097 binding to plasma proteins was determined using a validated equilibrium dialysis method. Pharmacokinetics of total and unbound RO4929097 was evaluated in eight patients with breast cancer receiving RO4929097 alone and in combination with the Hedgehog inhibitor GDC-0449. The impact of protein binding on RO4929097 pharmacodynamics was assessed using an *in vitro* Notch cellular assay.

Results—RO4929097 was extensively bound in human plasma, with the total binding constant of 1.0×10^6 and 1.8×10^4 L/mol for α 1-acid glycoprotein (AAG) and albumin, respectively. GDC-0449 competitively inhibited RO4929097 binding to AAG. In patients, RO4929097 fraction unbound (Fu) exhibited large intra- and interindividual variability; GDC-0449 increased RO4929097 Fu by an average of 3.7-fold. Concomitant GDC-0449 significantly decreased total (but not unbound) RO4929097 exposure. RO4929097 Fu was strongly correlated with the total drug exposure. Binding to AAG abrogated RO4929097 *in vitro* Notch-inhibitory activity.

Conclusions—RO4929097 is highly bound in human plasma with high affinity to AAG. Changes in plasma protein binding caused by concomitant drug (e.g., GDC-0449) or disease states (e.g., $\uparrow$ AAG level in cancer) can alter total (but not unbound) RO4929097 exposure. Unbound RO4929097 is pharmacologically active. Monitoring of unbound RO4929097 plasma concentration is recommended to avoid misleading conclusions on the basis of the total drug levels.

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Introduction

The Notch signaling pathway is a critical component in the molecular circuits that control cell fate during development. Aberrant activation of this pathway contributes to tumor initiation and progression through promoting 3 tumor survival processes: tumor cell transformation, cancer stem cell survival, and tumor angiogenesis (1–3). Inhibition of the Notch signaling pathway is an area of intense research in oncology. The basic molecular players in this pathway are 5 ligands [named Jagged-1 and -2, and Delta-like (Dll)-1, -3 and -4], 4 Notch receptors (named Notch-1, -2, -3, and -4), and transcriptional factors (4). Notch signaling is initiated by binding of the Notch ligand to its receptor, resulting in release of the intracellular domain of the Notch receptor (Notch-1C) through a cascade of proteolytic cleavages, the last of which is mediated by γ -secretase (5). The released intracellular Notch-1C, a functionally active form of Notch, translocates into the nucleus where it forms a transcription-activating complex thereby activating transcription of target genes (1, 5). Blocking Notch signaling via inhibition of γ -secretase is an attractive strategy to target tumor cells, tumor stem cells, and tumor endothelial cells. Several small-molecule γ -secretase inhibitors are currently being evaluated in phase I/II studies, including RO4929097 (Roche), MK-0752 (Merck), and PF03084014 (Pfizer).

RO4929097 inhibits Notch signaling and produces a less transformed, flattened, slower growing phenotype in a variety of cancer cell lines. *In vivo* studies indicated good antitumor activity in xenograft mouse models of colon, pancreatic, and non-small cell lung cancer, and notably the antitumor effect was seen with both continuous and intermittent dosing and the effect persisted after cessation of treatment (6). Currently, more than 30 phase I/II clinical trials are underway with RO4929097 either as monotherapy or in combination with other cytotoxic or targeted agents in multiple solid tumors, myeloma, lymphoma, melanoma, leukemia, and pediatric central nervous system tumors (<http://www.cancer.gov/drugdictionary/?Cdrid=662240>). Recent data from a phase I trial involving 89 patients suggested that RO4929097 was well tolerated and it showed encouraging signs of antitumor activity and prolonged stable disease in patients with melanoma, neuroendocrine, sarcoma, and ovarian cancers (7).

RO4929097 is currently being evaluated as the combination with the Hedgehog inhibitor GDC-0449 (vismodegib) in patients with metastatic breast cancer in a phase I clinical trial at the Karmanos Cancer Institute (Detroit, MI; NCI study #8420). The primary objective of this phase I study was to determine the safety and maximum tolerated dose of RO4929097 administered orally (starting dose at 20 mg/d) on a schedule of 3-day on/4-day off every 3 weeks in combination with continuous daily oral administration of GDC0449 (150 mg/d). Real-time pharmacokinetic analysis indicated that concomitant administration of GDC-0449 dramatically decreased the systemic (plasma) exposure to RO4929097. Elucidation of the mechanism underlying this pharmacokinetic interaction will aid in decision making on whether and how RO4929097 dosing should be adjusted in the combination with GDC-0449 for further clinical study.

It is well known that changes in plasma protein binding due to drug displacement interactions, disease effects, genetic factors, or formulation factors could affect drug pharmacokinetics (8), including anticancer drugs such as paclitaxel, imatinib, and UCN-01 (9–11). Nevertheless, changes in plasma protein binding may or may not be clinically relevant. This depends on whether unbound drug exposure is changed with alterations of protein binding, given the notion that only the unbound drug fraction is the pharmacologically active form (8). Determination of drug binding in plasma and factors affecting this process will provide important mechanistic insights into drug displacement interactions or unusual pharmacokinetic characteristics. Understanding of implications of

plasma protein binding for RO4929097 pharmacokinetics and pharmacodynamics will be useful in guiding dose selection or dosing adjustment for further clinical studies. The objectives of this study were to determine RO4929097 binding to plasma proteins and to investigate the impact of plasma protein binding on the pharmacokinetics and pharmacologic activity of this novel anticancer agent.

Materials and Methods

Chemicals and reagents

RO4929097 was provided by the Cancer Therapy Evaluation Program (CTEP), National Cancer Institute (Bethesda, MD). GDC-0449 for the clinical study was provided by Genentech, Inc., and the reference standard GDC-0449 (purity > 99%) used for the *in vitro* protein binding experiments was obtained from LC Laboratories. Human serum albumin (HSA; catalog #A9511; purity 97%–99%) and human α 1-acid glycoprotein (AAG, catalog #G9885; purity 99%) were obtained from Sigma-Aldrich. Human, rat, mouse, beagle plasma, and human blood were obtained from Innovative Research Inc. pGL3-Basic vector, pRL-SV40 expression construct, and the Dual-Luciferase Reporter Assay System were obtained from Promega. Lipofectamine 2000 was obtained from Invitrogen. Dulbecco's Modified Eagle's Medium (DMEM) and FBS were obtained from Atlantic Biologicals.

Optimization and validation of equilibrium dialysis method

RO4929097 fraction unbound in plasma or in isolated protein solution was determined by an equilibrium dialysis method as described previously (12). Briefly, equilibrium dialysis was conducted on a 96-well Equilibrium DIALYZER with a 5-kDa cut-off regenerated cellulose membrane (Harvard Apparatus) on a rotator (Harvard Apparatus) at 37° C. Experiments were carried out with 200 μ L of plasma containing a wide concentration range of RO4929097 (20–10,000 ng/mL or 43–21,304 nmol/L) against an equal volume of PBS (pH = 7.2). At equilibrium, a 150- μ L aliquot of each compartment was collected and stored at –80° C until analysis. The total concentration of RO4929097 in the plasma compartment (C_p) and the unbound drug concentration in the PBS compartment (C_u) were determined using a validated liquid chromatography/tandem mass spectrometry (LC/MS-MS) method (13). The fraction unbound (F_u) was calculated as: $F_u = C_u/C_p$.

The optimal time to equilibrium was determined by assessing the F_u of RO4929097 in human plasma at the concentrations of 20, 400, and 8,000 ng/mL (43, 852, and 17,043 nmol/L) at the equilibrium time of 2, 4, 6, 24, and 48 hours. The equilibrium dialysis method was validated in pooled healthy human plasma with quality control (QC) samples containing 20, 400, and 8,000 ng/mL of RO4929097. The intra- and interday precisions were assessed by determination of RO4929097 F_u in the QCs on 3 days (each QC in triplicate), using one-way ANOVA as described previously (14). The recovery was assessed as the percentage of the sum of RO4929097 concentrations in the postdialysis plasma and buffer compartments to the original concentration in the predialysis plasma compartment.

In vitro binding experiments

To determine RO4929097 binding in different species plasma (i.e., human, dog, rat, and mouse plasma), RO4929097 F_u was determined in plasma at varying drug concentrations (20–10,000 ng/mL or 43–21,304 nmol/L), using the validated equilibrium dialysis method with the optimal equilibrium time at 24 hour. To determine the binding affinity and binding capacity of 2 major human plasma proteins albumin and AAG for RO4929097, the F_u was determined in isolated protein solution (HSA or AAG in PBS, pH 7.4) with a fixed protein concentration (i.e., 40 mg/mL of HSA or 1.4 mg/mL of AAG) at varying total drug concentrations; binding to HSA or AAG was also assessed with varying protein

concentrations (i.e., HSA, 5–50 mg/mL; AAG, 0.2–3.2 mg/mL) at the clinically relevant total drug concentration of 500 ng/mL (1,065 nmol/L). To examine the effect of GDC-0449 on RO4929097 binding to plasma proteins, RO4929097 *Fu* was determined in human plasma or in isolated protein solution (i.e., 40 mg/mL of HSA or 1.4 mg/mL of AAG) in the absence or presence of clinically relevant concentrations (5, 25, and 125 μ mol/L) of GDC-0449. In addition, the effect of RO4929097 on GDC-0449 binding to AAG was assessed by determining GDC-0449 *Fu* in the isolated AAG solution (1.4 mg/mL) in the absence or presence of clinically relevant concentrations of RO4929097 (213, 1,065, and 4,260 nmol/L).

The partitioning of RO4929097 to blood cells was examined according to the following procedure. One-half (0.5) milliliter of fresh human blood samples was spiked with RO4929097 over a concentration range of 20 to 10,000 ng/mL and equilibrated at 37°C with gentle orbital shaking for 1 hour. At equilibrium, a 100- μ L aliquot of the whole blood was taken for the determination of RO4929097 concentration in whole blood (C_b); the remaining blood sample was subjected to centrifugation ($2,000 \times g$, 37°C, 10 minutes) to separate plasma for determination of the drug concentration in plasma (C_p). Before the extraction procedure, a 100- μ L blood sample was bleached by adding 500 μ L of sodium hypochloride, vortex-mixing for 10 seconds, and standing at room temperature for 10 minutes. The plasma and bleached blood samples were extracted with ethyl acetate and RO4929097 concentrations were determined using a validated LC/MS-MS method, as described previously (13).

Clinical pharmacokinetic studies

To detect potential drug interaction, plasma pharmacokinetics of RO4929097 and GDC-0449, each given alone and in the combination, was evaluated in the context of a phase I clinical trial (NCI study #8420) in patients with advanced breast cancer. The protocol was approved by the Institutional Review Board (IRB) of the Karmanos Cancer Institute at Wayne State University (IRB # 085709H1F). All the patients provided written informed consent.

RO4929097 was administered orally once (20 mg) as single agent on day 1 to allow for single-agent pharmacokinetic sampling. GDC-0449 was administered orally daily (150 mg/d) as single agent beginning on day 8 and was given on a continuous daily schedule as monotherapy for 2 weeks. Starting day 22 (cycle 2, day 1), RO4929097 was administered 20 mg/d on days 1 to 3, 8 to 10 every 21 days (i.e., 3-day on/4-day off-schedule), and GDC-0449 was administered 150 mg/d. To characterize the single-agent pharmacokinetics of RO4929097, blood samples were collected in heparinized tubes at predose (within 10 minutes before the dosing) and 0.5, 1, 1.5, 2, 3, 4, 8, 24, and 48 hours after oral administration of the first dose RO4929097 (20 mg) on cycle 1, day 1. To characterize the single-agent pharmacokinetics of GDC-0449, blood samples were collected at predose and 0.5, 1, 1.5, 2, 3, 4, 8, and 24 hours after oral administration of GDC-0449 (150 mg) on cycle 1, day 21. To characterize the pharmacokinetics of RO4929097 and GDC-0449 in the combination, blood samples were collected at predose and 0.5, 1, 1.5, 2, 3, 4, 8, and 24 hours after co-administration of RO4929097 (20 mg) and GDC-0449 (150 mg) on cycle 2, day 9. The dosing schedule and pharmacokinetic sampling scheme are illustrated in Supplementary Fig. S1.

Within 1 hour of sample collection, the blood sample was centrifuged at 4°C, at 3,000 rpm for 10 minutes, and plasma was collected and stored at –80°C until analysis. The total plasma concentrations of RO4929097 were determined using a validated LC/MS-MS method in the Karmanos Cancer Institute Pharmacology Core (13). The fraction unbound and unbound plasma concentrations of RO4929097 were determined using the validated

equilibrium dialysis method as described above. The total plasma concentrations of GDC-0449 were determined using a validated LC/MS-MS method in the Genentech Tandem Laboratory, as described previously (15).

***In vitro* Notch cellular assay**

To show the impact of plasma protein binding on RO4929097 pharmacodynamics, the *in vitro* Notch-inhibitory activity of RO4929097 was determined in the absence and presence of physiologic/pathologic concentrations of AAG (0.5, 1.4, and 3.2 mg/mL), using a NOTCH1-HES1 reporter gene assay as described previously (16). A constitutively active NOTCH1 mutant [DEL 1676 (V)] in pcDNA3 and a reporter construct with an artificial luciferase reporter gene under the control of an HES1 promoter containing the CSL/ICN1-binding site (HES1-Luc) in pGL3-Basic vector were prepared as described previously (16). ICN-pcDNA3 (active form of Notch1 served as the positive transfection control) was a gift of Dr. Lucio Miele (Loyola University, Chicago, IL). Human U2OS osteosarcoma cells (American Type Culture Collection) were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C under 5% CO₂. Empty pcDNA3 vector, mutant NOTCH1-pcDNA3, or ICN1-pcDNA3 construct was transiently transfected into U2OS cells with HES1-Luc (a downstream Notch1 target) and pRL-SV40 (encoding *Renilla* luciferase activity as the internal standard), using Lipofectamine 2000 as described previously (16). At 24 hours after transfection, the cells were treated with RO4929097 at concentration of 50, 500, or 5,000 nmol/L in the absence or presence of AAG (0.5, 1.4, or 3.2 mg/mL) in serum-free medium. After 24 hours of treatment, the cells were lysed and luciferase activities were assayed using Dual Luciferase Reporter Assay Kit (Promega) on TD-20/20 Luminometer (Turner Designs). The luciferase activity in each cell lysate sample was normalized to *Renilla* luciferase activity (encoded by pRL-SV40). The Notch-inhibitory activity of RO4929097 was expressed as the percentage of normalized luciferase activity in the treated cells relative to that in the untreated cells. Two independent experiments (with duplicate in each experiment) were carried out.

Data analyses

Estimation of binding parameters—The binding parameters for RO4929097 interaction with isolated plasma protein HSA or AAG were estimated by fitting the observed data to Equations (A–C), as described below. The total binding constant (nK) for HSA or AAG was estimated by fitting the observed F_u against protein concentrations to Equation (A) using nonlinear regression analysis with S-PLUS software (version 7.0; TIBCO Insightful Corp.). The binding parameters were also estimated by fitting modified Scatchard plots, which were constructed using the observed unbound drug concentration (C_u) against the bound drug concentration (C_{bd}), to either Equation (B) using linear regression (if the binding is nonsaturable) or Equation (C) using nonlinear regression (if the binding is saturable; ref. 12).

$$F_u = \frac{1}{1 + nK P} \quad (A)$$

$$C_{bd} = nK P C_u \quad (B)$$

$$C_{bd} = \frac{n P C_u}{K_d + C_u} \quad (C)$$

where P , C_u , and C_{bd} are the molar concentrations of protein (HSA or AAG), unbound drug, and bound drug, respectively; n is the number of binding site per molecular of protein; nK

(product of number of binding sites by affinity constant) denotes the total binding constant; and K_d is the dissociation constant, which is the reciprocal of the association constant (K_a).

To illustrate the effects of varying AAG or HSA concentrations on RO4929097 binding in plasma, modified Scatchard plots of the observed C_u versus C_{bd} were simulated in plasma with varying clinically relevant AAG concentrations (i.e., 0.2, 1.4, and 3.2 mg/mL) and a fixed HSA concentration (40 mg/mL) or with varying clinically relevant HSA concentrations (i.e., 20, 40, and 50 mg/mL) and a fixed AAG concentration (1.4 mg/mL), using a 2-binding site model [Equation (D)] assuming the drug binds to AAG with a saturable binding kinetics and to HSA with a linear (nonsaturable) binding kinetics.

$$C_{bd} = \frac{n_{AAG} P_{AAG} C_u}{K_d + C_u} + (nK)_{HSA} P_{HSA} C_u \quad (D)$$

where the K_d and n_{AAG} are AAG binding parameters obtained from Equation (C); $(nK)_{HSA}$ is the total binding constant of HSA obtained from Equation (B); and P_{AAG} and P_{HSA} are the molar concentrations of AAG and HSA, respectively.

The extent of blood cell partitioning is assessed by the ratio of drug concentration in blood cells (C_{bc}) to unbound drug concentration in plasma (C_u), which is expressed as ρ (17). The fractional amount of drug unbound (A_u), bound to plasma proteins (A_{bd}), and bound to blood cells (A_{bc}), relative to the total amount of drug in whole blood (A_b) were estimated using the equations as described previously (12).

Pharmacokinetic data analysis

Pharmacokinetic parameters of RO4929097 and GDC-0449 for individual patients were estimated using noncompartmental analysis with the computer software program WinNonlin version 5.2 (Pharsight Corporation). The maximum plasma concentration (C_{max}) and the time achieving the maximum concentration (T_{max}) were obtained by visual inspection of the plasma concentration–time curves. The area under the plasma concentration–time curve from 0 to 24 hours (AUC_{0-24h}) was calculated using the linear and logarithmic trapezoidal method for ascending and descending plasma concentrations, respectively. The total area under the plasma concentration–time curve from time zero to infinity ($AUC_{0-\infty}$) was calculated as the sum of AUC_{0-t} and the extrapolated area, which was calculated by the last observed plasma concentration divided by the terminal rate constant (λ_z), where λ_z was estimated by terminal log-linear portion of the plasma concentration–time curve using $1/X^2$ weighted linear regression. Terminal plasma half-life ($t_{1/2,z}$) was calculated as $0.693/\lambda_z$. Apparent oral clearance (CL/F) was calculated as dose/ $AUC_{0-\infty}$.

Statistical analysis

One-way ANOVA with Tukey honestly significant difference (HSD) test as *post hoc* multiple comparison was used for comparing RO4929097 fraction unbound in plasma from different species or comparing RO4929097 *in vitro* Notch-inhibitory activity in the absence or presence of varying concentrations of AAG. Interindividual variability in RO4929097 pharmacokinetic parameters (i.e., C_{max} , AUC_{0-24h} , and CL/F) was described as fold difference (determined from the ratio of the maximum and minimum value of each parameter). The Wilcoxon signed-rank test was used for comparing RO4929097 fraction unbound or pharmacokinetic parameters when RO4929097 was given alone and in combination with GDC-0449. The Spearman rank correlation test was used for assessment of association between RO4929097 fraction unbound and pharmacokinetic parameter (C_{max} , AUC_{0-24h} , or CL/F). Statistical analyses were conducted with SPSS (Version 10.0, SPSS

Inc.). All P values were based on 2-sided statistical tests, and $P < 0.05$ was considered as statistical significance unless specified otherwise.

Results

RO4929097 binding in plasma and blood

A semi-high-throughput equilibrium dialysis method was optimized and validated for determination of RO4929097 fraction unbound in plasma. The optimal equilibrium time was established at 24 hours, at which the dialysis reached equilibrium (Supplementary Fig. S2). The assay was accurate and reproducible, with the intra- and interday precisions of less than 15% and average recovery of 97% from assessment of QC samples in triplicate on 3 separate days.

RO4929097 was extensively bound in plasma, with some differences across species. Specifically, the binding was significantly lower in rat plasma (mean F_u , 9.7%; $P < 0.05$) than in human (2.3%), mouse (1.2%), and dog (1.8%) plasma (Supplementary Table S1). The binding was drug concentration independent at the clinically relevant drug concentrations (20–2,000 ng/mL or 43–4,260 nmol/L) while appearing nonlinear (saturable) at higher drug concentrations (Supplementary Table S1). Given the species difference in RO4929097 plasma protein binding, consideration of protein binding is essential in the scale-up of RO4929097 pharmacokinetic and pharmacodynamic parameters from animal models, in particular from the rat model, to humans.

Along with binding to plasma proteins, RO4929097 was bound to blood cells in a drug concentration-independent manner over RO4929097 blood concentrations of 20 to 10,000 ng/mL (43–21,304 nmol/L). By simultaneous determinations of plasma and blood concentrations of RO4929097, the ratio of blood-to-plasma concentration (C_b/C_p) was determined as 0.73 ± 0.06 ($n = 15$), suggesting that the drug mainly binds to plasma proteins and to a lesser degree to blood cells. The affinity of RO4929097 to blood cells (i.e., ρ) was estimated as 14.1, assuming the mean blood hematocrit (H) of 0.4, mean F_u of 2.3% in human plasma, and mean C_b/C_p ratio of 0.73. Taken together, given RO4929097 binding to both plasma proteins (F_u , 2.3%) and blood cells (ρ , 14.1), it was estimated that in whole blood, only 2.0% of RO4929097 existed as the free drug, whereas 80.0% and 18.0% was bound to plasma proteins and blood cells, respectively.

RO4929097 binding to HSA and AAG

In the isolated protein solution at the clinically relevant total drug concentration of 500 ng/mL (1,065 nmol/L), RO4929097 binding to HSA or AAG was protein concentration-dependent. The mean F_u was decreased from 33.3% to 12.6% as the HSA concentration increased from 5 to 50 mg/mL, whereas it was decreased from 19.0% to 1.1% as the AAG concentration increased from 0.2 to 3.2 mg/mL (Fig. 1A and B). By fitting the F_u versus protein concentration data to Equation (A) using nonlinear regression analysis, the total binding constant (nK) was estimated as 1.8×10^4 L/mol and 1.0×10^6 L/mol to HSA and AAG, respectively. In the isolated protein solution with a fixed protein concentration (i.e., 40 mg/mL of HSA or 1.4 mg/mL of AAG), RO4929097 binding to HSA was linear (nonsaturable) whereas binding to AAG was nonlinear (drug concentration-dependent) as the total drug concentration increased from 20 to 10,000 ng/mL (43–21,304 nmol/L; Fig. 1C and D). By fitting the observed unbound versus bound drug concentrations in 40 mg/mL of HSA solution to Equation (B) using linear regression analysis, the nK for HSA was estimated as 1.1×10^4 L/mol, which was in agreement with the nK value (1.8×10^4 L/mol) estimated from Equation (A). By fitting the observed unbound versus bound drug concentrations in 1.4 mg/mL of AAG solution to a saturable binding model [Equation (C)],

it was estimated that the maximum binding concentration (nP) of AAG was 23.1 $\mu\text{mol/L}$, the number of binding site per molecule of AAG (n) was 0.73, and the dissociation constant (K_d) of the AAG–RO4929097 interaction was 9.0×10^{-7} mol/L, corresponding to an association constant K_a of 1.1×10^6 L/mol [which was in agreement with the nK of 1.0×10^6 L/mol estimated from Equation (A)]. Collectively, these data indicate that RO4929097 binds to AAG with a significantly higher affinity (>50-fold) than to HSA.

The influence of varying AAG or HSA concentrations on RO4929097 binding in plasma was illustrated by simulations of modified Scatchard plots and unbound fractions of RO4929097 in human plasma containing different HSA or AAG concentrations (Fig. 1E and F) using a 2-binding site model [Equation (D)]. At a fixed HSA concentration of 40 mg/mL, RO4929097 unbound fraction in plasma ranged from approximately 2% to 10% (varied 5-fold) as the AAG concentration decreased from 3.2 to 0.2 mg/mL (Fig. 1F). On the other hand, variation of HSA concentrations showed an insignificant influence on RO4929097 unbound fraction (Fig. 1E). These data suggest that AAG was the main factor attributable to the variation of RO4929097 unbound fraction in patient plasma.

Effect of GDC-0449 on RO4929097 binding in plasma, *in vitro*, and in cancer patients

The effect of GDC-0449 on RO4929097 binding in human plasma and in the isolated AAG or HSA solution was shown in Table 1. In the presence of clinically relevant concentrations of GDC-0449 (5, 25, and 125 $\mu\text{mol/L}$), RO4929097 fraction unbound in human plasma was increased 1.5-, 2.1-, and 2.7-fold, respectively, compared with that in the absence of GDC-0449. GDC-0449 increased RO4929097 fraction unbound to the most significant extent in AAG solution while to a negligible extent in HSA solution (Table 1). The effect of GDC-0449 on RO4929097 binding to AAG was further examined by comparison of the binding affinity (assessed by K_d) and binding capacity (assessed by nP) of RO4929097 for AAG in the absence or presence of GDC-0449 in isolated AAG solution (1.4 mg/mL). By fitting modified Scatchard plots of the observed unbound versus bound concentrations of RO4929097 in the presence of 0, 5, 25, and 125 $\mu\text{mol/L}$ of GDC-0449 to Equation (C), it was estimated that the K_d (expressed as estimate $\pm$ SE) was 0.9 ± 0.1 , 1.3 ± 0.1 , 8.7 ± 7.7 , and 8.2 ± 2.2 $\mu\text{mol/L}$, respectively, and the nP (expressed as estimate $\pm$ SE) was 23.1 ± 0.9 , 25.3 ± 1.1 , 34.9 ± 2.0 , and 25.2 ± 2.9 $\mu\text{mol/L}$, respectively (Fig. 2). These data suggested that in the presence of GDC-0449, RO4929097-binding affinity for AAG was decreased whereas the binding capacity remained unchanged, indicating that GDC-0449 replaced RO4929097 bound to AAG by competitive binding. On the contrary, the presence of clinically relevant concentrations of RO4929097 (213–4,260 nmol/L) did not influence GDC-0449 binding to AAG (Supplementary Fig. S3).

In accordance with *in vitro* binding data, the increase of RO4929097 fraction unbound in plasma by concomitant GDC-0449 was observed in patients with cancer. RO4929097 fraction unbound exhibited a large intraindividual and interindividual variability (Fig. 3E and F), with individual mean values ranging from 0.2% to 2.0% and from 1.7% to 6.7% when RO4929097 was given alone and in combination with GDC-0449, respectively (Table 1). Co-administration of GDC-0449 increased RO4929097 fraction unbound by an average of 3.7-fold (ranging from 1.7- to 8.5-fold among individual patients; $P = 0.016$; Table 1).

Pharmacokinetics of RO4929097 and GDC-0449, given alone and in the combination

Figure 3 shows the observed total and unbound RO4929097 plasma concentration–time profiles in individual patients following oral administration of RO4929097 20 mg alone (cycle 1) and in combination with GDC-0449 150 mg orally (cycle 2). The pharmacokinetic parameters of RO4929097 and GDC-0449, given alone and in the combination, are summarized in Table 2.

The interindividual variability in the systemic exposure to total RO4929097 was extensive. Following a single oral dose of 20 mg, the $C_{\max}$ and AUC_{0-24h} of total RO4929097 varied 15-fold (median, 498.1 nmol/L; range, 96.0–1,400.3 nmol/L) and 16-fold (median, 6,034.5 nmol/L h; range, 1,530.0–24,804.1 nmol/L h), respectively. Notably, the interindividual variability in the unbound drug exposure was reduced: the $C_{\max}$ and AUC_{0-24h} varied 3-fold (median, 4.3 nmol/L; range, 1.9–6.1 nmol/L) and 4-fold (median, 52.8 nmol/L h; range, 28.1–113.0 nmol/L h), respectively. As assessed by the coefficient variation of the AUC_{0-24h} following administration of a single oral dose of 20 mg in 8 patients, the interindividual variability in total and unbound RO4929097 exposure was 99% and 49%, respectively (Table 2), implicating that plasma protein binding may account for a substantial portion (~50%) of the unexplained interindividual variation in the systemic exposure to total RO4929097. The impact of plasma protein binding on total RO4929097 pharmacokinetics was further showed by a highly statistically significant correlation between RO4929097 fraction unbound and the $C_{\max}$ ($\rho=-0.965$, $P < 0.001$), AUC_{0-24h} ($\rho=-0.947$, $P < 0.001$), or CL/F ($\rho=0.793$, $P < 0.001$) of total RO4929097 (Fig. 4A–C). On the other hand, the correlations between RO4929097 fraction unbound and the unbound drug pharmacokinetics ($C_{\max}$, AUC_{0-24h} , or CL/F ; Fig. 4D–F) were much weaker and less significant.

Co-administration of GDC-0449 significantly decreased the systemic exposure ($C_{\max}$ and AUC_{0-24h}) to total RO4929097 (Table 2). Paired comparisons in individual patients suggested that the $C_{\max}$ and AUC_{0-24h} in the combination were 13% to 69% (median, 18%) and 9% to 42% (median, 14%) of those achieved following administration of RO4929097 alone, respectively. On the other hand, co-administration of GDC-0449 did not show a statistically significant impact on the systemic exposure to unbound RO4929097 (Table 2). Co-administration of RO4929097 did not influence the pharmacokinetics of GDC-0449 (Table 2).

Impact of plasma protein binding on RO4929097 *in vitro* Notch-inhibitory activity

The *in vitro* Notch-inhibitory activity of RO4929097 in the absence or presence of varying concentrations of AAG was evaluated in human U2OS cells transiently cotransfected with a constitutively active NOTCH1 construct and a HES1-Luc (a downstream Notch1 target) reporter construct. As shown in Fig. 5A, 24-hour treatment with 50, 500, and 5,000 nmol/L of RO4929097 inhibited 2.1%, 62%, and 73% of the cellular Notch activity, respectively; the presence of 0.5, 1.4, and 3.2 mg/mL of AAG significantly reduced RO4929097 Notch-inhibitory activity at total RO4929097 concentrations of 500 and 5,000 nmol/L; 0.5, 1.4, or 3.2 mg/mL of AAG alone did not show any Notch-inhibitory effect.

Because RO4929097 binding to AAG was linear as the total drug concentration ranged from 50 to 5,000 nmol/L (Fig. 1D), RO4929097 fraction unbound was estimated as 8%, 3%, and 1.4% in 0.5, 1.4, and 3.2 mg/mL of AAG solution, respectively, based on Equation (A) and given the nK of 1.0×10^6 L/mol. Thus, the unbound RO4929097 concentrations in AAG solutions at varying total drug concentrations (50, 500, and 5,000 nmol/L) were estimated, as shown in Fig. 5A. As shown in Fig. 5B, the observed RO4929097 Notch-inhibitory activities as a function of the estimated unbound drug concentrations were well fit by an inhibitory $E_{\max}$ model, where the unbound RO4929097 concentration for 50% inhibition of the maximum Notch activity (IC_{50}) was estimated as 109 ± 23 nmol/L (expressed as the estimate $\pm$ SE of estimation).

Discussion

RO4929097, a γ -secretase inhibitor capable of blocking Notch signaling, is currently under intense clinical investigation either as monotherapy or in combination with other cytotoxic or targeted agents for treating a broad spectrum of human cancers. Here, we described for

the first time the plasma pharmacokinetics of both total and unbound RO4929097 in patients with breast cancer receiving RO4929097 alone and in combination with the Hedgehog inhibitor GDC-0449. RO4929097 exhibited a large inter-individual pharmacokinetic variability (for example, the AUC_{0-24h} after a single oral dose of 20 mg varied up to 16-fold). Concomitant administration of GDC-0449 dramatically decreased systemic exposure to total RO4929097 while showing insignificant influence on the unbound drug exposure. We elucidated that the observed altered pharmacokinetic profiles of RO4929097 in the combination with GDC-0449 could, for a large part if not all, be explained by GDC-0449 displacement of RO4929097 bound to AAG.

RO4929097 was extensively (>97%) bound in human plasma, with a high binding affinity for AAG ($nK\ 1.1 \times 10^6\ L/mol$) and a lower affinity for HSA ($nK\ 1.8 \times 10^4\ L/mol$). AAG is an acute-phase protein that is synthesized in the liver. Plasma concentrations of AAG are normally around 0.28 to 0.92 mg/mL, whereas it can vary considerably in pathologic or stress conditions such as chronic inflammation, myocardial infarction, and advanced cancer (18). It has been reported that AAG plasma concentrations in the cancer population vary between 0.45 and 2.85 mg/mL (mean, $1.12 \pm 0.51\ mg/mL$; ref. 19). Thus, variations in this protein could readily account for inter- or intraindividual variability in drug plasma protein binding. Simulations of RO4929097 binding (fraction unbound) in plasma with varying physiologic/pathologic relevant concentrations of AAG (0.2–3.2 mg/mL; Fig. 1E and F) clearly suggested that AAG was a significant determinant of RO4929097 fraction unbound in plasma. In this study, substantial interindividual (varying up to 10-fold) and intraindividual (varying up to 5-fold during the treatment in individual patient) variability in RO4929097 fraction unbound were observed in patients with cancer receiving RO4929097 orally alone or in combination with GDC-0449 (Fig. 3E and F). This variability could be explained, at least in part, by the variation of AAG levels in patients with cancer.

Binding to AAG has been identified as a significant covariate accounting for unusual pharmacokinetic profiles or large interindividual pharmacokinetic variability for several anticancer drugs including imatinib, UCN-01, and GDC-0449 (10, 11, 20). The expression of AAG or orosomucoid (ORM) is controlled by 2 genes, *ORM1* and *ORM2*, which are closely linked on chromosome 9 and encode for 2 proteins (i.e., AAG1 and AAG2, which differ by 22 amino acids; ref. 21). While the *ORM2* is monomorphic in most populations, the *ORM1* is characterized by 3 alleles (*ORM1*F1*, *ORM1*F2*, and *ORM1*S*; ref. 22). The *ORM1*F1* is generally considered as the “wild-type” variant; the *S* variant has an allelic frequency of approximately 0.345, whereas the *F2* is presented at a low allele frequency (19). The *ORM1*F1* and *S* variants differ by fewer than 5 residues, with glutamine and arginine in position 20 characterizing the *F1* and *S* variant, respectively (22). Genetic variants of AAG may have different binding properties (23). Indeed, specific binding to the AAG1 or AAG2 variants have been shown for several drugs (24), including the tyrosine kinase inhibitor imatinib that shows a high binding preference for the AAG1 F1-S variants but weaker and less specific binding for the AAG2 variant (25). The specific binding and variation in the relative levels of AAG variants can be of pharmacologic relevance. It has been shown that AAG levels and genotype/phenotype influence the pharmacokinetics of several drugs such as imatinib, indinavir, and atazanavir (26, 27). For instance, indinavir apparent clearance was significantly higher in patients with F1F1 phenotype than in those with F1S and SS phenotype (26). The binding preference of RO4929097 for different AAG variants has not been studied so far and needs further investigation. Given the fact that RO4929097 was highly bound in plasma with a similar binding affinity for AAG ($K_a\ 1.1 \times 10^6\ L/mol$) as imatinib ($K_a\ 1.7 \times 10^6\ L/mol$; ref. 25), it is plausible that AAG level and genotype/phenotype may modulate the pharmacokinetics of total RO4929097. Measurement of AAG plasma concentration and analysis of AAG genotype/phenotype are currently being implemented in the amended clinical protocol to confirm this possibility.

It should be noted that although changes in plasma protein caused by variation of AAG levels or AAG genotype/phenotype or protein-binding displacement can influence the total drug pharmacokinetics, they rarely modify the unbound drug pharmacokinetics. An analysis based on the well-stirred model has theoretically revealed that changes in plasma protein binding do not influence the unbound drug exposure for all low extraction ratio drugs irrespective of the route of administration and for all drugs administered orally irrespective of the magnitude of extraction ratio, despite the fact that changes in plasma protein binding can significantly alter the total drug exposure (8, 28). Changes in plasma protein binding could alter the unbound drug exposure only for those drugs with high extraction ratio and administered intravenously, and there are few examples of this in clinical practice (8, 28).

In this study, concomitant administration of GDC-0449 dramatically decreased systemic exposure to total RO4929097 while showing insignificant influence on the unbound drug exposure (Table 2 and Fig. 3). On the other hand, co-administration of RO4929097 did not influence the pharmacokinetics of total GDC-0449 (Table 2). The observed pharmacokinetic profiles of RO4929097 and GDC-0449 in the combination could be explained, for a large part if not all, by protein-binding displacement interaction between RO4929097 and GDC-0449. An average of 3.7-fold (range, 1.7- to 8.5-fold) increase in RO4929097 fraction unbound in plasma was observed in patients with cancer receiving combination of RO4929097 20 mg orally and GDC-0449 150 mg orally (Table 1). It has been reported that GDC-0449 is highly bound in human plasma (>95%) with high affinity binding for AAG and lower affinity for albumin (29). Following daily oral administration of 150 mg/d in patients with cancer, the average steady-state plasma concentration of total GDC-0449 was approximately 25 $\mu\text{mol/L}$ (ranging from 5.5 to 56 $\mu\text{mol/L}$), and the unbound drug levels were less than 1% of the total drug (20). Notably, AAG levels were strongly correlated with total GDC-0449 levels (20, 29). Because of the high binding affinity of GDC-0449 for AAG, it is likely that concomitant GDC-0449 could competitively inhibit RO4929097 binding to AAG. *In vitro* binding experiments supported this hypothesis. As shown in Table 1, GDC-0449 increased RO4929097 fraction unbound in plasma mainly by displacing RO4929097 bound to AAG. Further examination of the interaction between RO4929097 and GDC-0449 in the isolated AAG solution indicated that clinically relevant concentrations of GDC-0449 (5–125 $\mu\text{mol/L}$) competitively inhibited RO4929097 binding to AAG (Fig. 2); whereas clinically relevant concentrations of RO4929097 (213–4,260 nmol/L) had no apparent impact on GDC-0449 binding to AAG (Supplementary Fig. S3). It should be mentioned that RO4929097 and GDC-0449 exhibited a similar binding affinity for AAG, with the K_d values of 0.9 and 1.5 $\mu\text{mol/L}$ determined by *in vitro* equilibrium dialysis, respectively (29). Thus, the observed *in vivo* protein-binding displacement interaction between RO4929097 and GDC-0449 was mainly driven by the plasma concentrations of each agent. RO4929097 would be expected to displace GDC-0449 bound to AAG when RO4929097 plasma concentrations were higher than GDC-0449. However, this cannot happen in patients because the clinically achievable plasma concentrations of GDC-0449 are 20-fold excess of RO4929097 concentrations.

Interestingly, a reversible, time-dependent pharmacokinetics of RO4929097 (i.e., increased apparent oral clearance and reduced elimination half-life after chronic treatment) has been observed in patients with cancer receiving 7-day daily treatment at higher dose levels (60 mg) in a phase I study (7). This limited information implicated that RO4929097 could possibly induce its own metabolism. In the present study, the pharmacokinetics of RO4929097 given alone was evaluated after a single dose of RO4929097 when autoinduction was not expected, whereas the pharmacokinetics of RO4929097 in the combination was assessed after 2-day treatment on the 3-day on/4-day off-schedule when potential autoinduction could be possible. However, we reasoned that the observed decreased systemic exposure to total RO4929097 in the combination was not due to

potential autoinduction of RO4929097 metabolism for 2 reasons. First, in the present study, RO4929097 was given at a daily dose of 20 mg. At such a low dose level, autoinduction would not be expected. Indeed, in the previous phase I study involving 89 patients, 1.3- to 2.5-fold accumulated systemic exposure to total RO4929097 was achieved following 7-day or 3-day daily treatment at the lower dose levels (24 mg), suggesting that autoinduction unlikely occurs at the lower dose levels (Investigator Brochure). Second, on the basis of the well-stirred model, for all drug administered orally (such as RO4929097) irrespective of the magnitude of clearance, the total drug exposure (AUC) is determined by the dose, fraction unbound, and intrinsic clearance, whereas the unbound drug exposure (AUC) is determined by the dose and intrinsic clearance (8, 28). Autoinduction or induction of metabolism by concomitant drug is expected to increase the intrinsic clearance due to the induction of metabolizing enzyme activity. Therefore, for all drug administered orally, enzyme induction will result in reduced elimination half-life and decreased systemic exposure of both total and unbound drug to a similar extent. Nevertheless, when RO4929097 was co-administered with GDC-0449, while there was 8.3-fold decrease ($P = 0.016$) in the average systemic exposure to total RO4929097, the average unbound drug exposure was decreased insignificantly (1.7-fold, $P = 0.453$); moreover, there was no apparent change in the elimination half-life of total or unbound drug (Table 2). Apparently, these pharmacokinetic observations could not be explained by the induction of RO4929097 metabolism due to either autoinduction (if any) or concomitant GDC-0449 (if any).

It is generally believed that only unbound drug can cross the cell membrane and result in pharmacologic effects. In agreement with this free drug hypothesis, the *in vitro* Notch cellular assay showed that binding to plasma proteins such as AAG abrogated the Notch-inhibitory activity of RO4929097 (Fig. 5A). Indeed, the RO4929097 Notch-inhibitory effect as a function of the unbound drug concentration was well described by the inhibitory $E_{\max}$ model with an estimated IC_{50} of 109 nmol/L (Fig. 5B). These data strongly support the notion that unbound RO4929097 is pharmacologically active. Although concomitant GDC-0449 dramatically decreased total RO4929097 exposure, the pharmacodynamic effect and efficacy of RO4929097 is not expected to vary because the unbound (pharmacologically active) drug exposure remains unchanged. Therefore, no adjustment of RO4929097 dosing should be required in the combination with GDC-0449.

In conclusion, RO4929097 is highly bound in plasma with high affinity to AAG. Changes in plasma protein binding caused by concomitant drugs (e.g., GDC-0449) or disease states (e.g., increased and varied AAG levels in patients with cancer) can alter the systemic exposure to total RO4929097 but without modifying the unbound drug exposure. Given the notion that the unbound drug is pharmacologically active, monitoring of unbound RO4929097 plasma concentration is recommended to avoid misleading conclusions based on the total drug levels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

Aberrant activation of the Notch pathway contributes to tumor initiation and progression. RO4929097, a γ -secretase inhibitor blocking Notch signaling, is being evaluated as combination with the Hedgehog inhibitor GDC-0449 (vismodegib) for treating metastatic breast cancer. Co-administration of GDC-0449 dramatically decreased the systemic exposure to total RO4929097. It was found that RO4929097 was highly bound in plasma with high affinity to α 1-acid glycoprotein (AAG), and concomitant GDC-0449 replaced RO4929097 bound to AAG. Plasma protein binding was identified as a significant covariate on total RO4929097 pharmacokinetics. This study suggests that changes in plasma protein binding caused by concomitant drug (e.g., GDC-0449) or disease states (e.g., $\uparrow$ AAG level in cancer) can alter total RO4929097 exposure while having insignificant influence on the unbound (pharmacologically active) drug exposure. This study provides an example underscoring the importance of measuring plasma protein binding and unbound drug concentration in the clinical development of novel anticancer drugs.

Figure 1.

RO4929097 binding to plasma proteins HSA and AAG. A and B, RO4929097 fraction unbound (Fu) as a function of HSA (5–50 mg/mL) and AAG (0.2–3.2 mg/mL) concentration. C and D, modified Scatchard plots of the observed unbound versus bound drug concentration as the total drug concentration ranging from 20 to 10,000 ng/mL (43–21,268 nmol/L) in isolated HSA (40 mg/mL or 615.4 μ mol/L; C) and AAG (1.4 mg/mL or 31.8 μ mol/L; D) solution. E and F, simulated RO4929097 Fu in plasma with varying clinically relevant AAG concentrations (i.e., 0.2, 1.4, 3.2 mg/mL) and a fixed HSA concentration (40 mg/mL) or with varying clinically relevant HSA concentrations (i.e., 20, 40, 50 mg/mL) and a fixed AAG concentration (1.4 mg/mL), using a 2-binding site model [Equation (D)].

Figure 2.

Effect of GDC-0449 on RO4929097 binding to AAG in isolated AAG solution (1.4 mg/mL or 31.8 μ mol/L). A, modified Scatchard plots of the observed unbound versus bound RO4929097 concentration and (B) RO4929097 fraction unbound (F_u), as the total drug concentration ranging from 43 to 21,304 nmol/L in AAG solution in the presence of 0 (■), 5 (○), 25 (▲), and 125 (□) μ mol/L of GDC-0449.

Figure 3.

Observed RO4929097 total and unbound plasma concentration versus time profiles and the unbound fraction (F_u) as a function of time in individual patients receiving oral administration of RO4929097 20 mg alone (cycle 1) and in combination with oral administration of GDC-0449 150 mg (cycle 2). A and B, individual observed total RO4929097 plasma concentration–time profiles. C and D, individual observed unbound RO4929097 plasma concentration–time profiles. E and F, individual observed RO4929097 fraction unbound as a function of time. The different symbols in all graphs consistently represent individual patients.

Figure 4.

Correlations between RO4929097 unbound fraction (Fu) and the pharmacokinetic parameters (C_{max} , AUC_{0-24h} , or CL/F) of total (A–C) and unbound (D–F) RO4929097 in patients receiving RO4929097 20 mg orally alone or in combination with GDC-0449 150 mg orally. Bivariate correlations were examined with the Spearman rank correlation test; ρ is the Spearman rank correlation coefficient; correlation is considered significant at $P < 0.01$ (2-tailed).

Figure 5.

Impact of protein binding on *in vitro* Notch-inhibitory activity of RO4929097. A, the Notch-inhibitory activity of RO4929097 was expressed as the percentage of normalized luciferase activity in the treated cells relative to that in the untreated cells. Human U2OS cells were transiently cotransfected with a constitutively active NOTCH1 mutant construct and HES1-Luc reporter construct. The cells were treated with RO4929097 at 50, 500, and 5,000 nmol/L in the absence or presence of 0.5, 1.4, and 3.2 mg/mL AAG for 24 hours. The bars represent the mean $\pm$ SD from 2 independent experiments, with duplicate in each experiment. B, RO4929097 *in vitro* Notch-inhibitory activity as a function of the estimated unbound drug concentration was described by the inhibitory $E_{\max}$ model with an estimated IC_{50} value of 109 nmol/L. The observed values are shown as ●, and the solid line represents the model-fitted curve.

*, significantly different than RO4929097 treatment in the absence of AAG.

Table 1

Effect of GDC-0449 on RO4929097 unbound fraction (Fu) in human plasma, in the isolated AAG (1.4 mg/mL) and HSA (40 mg/mL) solution, and in patients with cancer

<i>In vitro</i> experiment		RO4929097 Fu, ^a %			
Total RO4929097, nmol/L	GDC-0449 (0 μmol/L)	GDC-0449 (5 μmol/L)	GDC-0449 (25 μmol/L)	GDC-0449 (125 μmol/L)	
In plasma					
43	1.7	2.7	4.3	5.7	
426	2.1	3.3	4.4	5.9	
4,260	2.8	3.6	5.4	6.0	
43–4,260 ^b	2.2 ± 0.5	3.2 ± 0.5 (1.5)	4.7 ± 0.6 (2.1)	5.9 ± 0.4 (2.7)	
In AAG solution					
43	2.1	2.7	18.2	40.4	
426	2.2	2.9	19.4	35.0	
4,260	2.8	4.6	21.9	30.7	
43–4,260 ^b	2.0 ± 0.3	3.7 ± 0.9 (1.8)	20.6 ± 2.2 (10.2)	48.2 ± 5.7 (24.0)	
In HSA solution					
43	12.1	9.7	12.6	13.9	
426	10.7	10.6	12.1	13.6	
4,260	10.6	10.7	12.9	13.2	
43–4,260 ^b	11.1 ± 1.0	10.3 ± 1.2 (0.9)	12.5 ± 0.6 (1.1)	13.6 ± 1.0 (1.2)	

<i>In cancer patients</i>		RO4929097 Fu, ^c %		
Patient no.	Given alone	In combination with GDC-0449	Fold change ^d	
1	1.4	4.8	3.4	
2	1.8	6.7	3.7	
3	0.8	n/a	n/a	
4	0.2	1.7	8.5	
5	1.5	3.0	2.0	
6	2.0	3.3	1.7	
7	0.4	2	5.0	
8	1.8	3.3	1.8	
Mean ± SD	1.2 ± 0.7	3.5 ± 1.7	3.7 ± 2.4	

^aFu value at each RO4929097 concentration level are shown as the mean of triplicate measurements.

^bFu values are shown as the mean±SD of measurements from 3 drug concentration levels (*n*=9); the fold change of the mean Fu in the presence of GDC-0449 relative to that in the absence of GDC-0449 are shown in the parenthesis.

^cPatients were treated with RO4929097 20 mg orally alone and in combination with oral GDC-0449 150 mg. Fu values for each individual patient are shown as the mean Fu observed in the treatment cycle.

^dFold change of the Fu when RO4929097 was combined with GDC-0449 relative to that when it was given alone.

Table 2

Plasma pharmacokinetic parameters of RO4929097 and GDC-0449 when each agent was given alone and in combination (RO4929097 20 mg orally, GDC-0449 150 mg orally) in patients with breast cancer

Parameter ^a	Given alone	In combination	P ^b
Total RO4929097			
T _{max} , h	2.5 (1.0–4.0)	1.1 (1.1–1.6)	0.688
C _{max} , nmol/L	498.1 (134.2–895.7)	70.9 (58.9–144.7)	0.016 ^c
AUC _{0–24h} , nmol/L h	6,034.5 (1,840.3–14,004.5)	723.3 (483.1–1,425.6)	0.016 ^c
T _{1/2} , h	24.5 (22.5–29.8)	24.1 (22.8–34.1)	1.000
CL/F, L/h	2.3 (1.0–10.4)	26.4 (9.0–36.6)	0.031 ^c
Unbound RO4929097			
T _{max} , h	1.5 (1.0–3.7)	1.1 (1.0–2.0)	1.000
C _{max} , nmol/L	4.3 (2.7–5.6)	3.0 (2.2–5.3)	1.000
AUC _{0–24h} , nmol/L h	52.8 (33.2–64.9)	31.6 (20.3–38.3)	0.453
T _{1/2} , h	21.1 (11.0–24.3)	16.4 (13.5–23.8)	0.688
CL/F, L/h	400.1 (350.6–743.2)	666.0 (546.6–1,337.8)	0.219
Total GDC-0449 ^c			
T _{max} , h	1.5 (1.0–24.4)	2.0 (1.5–24.0)	1.000
C _{max} , μmol/L	24.0 (21.9–37.7)	22.6 (20.4–33.9)	0.125
AUC _{0–24h} , μmol/L h	500.3 (472.5–703.8)	500.8 (448.9–746.2)	1.000

^a Parameters are shown as the median with interquartile range in the parenthesis, *n* = 8.

^b Wilcoxon signed-rank test

^c Statistically significantly different, *P* < 0.05.