Pharmacokinetic and Pharmacodynamic Evaluation of the Glycinamide Ribonucleotide Formyltransferase Inhibitor AG2034

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INTRODUCTION

GARFT is an essential enzyme in the de novo purine synthesis pathway. Most normal tissues, with the exception of liver and activated T lymphocytes, are thought to obtain purines primarily from the salvage pathway (1). Tumor cells generally have elevated activities of the de novo pathway and often have decreased activity of purine salvage enzymes. This has led to the hypothesis that tumors have a reliance on de novo purine biosynthesis, providing a mechanism for selective targeting of tumor compared with normal tissue using GARFT inhibitors (1).

The first GARFT inhibitor to undergo clinical evaluation was lometrexol. Lometrexol exhibited promising preclinical activity and evidence for clinical objective responses in a variety of solid tumors (2, 3). However, its clinical development was limited by serious cumulative hematopoietic toxicity (3, 4). Dietary folic acid supplementation was shown to modulate lometrexol toxicity, without diminishing antitumor activity in preclinical models (5). In clinical studies, concurrent folic acid supplementation increased the MTD of lometrexol and seemed to attenuate some cumulative toxicities (6–9). Pharmacokinetic studies of lometrexol observed that RBC, but not plasma, concentrations correlated with the observed cumulative hematological toxicity (10).

AG2034 is a second generation GARFT inhibitor, designed using knowledge of the X-ray crystal structure of GARFT from Escherichia coli and of the GARFT domain of the human trifunctional enzyme (11). Computational analysis of the GARFT active site suggested that sulfur atoms should have particular affinity with two regions of the folate cofactor binding site. Therefore, AG2034 was designed to fulfill this condition, while retaining substrate activity for the reduced folate carrier and FPGS. Preclinical studies, AG2034 was similar to lometrexol, in terms of GARFT inhibition and substrate specificity.

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3 The abbreviations used are: GARFT, glycaminamide ribonucleotide formyltransferase; AUC, area under the concentration versus time curve; CL, systemic clearance; eMF, erythrocyte 5-methyltetrahydrofolate; FPGS, folylpolyglutamate synthetase; HCY, homocysteine; HPLC, high-performance liquid chromatography; MMA, methylmalonic acid; MTD, maximum tolerated dose; PBS, phosphate-buffered saline; pMF, plasma 5-methyltetrahydrofolate; RBC, erythrocyte.
for FPGS, but had higher binding affinity for the membrane folate-binding protein (11). AG2034 demonstrated a broad spectrum of antitumor activity in *in vitro* and *in vivo* model systems, with greater potency than lometrexol (11). In addition, AG2034 demonstrated preferential cytotoxicity against tumor cells with mutant p53 (12).

On the basis of these findings, a Phase I program was designed to evaluate AG2034 as a short i.v. infusion every 3 weeks. Phase I studies were performed at clinical centers in the United States of America (Massey Cancer Center, Virginia Commonwealth University, Richmond, VA; The City of Hope Comprehensive Cancer Center; and University of Southern California) and Europe (University of Aberdeen, Scotland). The Phase I studies utilized a modified Fibonacci approach to define the MTD of AG2034 in patients with advanced cancer, with a recommended Phase II dose of 5 mg/m². In this study, we describe pharmacology studies conducted in conjunction with the Phase I trials, to define the plasma pharmacokinetics of AG2034. In addition, the influence of patient folate status on pharmacokinetics and pharmacodynamics was evaluated.

**MATERIALS AND METHODS**

**Patients.** Pharmacological evaluation was conducted in 54 patients in the Phase I development program (Table 1).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Median value (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of patients</td>
<td>54</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>58 (26–80)</td>
</tr>
<tr>
<td>Gender</td>
<td>M = 36, F = 18</td>
</tr>
<tr>
<td>pMF (nm)</td>
<td>14.9 (3.7–90.4)</td>
</tr>
<tr>
<td>eMF (nm)</td>
<td>327.9 (37.5–1305.5)</td>
</tr>
<tr>
<td>HCY (µM)</td>
<td>8.7 (4.2–45.8)</td>
</tr>
<tr>
<td>MMA (ng/ml)</td>
<td>26.3 (9.7–70.3)</td>
</tr>
<tr>
<td>Tumor type</td>
<td></td>
</tr>
<tr>
<td>Colorectal</td>
<td>19</td>
</tr>
<tr>
<td>Lung</td>
<td>5</td>
</tr>
<tr>
<td>Mesothelioma</td>
<td>6</td>
</tr>
<tr>
<td>Other</td>
<td>24</td>
</tr>
</tbody>
</table>

Further information on the clinical and toxicological findings of these studies are published elsewhere (8, 9). Patients received AG2034 as a 5-min i.v. infusion, with doses escalated from 1 to 11 mg/m² (Table 2). Blood sampling for pharmacokinetic analysis was performed prior to and 5, 15, 30, 45 min and 1, 1.5, 2, 4, 6, 8, 12, 24, 48, 72, and 96 h after bolus injection during course 1. During course 3, limited blood sampling was performed, with samples obtained before injection and 5 min, 1 h, and 24 h after injection. Blood samples for analysis of AG2034 RBC concentrations were obtained on days 1, 8, and 15 after each course from seven patients treated at the 5 mg/m² and 7.5 mg/m² dose level. Following separation of plasma from whole blood and removal of the buffy coat, the remaining red cell pellet was washed twice with PBS and stored at −70°C until analysis.

**Assay Development for Plasma AG2034.** Analysis of AG2034 in human plasma was performed using a sensitive and reproducible ELISA assay, which was modified from a Good Laboratory Practice ELISA assay developed for analysis of AG2034 in dog plasma at Agouron Pharmaceuticals. An AG2034 BSA conjugate was generated and used for coating microtiter plates. Before each assay, plates were incubated with blocking solution (PBS with 1% Casein). Plasma standards, quality control samples, and patient samples were incubated with a rabbit polyclonal antibody generated against AG2034 prior to incubation on the microtiter plate. Each 96-well plate contained a standard curve in triplicate (0.1–1000 ng/ml) and triplicate quality control samples (4, 20, and 400 ng/ml). A total binding (antibody alone) and blank nonspecific binding (no antibody) control was included with each plate. After incubation, plates were washed three times in PBST, incubated with a secondary antibody conjugated to horseradish peroxidase, and color developed using TMB peroxidase substrate. Microtiter plates were read at 450 nm. Intra-assay coefficients of variation at 4, 20, and 400 ng/ml AG2034 ranged from 6.0–7.7%. The interassay coefficients of variation at the same concentrations ranged from 6.7–8.2%. The lower limit of detection was 0.5 ng/ml, and the lower limit of quantitation was 1 ng/ml (2 nm).

The assay was linear from 1–500 ng/ml (*r*² = 0.99). Evaluation of plasma dilution protocols determined that linear sample prediction was achieved up to 1:200 dilution. Samples diluted 1:1000 gave a predicted AG2034 concentration >20% of actual value (21.7%). There were no significant changes in AG2034 concentration when left at room temperature for up to 4 h, or after three freeze-thaw cycles. The assay was not cross-reactive with folic acid or 5-methyltetrahydrofolate.

**Pharmacokinetic Analysis**

Plasma concentrations after 24 h were low and relatively constant (8), with negligible contribution to the overall AUC. Plasma AG2034 data from 0–24 h was analyzed using a three-compartment linear pharmacokinetic model, implemented on ADAPT II software (13). An iterative two-stage approach was used with initial Bayesian starting primary pharmacokinetic parameters (*e.g.* V₀, K₁₀, K₂₁, K₁₂, K₁₃, and K₃₁) from that recently described for lometrexol (10). Primary estimates were generated following three iterations, and secondary pharmacokinetic parameters [CL, volume of distribution (Vₐ), plasma half-life (1/2), and AUC] were derived from the model-generated primary estimates.

**Analysis of HCY, MMA, pMF, and eMF.** Blood samples for analysis of patient folate status were obtained immediately before the first dose of AG2034. Plasma HCY was measured using a HPLC assay (14), whereas a gas chromatography-mass spectroscopy assay was used for determination of serum MMA (15). Plasma and RBCs were analyzed for 5-methyltetrahydrofolate content using an assay based on enzymatic cycling of this reduced folate form to 5,10-methylenetetrafolate, followed by entrapment into a stable ternary complex with thymidylate synthase and tritiated fluorodeoxyuridylicate (16).

**Analysis of RBC AG2034 Concentrations.** RBC AG2034 concentrations were measured using a newly developed HPLC/MS electrochemical detection assay. The assay mea-

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4 M. A. Zorbas, unpublished data.
sures the desglutamate form of AG2034, following enzymatic digestion with carboxypeptidase G. The HPLC system consisted of a Shimadzu LC-10AS solvent delivery pump (Shimadzu Inc., Columbia, MD), a SIL-10A automatic injector, and a Coulochem II electrochemical detector (ESA Inc., Chelmsford, MA). Separation was achieved using a C18 250 × 4.6-mm analytical column (Regis Technologies, Morton Grove, IL) and a NovaPak C18 guard column (Waters Chromatography, Milford, MA). The mobile phase was 17% acetonitrile in 0.05 M potassium phosphate buffer (pH 3.5) with 0.1 mM EDTA at a flow rate 1.2 ml/min. The total run time for each sample was 26 min. The electrochemical settings were 1.0 V for the guard cell, 1.0 V for electrode 1, and 1.0 V for electrode 2.

To determine intracellular levels of both AG2034 and its polyglutamates, RBC samples were first lysed using three rapid freeze-thaw cycles. The lysed cells were then treated with carboxypeptidase G (Sigma Chemical Co., St. Louis, MO) to cleave all intracellular drug species down to their common desglutamate form (AG2057). Following enzymatic digestion, samples were extracted using 3 cc sulfonic acid cation-exchange cartridges (J. T. Baker, Phillipsburg, NJ). The pH of the extracted samples was then adjusted to 4 using acetic acid, and 100 μl was injected on the HPLC. The percentage conversion of AG2034 and AG2034-pentaglutamate to AG2057 was 115% and 92%, respectively. The standard curve for AG2057 was linear in the range of 5–250 ng/ml (rs = 0.999). Intra-assay coefficients of variation at 7.5, 75 and 200 ng/ml AG2057 ranged from 3.6 –5.8%, whereas the interassay coefficients of variation at the same concentrations ranged from 2.9 –6.6%. The lower limit of quantitation of the assay was 5 ng/ml, with a lower limit of detection of 2 ng/ml.

**Pharmacodynamic Analysis.** Both hematological and nonhematological toxicity (diarrhea, fatigue, hyperbilirubinemia) were observed after treatment with AG2034 (8, 9). This toxicity was both acute and cumulative. Therefore, the highest grade of toxicity during courses 1–3 were used for each patient in the pharmacodynamic analysis.

**Statistical Analysis.** Evaluation of the relationship between age, folate status (HCY, MMA, pMF, eMF), or dose and either Vd or CL were made using the Spearman’s rank test. The

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**Table 2** Course 1 AG2034 pharmacokinetics

<table>
<thead>
<tr>
<th>AG2034 dose (mg/m²)</th>
<th>Clearance (ml/min/m²)</th>
<th>Vd (ml/m²)</th>
<th>t1/2a (min)</th>
<th>T1/2a (min)</th>
<th>t1/2x (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>70.7</td>
<td>4128.4</td>
<td>8.4</td>
<td>51.4</td>
</tr>
<tr>
<td>1.5</td>
<td>8</td>
<td>51.0</td>
<td>5060.6</td>
<td>7.9</td>
<td>65.9</td>
</tr>
<tr>
<td>2.25</td>
<td>5</td>
<td>33.6</td>
<td>3090.7</td>
<td>7.9</td>
<td>84.7</td>
</tr>
<tr>
<td>3.4</td>
<td>6</td>
<td>39.8</td>
<td>2842.2</td>
<td>8.9</td>
<td>55.6</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>42.8</td>
<td>3655.6</td>
<td>8.5</td>
<td>70.1</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>30.2</td>
<td>3492.6</td>
<td>9.4</td>
<td>116.5</td>
</tr>
<tr>
<td>7.5</td>
<td>7</td>
<td>60.4</td>
<td>3662.9</td>
<td>8.3</td>
<td>71.3</td>
</tr>
<tr>
<td>11</td>
<td>6</td>
<td>28.9</td>
<td>3479.2</td>
<td>10.9</td>
<td>99.9</td>
</tr>
<tr>
<td>All doses</td>
<td>54b</td>
<td>38.1</td>
<td>3622.4</td>
<td>8.7</td>
<td>72.6</td>
</tr>
</tbody>
</table>

(9.4–144.5) (1231.9–7652.9) (5.9–23.2) (25.3–190.4) (129.7–539.4)

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Fig. 1 AG2034 pharmacokinetics over 24 h in a patient receiving 6 mg/m² i.v. over 5 min.
influence of gender on $V_d$, CL, HCY, MMA, pMF, and eMF was assessed using the Mann-Whitney test. Differences between pharmacokinetic parameters or folate status in patients from the United States or United Kingdom were evaluated by using the Mann-Whitney test. The change in AG2034 AUC between course 1 and course 3 was analyzed with the Wilcoxon signed ranks test. The Mann-Whitney test was also used to evaluate differences in AUC, CL, HCY, MMA, pMF, eMF or age in patients with grade II toxicity or less or grade III/IV toxicity.

**RESULTS**

**Course 1 Plasma Pharmacokinetics.** Plasma pharmacokinetic data from the first course of AG2034 administration was available for 54 patients (Table 2). Plasma CL was rapid with a median value of 38.1 ml/min/m² (Table 2). AG2034 pharmacokinetics demonstrated a triphasic elimination profile, with a rapid initial distribution phase (median $t_{1/2} = 8.7$ min) and a prolonged terminal elimination phase (median $t_{1/2} = 364.2$ min; Fig. 1 and Table 2). AG2034 AUC demonstrated a linear relationship with dose (Fig. 2; $r_s = 0.86; P < 0.001$).

AG2034 CL decreased with increasing age ($r_s = -0.32; P = 0.018$) and increasing pMF concentration ($r_s = -0.47; P = 0.001$) (Fig. 3). CL did not seem to be influenced by HCY or MMA concentrations. A 5.6-fold range in $V_d$ was observed (Table 2). $V_d$ was not associated with patient age, BSA,
AG2034 dose, HCY, MMA, or eMF. However, a significant inverse relationship between V_d (ml/m^2) and pMF was observed (r_s = -0.44; P = 0.004).

This Phase 1 study was conducted at three United States centers and one United Kingdom center. Differences in folate status was apparent, with lower pMF and eMF and higher HCY in United Kingdom patients (Table 3). Plasma MMA was not different between the two countries. The impact of altered folate status on AG2034 was less apparent, because there was no significant difference between AG2034 CL (P = 0.22) or V_d (P = 0.25) between the two countries.

AG2034 Accumulation. Information on plasma AG2034 pharmacokinetics was available for course 3 in 23 patients. Evidence for drug accumulation was apparent, because AG2034 AUC demonstrated a median 212.7% increase from course 1 to course 3 (P < 0.001; Fig. 4). The course 3 AUC was higher than course 1 in 23 of 23 patients. A strong correlation between course 1 and course 3 AG2034 AUC was observed (r_s = 0.88, P < 0.001). There was no evidence for disproportional intracellular accumulation of AG2034. RBC AG2034 concentrations during the first three courses were low (<4.5 to 13.9 ng/ml) and mirrored that observed in plasma.

AG2034 Pharmacodynamics. Grade III/IV toxicity was observed in 14 of 23 total patients (United States, 11; United Kingdom, 12) receiving the top three dose levels (6, 7.5, and 11 mg/m^2). Grade III/IV hematological toxicity occurred in seven patients, with grade III/IV nonhematological toxicity in 14 patients. Unlike lometrexol, anemia was not a dominant hematological toxicity of AG2034 (8, 9). The patients were equally distributed by country for nonhematological toxicity (United States 7 of 11; United Kingdom, 7 of 12). Grade III/IV hematological toxicity seemed to occur more frequently among the United Kingdom patients (United Kingdom, 5 of 12, and United States, 2 of 11 patients at 6, 7.5, and 11 mg/m^2), although this did not reach statistical significance (P = 0.22). The incidence of systemic toxicity was significantly higher among patients with greater than the median course 1 AG2034 AUC value (12 of 27 patients with toxicity versus 2 of 27 patients whose AUC was less than the course 1 median P = 0.005). Course 1 AG2034 AUC was significantly higher in patients with hematological (P = 0.001) and nonhematological (P < 0.001) toxicity. No other pretherapy demographical or biochemical variable was associated with nonhematological toxicity, whereas a higher pretherapy HCY concentration was observed in patients with grade III/IV hematological toxicity (P = 0.024). When toxicity in any tissue was considered, course 1 median AG2034 AUC was significantly higher in patients with grade III/IV toxicity compared with other patients (toxic 198,441.2 ng/ml*min, P < 0.001; Fig. 5). The influence of AG2034 systemic exposure on toxicity was also evident among the 23 patients with course 3 AUC measurements (P = 0.001, Fig. 5). The degree of increase in AG2034

<table>
<thead>
<tr>
<th>Country</th>
<th>pMF (nm)</th>
<th>eMF (nm)</th>
<th>HCY (µM)</th>
<th>MMA (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>United States</td>
<td>30.5 (5.7–90.4)</td>
<td>418.3 (111.7–1214.2)</td>
<td>7.3 (4.2–12.7)</td>
<td>25.8 (16.6–54.7)</td>
</tr>
<tr>
<td>United Kingdom</td>
<td>9.4 (3.7–37.3)</td>
<td>195.7 (37.5–1305.5)</td>
<td>10.6 (5–45.8)</td>
<td>27.0 (9.7–70.3)</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.001</td>
<td>0.012</td>
<td>0.009</td>
<td>0.53</td>
</tr>
</tbody>
</table>

Table 3 Influence of country of residence on median (range) patient folate status
AUC between courses 1 and 3 was not different between nontoxic and toxic patients ($P = 0.88$).

**DISCUSSION**

AG2034 pharmacokinetics were best described by a three-compartment model over the first 24 h after initial injection. A prolonged terminal elimination phase was observed with a median $t_{1/2}$ of 364.2 min, and AG2034 AUC was linear with dose. CL was highly variable and seemed to be influenced, at least in part, by patient age and pretherapy pMF concentration. Overall, AG2034 pharmacokinetics were similar to the first generation GARFT inhibitor lometrexol (10). Lometrexol has demonstrated a triphasic pattern of elimination, which is not influenced by folic acid or leucovorin pretreatment (10, 17). AG2034 and lometrexol have a similar CL value (median 38.1 versus 22.6–26.7 ml/min/m$^2$) and $V_d$ (median 3.6 versus 8.6–8.9 liter/m$^2$) (10, 17). Because previous studies of lometrexol have not included measures of patient folate status, regulation of pharmacokinetics by pMF cannot be compared between the two agents.

AG2034 pharmacokinetics seem to be influenced by both age and pMF. CL illustrated an inverse relationship between both age and pMF, whereas $V_d$ was inversely related to pMF alone. AG2034 is a substrate for both the reduced folate carrier and FPGS and has high binding affinity for membrane folate-binding protein (11). Therefore, it can be hypothesized that patients with high pMF will have a greater cellular competition for both intercellular uptake and efflux of AG2034. The linear relationship between CL and $V_d$ may reflect coregulation by patient folate status. A precise physiological basis for the interaction between pMF and both AG2034 CL and $V_d$ remains to be defined. The influence of age on AG2034 CL is likely a surrogate of renal function. AG2034 seems to be eliminated unchanged in the urine, and no metabolites were apparent in plasma with HPLC analysis. Because there is a well-defined relationship between increasing age and decreasing kidney function, the correlation between age and CL is likely explained. The definitive measures of patient creatinine clearance (24-h urine collection or radioisotope evaluation) were not available from patients on this Phase 1 study.

Significant differences in pMF, eMF, and HCY were observed between United States and United Kingdom patients. This is not surprising because folic acid fortification of bread and other foodstuffs is not a common practice in the United Kingdom. In addition, vitamin supplementation is less frequently used in the United Kingdom than United States. There was no significant difference in AG2034 pharmacokinetic parameters between countries. However, the incidence of hematological toxicity was higher in United Kingdom patients, although this did not reach statistical significance. The MTD was also identical between the two countries (8, 9). This makes the significance of differences in folate status unclear. Caution may need to be used when extrapolating drug dosage across populations, especially for anticancer agents that involve folate pathways.

Drug accumulation was apparent for AG2034 between course 1 and course 3. AG2034 AUC increased from course 1 to course 3 in all 23 evaluable patients. The median change in AG2034 AUC was 212.7%, with a range from 20–389% increase from course 1 to course 3. This is similar to that previously described for lometrexol. However, unlike lometrexol, the accumulation of AG2034 in RBCs was not apparent (10). This may suggest that delayed elimination from plasma, rather than

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**Fig. 5** AG2034 AUC$_{0-24}$ has from both course 1 and course 3 are significantly higher in patients experiencing grade III/IV systemic toxicity.
enhanced tissue retention, may be responsible for the alteration in AG2034 AUC. Alternatively, RBC accumulation may not be a good surrogate for tissue accumulation of AG2034.

AG2034 pharmacokinetics demonstrated a significant influence on toxicity. AG2034 AUC after courses 1 or 3 were significantly higher in patients demonstrating either grade III/IV hematological or nonhematological toxicity (Fig. 5). Indeed, 12 of 14 patients with systemic toxicity had a course 1 AG2034 AUC greater than the median value. All toxic patients were treated at the three highest dose levels (6, 7.5, and 11 mg/m²), reflecting the linearity of the AG2034 dose-AUC relationship. A correlation between AUC and toxicity is not surprising, because lometrexol systemic exposure has been associated with the degree of hematological toxicity (10). Similar pharmacodynamic relationships have also been described for other antifolates, including methotrexate (18, 19). Although drug accumulation and cumulative toxicity was evident for AG2034, the high correlation of AUC between the courses made course 1 AUC a strong predictor for systemic toxicity. This provides a useful tool for future development of this class of compounds.

The results of the AG2034 pharmacodynamic analysis provide further support for the selection of 5 mg/m² AG2034 as the Phase II dose, based on clinical toxicity information. Based on the AG2034 pharmacokinetic data in Table 2, 5 mg/m² would give an estimated median AUC 131,580 ng/ml*min, whereas 6 mg/m² would result in a median 157,900 ng/ml*min. The estimated AG2034 AUC (±10%) from 5 mg/m² would be predicted to be well tolerated, as none of four patients in that range experienced toxicity. The 6 mg/m² AUC estimate was associated with toxicity in two of four patients. The schedule dependence of antimetabolite anticancer agents do not allow the use of simulation experiments based on this data to predict the toxicity profile that would be observed with alternative administration schedules.

Whereas higher AG2034 AUC is associated with increased systemic toxicity, it does not seem to be the only factor regulating AG2034 pharmacological activity. A degree of overlap in AG2034 AUC values was observed between toxicity groups (Fig. 5). A high pretherapy HCY value was associated with greater hematological toxicity, but did not seem to influence nonhematological toxicity. This may reflect the finding that HCY was higher in United Kingdom patients than United States patients, and a high proportion of patients with hematological toxicity were from the United Kingdom center (five of seven patients). Demographic variables, including prior treatment with leucovorin, did not have an apparent influence on the incidence of AG2034 toxicity. However, preclinical studies have identified other variables that influence AG2034 cytotoxicity, including hypoxanthine concentrations, FPGS activity, and expression of reduced folate carrier and membrane folate-binding proteins (11, 20). In addition, AG2034 seems to be preferentially cytotoxic to cells with mutant p53 protein (12). Variability in the p53-mediated apoptosis pathway (or a related p53-mediated checkpoint) between normal tissues may be an additional influence on AG2034 systemic toxicity.

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