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EVALUATING *IN VITRO* DOSING OF TYROSINE KINASE INHIBITORS FOR CLINICAL REPOSITIONING: A SYSTEMATIC REVIEW

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ABSTRACT

In vitro experimentation plays an essential role in the early stages of drug development, though its environments vary greatly from physiological systems. Poor success in recent clinical trials suggests a systematic overestimation of preclinical drug efficacy, possibly due to inadequate consideration for the physiological effects influencing drug concentrations. For example, the pharmacologic activity of kinase inhibitors, a class of targeted cancer therapies, is influenced by plasma protein binding and other interactions less impactful in *in vitro* environments than physiologically. This study employs a systematic review of the preclinical literature comparing clinically relevant kinase inhibitor concentrations with those being applied *in vitro*. Additionally, we evaluate how these concentrations are justified and discussed in the literature with respect to pharmacokinetics and physiological interactions. Our results show little consideration for clinical pharmacokinetics in the published discussion of *in vitro* results. Median *in vitro* concentrations for all six kinase inhibitors in the study's focus were also found to be greater than their effective mean plasma concentrations, with heightened disparities when publications fail to reference pharmacokinetic parameters. These results present crucial evidence of preclinical reports communicating kinase inhibitor efficacy at clinically unrealistic concentrations; such reports likely drawing attention to drug repositioning efforts with poor prospects for clinical success. Despite recent efforts in the scientific community to improve standards for basic science experimentation, further consideration may be necessary to accurately reconcile clinical observations with methods of *in vitro* drug profiling.

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Chapter 1

Introduction

Protein kinases are excellent targets for anticancer therapeutics. Thoroughly characterized as driving the progression of numerous malignant diseases, overexpressed and mutant protein kinases have received much focus in the development of small molecule targeted therapies[1,2]. Drugs targeting kinases aberrantly expressed in several cancers, such as the ABL1 kinase, a segment of the BCR-ABL fusion gene product, and the epidermal growth factor receptor (EGFR), have respectively led the treatment of liquid and solid tumors with great clinical efficacy.

Before targeted therapies' rise to prominence in clinical oncology, a limited range of treatment options were available to patients with advanced disease. Treatment for chronic myeloid leukemia (CML), a hematological malignancy characterized by a chromosomal translocation producing the BCR-ABL oncogene, was especially narrow prior to FDA approval of imatinib, its first kinase inhibitor therapy, in 2001[3]. Treatment courses for CML involved allogeneic stem cell transplantation, posing the serious risk of graft-versus-host disease, as well as interferon-alpha treatment which carries substantial toxicity risks[4]. Non-small cell lung cancer (NSCLC), a disease of the epithelial lung tissue, is associated with deletion or substitution mutations of the EGFR receptor tyrosine kinase in 31.6% of patients[5]. Following the 2003 FDA approval of gefitinib, an inhibitor effective against wild-type and mutant EGFR, further drug development produced erlotinib: an inhibitor providing selectivity for EGFR variants with

NSCLC driver mutations[6]. Clinical application of erlotinib added to the standard course of surgical lung tumor removal and radiation therapy, providing greater specificity in mechanisms for personalized NSCLC treatment[7].

Believed to head the newest generation of cancer therapeutics, kinase inhibitors displayed outstanding early effectiveness in clinical trials, with imatinib described by TIME Magazine as a cancer-targeting bullet[8]. Having specificity for kinase targets with oncogenic driver mutations, imatinib and erlotinib saw clinical success as first and second-line monotherapies, respectively. Imatinib radically shifted the landscape of BCR-ABL-positive myeloid disease outcomes, elevating 5-year CML survival rates from 30% to 89%[9]. Though experiencing modest clinical success relative to imatinib, erlotinib has shown extension of progression-free survival past that of cytotoxic chemotherapy[10].

Although such drugs have led to effective clinical responses for patients with a variety of solid and liquid tumors, molecular resistance mechanisms have emerged reducing therapeutic function. Despite *in vitro* experiments showing selectivity of erlotinib for EGFR with activating mutations: such as exon 19 deletions or exon 21 substitutions, secondary mutants have conferred clinical resistance to the kinase inhibitor[11]. T790M, the most common gatekeeper mutation causing steric activation in the EGFR kinase domain[11], drives resistance to erlotinib at low drug concentrations[12]. Similar mechanisms have diminished imatinib's antitumor function in CML patients, with several secondary mutations shown to drive drug resistance in chronic and advanced phases of disease. Single amino acid substitutions in the ABL1 kinase phosphate-binding loop reduce imatinib sensitivity most substantially, while resistance mutations in the kinase's activation loop and catalytic domain have also been validated[13].

The emerging obstacle of resistance in EGFR and BCR-ABL-positive cancers begs the question: how can the roles of imatinib and erlotinib, two potent kinase inhibitors, be reconsidered with such prevalent clinical resistance? The biology underlying human neoplasia may provide direction to a new generation of uses for kinase inhibitor drugs. In many cases, tyrosine kinases targeted by solid and liquid tumor therapies spark downstream cellular signaling upon activation. Kinases overexpressed or possessing activating mutations, such as EGFR and BCR-ABL, produce signals driving mitogenesis and proliferation while inhibiting apoptotic signals, allowing these malignant cells to outcompete healthy counterparts and proliferate uncontrollably[1,2]. Potent kinase inhibitors hold the potential for clinical repurposing to treat neoplasms characterized without resistance mutations from various tissue types.

Kinase inhibitors have experienced a recent development toward clinical repositioning past hematological and solid-tumor cancers, stretching as far as treatment for viral and bacterial infections. With focus on treating *Mycobacterium tuberculosis* infection, host tyrosine kinases show regulation of bacterial entry in preclinical models, boosting interest in both EGFR and ABL inhibitors to attenuate disease[14,15]. Imatinib, in particular, received attention early in the global COVID-19 pandemic for successfully treating SARS-CoV-2 infection in a 38-year-old woman[16] — initiated based on *in vitro* data showing imatinib efficacy inhibiting host cell entry by phylogenetically-related coronaviruses[17]. Although a range of kinase inhibitors display broad efficacy in case-specific and *in vitro* literature, these successes appear to be lacking in clinical trials. A 2019 analysis estimated the clinical trial success rate for oncology drugs at 3.4%, and current practices in translational science may highlight the roots of limited success in the field[18].

With such repurposing efforts, *in vitro* preclinical evaluations lack certain physiological considerations that may lead to more promising results than those produced clinically. A key

inadequacy in many *in vitro* assays is the diminished effect of plasma protein binding, through which drugs bind, often reversibly, to proteins ubiquitous in blood plasma[19]. Shown to possess high binding affinities for plasma proteins, kinase inhibitors primarily bind alpha-1-acid glycoprotein[20]; smaller fractions bind to serum albumin, human plasma's most abundant protein[21]. Imatinib and erlotinib are reported to be 95% and 93% bound, respectively, to human plasma proteins following administration of clinically relevant doses[22,23]. With standard cell culture media containing 10% bovine serum, binding effects of plasma proteins during *in vitro* kinase inhibitor assays cannot nearly replicate those of human plasma, vastly overstating these drugs' true clinical potential. For example, a 2010 study found sorafenib, a multi-kinase inhibitor, to inhibit 50% of FLT3 autophosphorylation at an *in vitro* concentration of 3 nM[24], approximately 100-times lower than the 500 nM necessary to have a similar effect in human plasma[25]. Exploration into how *in vitro* kinase inhibitor dosing compares with clinically relevant concentrations may elucidate the mechanism behind high failure rates in clinical trials.

In the scope of kinase inhibitor repurposing, a clearer understanding of the *in vitro* literature may inform how these drugs are applied in preclinical models. By reducing the volume of translational claims abundant in the preclinical literature with *in vitro* assertions destined for failure in clinical trials, the field has an incredible opportunity to preserve financial resources and invaluable patient well-being wasted in these endeavors. To investigate kinase inhibitor concentrations applied in the preclinical literature, we employed a systematic review similar to that used by Björkhem-Bergman et al., which explored *in vitro* statin dosing[26]. We centered this search on recent publications reporting *in vitro* efficacy of ABL and EGFR inhibitors, opening the door to guidance on relevant kinase inhibitor concentrations for future *in vitro* studies.

Chapter 2

Methods

Overview

To evaluate the *in vitro* application of ABL1 and EGFR kinase inhibitors, we performed a systematic review of the recent literature expressing optimistic outlooks for these drugs' translational use. The search focused on publications administering imatinib, erlotinib, and other similar kinase inhibitors through *in vitro* experiments with online or print publication dates in 2018. After employing these search criteria to gather all relevant publications available on PubMed, publications were iteratively reviewed using a set of inclusion, exclusion, and analysis points to assess experimental kinase inhibitor dosing. For inclusion in the analysis, publications were required to apply FDA-approved kinase inhibitors in *in vitro* experiments, as well as make conclusions from these experiments suggesting clinical potential. Publications were surveyed for experimentation on cell lines selected for drug resistance, alternative drug delivery methods, and *ex vivo* assays of *in vivo* dosing, among a set of other criteria imposing exclusion from our analysis. For each included publication, individual drugs' *in vitro* concentrations were recorded, with only one concentration per drug recorded for each publication. Publications were analyzed for discussion of experimental findings with reference to pharmacokinetics or clinical justification for its applied drug dose, as a marker for clinical data informing *in vitro* studies for repositioning. Analyzing references to pharmacokinetics provided evidence of feedback from the clinical side of drug translation to preclinical testing, under the assumption that consideration for

clinical pharmacokinetics provides more realistic environments when testing compounds for *in vitro* efficacy.

Compilation of Publications for Analysis

Search methods and initial criteria

The systematic review was initiated with a general search using the PubMed advanced search function on the National Library of Medicine online database. Two searches were completed: one for use of the term *imatinib* in all fields, the other with use of *erlotinib* in all fields. Both searches additionally required use of the term *in vitro* in any field, as well as publication dates in 2018. Data was collected and recorded for each publication, including the publication's identifiable information (PMID number), first author and publication date. See Supplementary File 1 for raw data collected.

Exclusion criteria were established, requiring that the analysis includes only publications with clear *in vitro* kinase inhibitor experimentation, and making translationally relevant claims based on these experiments' findings. First, studies without *in vitro* data, *ex vivo* assays of *in vivo* dosing, and those without clear use of kinase inhibitor treatment were excluded. Clinical case studies and studies without translational claims were also excluded, choosing only experiments extrapolating *in vitro* data to clinical use. Last, experiments on cell lines selected for kinase inhibitor resistance, as well as drug delivery studies (e.g. imatinib delivery via only lipid nanoparticles), were excluded from analysis to specify translational claims from free kinase inhibitor results on sensitive cell lines. Although the search keyed on publications including the terms *imatinib* or *erlotinib*, all included experiments with kinase inhibitor *in vitro* doses were

analyzed, rather than only imatinib or erlotinib dosing. Having sorted all publications for inclusion in the analysis, each publication's *in vitro* experiment was then considered for kinase inhibitor dosing and the translational claim based on its finding.

Assessment of publications for translational claims

To include individual experiments in the analysis, we required *in vitro* dosing to generate claims of clinical efficacy. Oftentimes, these arguments for kinase inhibitor translation from *in vitro* experimentation to clinical use reside in publications' abstract, discussion section, or conclusions — although this search thoroughly investigated entire publications for translational claims. To evaluate the content of such claims, we applied a binary scale: each kinase inhibitor *in vitro* experiment either having or not making an argument for clinical use.

Experiments were considered for analysis with positive language in claims for further experimentation to evaluate clinical efficacy. For example, publications describing *in vitro* findings for potential clinical application included the terms: "*may provide an attractive approach*" (PMID: 29165716), "*can be considered as interesting candidates*" (PMID: 29986185), and "*the findings are of potential therapeutic interest*" (PMID: 29856777). Conversely, experiments yielding subjectively weak or inadequate translational arguments were excluded from analysis. For example, *in vitro* experiments involving high-throughput drug target screens or kinase inhibition for signaling pathway investigation were considered to lack adequate translational claims, not directly promoting translational drug utilization based on their findings. Experiments generating novel experimental methods, such as spectrophotometry or imaging protocols, were also excluded from our analysis. For cases in which multiple kinase inhibitors were applied *in vitro*, only those referenced in translational arguments were included in our analysis.

Data Synthesis from Included Publications

Determining experiments' in vitro dose concentrations

Experiments verified for translational claims based on *in vitro* dosing were then examined for single dose concentrations. Though many publications apply kinase inhibitors at a range of concentrations, this review employed a stringent protocol to select single values for each drug per publication to simplify comparison across studies. Many *in vitro* experiments report *in vitro* inhibition as an IC_{50} value, the concentration at which 50% of a biological process is inhibited. IC_{50} values were recorded in our analysis yielding 50% inhibition of cell viability, with preference over experiments reporting only kinase inhibition in cell-free systems, often yielding IC_{50} values orders of magnitude lower than those measuring cell viability.

For cases in which a range of drug concentrations was employed for a single kinase inhibitor, the lowest *in vitro* concentration showing a significant effect was recorded in our analysis. A range of inhibitory effects may also have been measured across several cell lines, from which the lowest concentration leading to a translational claim was recorded. In our review, cases arose in which no single drug concentration was discussed or considered to show statistically-significant inhibition, with publications simply reporting effects at a range of concentrations on dose-response curves. In these cases, dose-response curves were investigated for points at which 50% inhibition was achieved — including notation in the raw data sheet explaining the imprecise measurement protocol. Several publications lacking a single lowest dose with significant effects included specific reference in the ensuing discussion on translational recommendations for that kinase inhibitor; in these cases, the drug concentration further

discussed was recorded. For example, Moslehi et al. applied three *in vitro* imatinib concentrations to inhibit *Leishmania major* viability (PMID: 31737578), the highest of which was discussed further in comparison with pre-existing treatments and recorded in our analysis.

Generation of effective C_{ave} values

In comparison with *in vitro* kinase inhibitor concentrations, effective mean plasma concentrations (C_{ave}) were generated for each drug to factor plasma protein binding effects into physiological bioavailability (SML). C_{ave} values serve as measures of steady state drug abundance in patient plasma, for comparison with concentrations used *in vitro*. Further reported by Leighow et al., IC_{50} values were produced for the most-used three EGFR and three ABL1 inhibitors, both in conditions containing and not containing plasma proteins[27]. C_{ave} values of FDA-approved doses were divided by each drug's experimental protein binding effect, an inhibition of kinase inhibitor activity, to produce individual inhibitor's effective C_{ave}.

Evaluation of pharmacokinetic references

Publications were examined for references to pharmacokinetics and clinical justification for their applied *in vitro* doses. In addition to a manual investigation for pharmacokinetics references, a search command was administered for pharmacokinetic metric terms, including C_{min} and C_{max}, parameters measuring a drug's minimum and maximum plasma concentration between dose administrations, respectively. Additionally, *plasma*, *serum*, *trough*, and *pharmacokinetics* were included in our search command. Upon investigation of these references, each included term was analyzed for its context in discussing the experimental data, often presented when considering a drug's physiological peak plasma level without correcting for protein binding effects.

Discussion of each experiments' findings were sorted into one of three categories: 1) lacking relevant references to pharmacokinetics, 2) referencing pharmacokinetics or clinical plasma values without correcting *in vitro* concentrations for protein binding, and 3) referencing a drug's pharmacokinetic profile with correction for protein binding. Publications were sorted into the category lacking pharmacokinetic references when including zero related terms or, oftentimes, applying these terms without any direct connection to the *in vitro* data, such as referencing pharmacokinetics in an introduction section. The second category consisted of publications referencing pharmacokinetics without correction for protein binding effects, often comparing experimental *in vitro* dose concentrations with C_{max} or C_{ave} values from the clinical literature. In the third category, we included publications that considered *in vitro* concentrations with pharmacokinetic parameters, in addition to correcting its dose for the physiological protein binding effect — considering plasma protein binding in its applied dose.

Analysis

Having compiled and evaluated *in vitro* experiments for their applied concentrations and references to clinical pharmacokinetics, all experiments were sorted by kinase target and ranked by experiment counts applying individual kinase inhibitors. Inhibitors targeting the analysis' two most prevalent kinases, ABL1 and EGFR, remained the focus of our analysis. Experiments applying the top three most-used inhibitors for ABL1 and EGFR were grouped by target kinase and evaluated for proportions referencing clinical pharmacokinetics. Drug concentrations in single experiments targeting these six kinases were compared with the inhibitor's concentration in human serum, as well as experimentally-validated effective C_{ave} values. Finally, experimental

concentrations for these six inhibitors were sorted further by inclusion of references to clinical pharmacokinetics and median concentrations for these groups were calculated and compared with effective C_{ave} values.

Chapter 3

Results

To generate the set of *in vitro* experimental doses from the preclinical literature, we employed a systematic review on the National Library of Medicine's PubMed database. Applying PubMed's advanced search tool, two searches centered on publications mentioning the terms *erlotinib* or *imatinib*, as well as *in vitro* with publication dates in 2018 (Figure 1). The erlotinib and imatinib searches, with results pooled together and duplicate publications extracted, yielded a total of 211 publications for analysis.

After filtering for studies making translational claims based on kinase inhibitor *in vitro* experiments, 209 unique experiments were found to use a total of 20 unique kinase inhibitors. Approximately three-quarters (158/209) of these experiments apply concentrations above each respective drug's threshold for clinical relevance, experimentally-derived mean serum concentrations (C_{ave}) based on FDA dose recommendations. Interestingly, publications applying kinase inhibitors at *in vitro* concentrations either above or below their physiological C_{ave} were found to reference clinical pharmacokinetics through uncorrected plasma values at similar rates, referencing pharmacokinetics in 24% and 25% of cases, respectively. A single publication was identified considering plasma protein binding effects (PMID: 30086285), comparing its imatinib IC_{50} (0.3 μM) with imatinib's cited maximum unbound hepatic input concentration (0.7 μM).

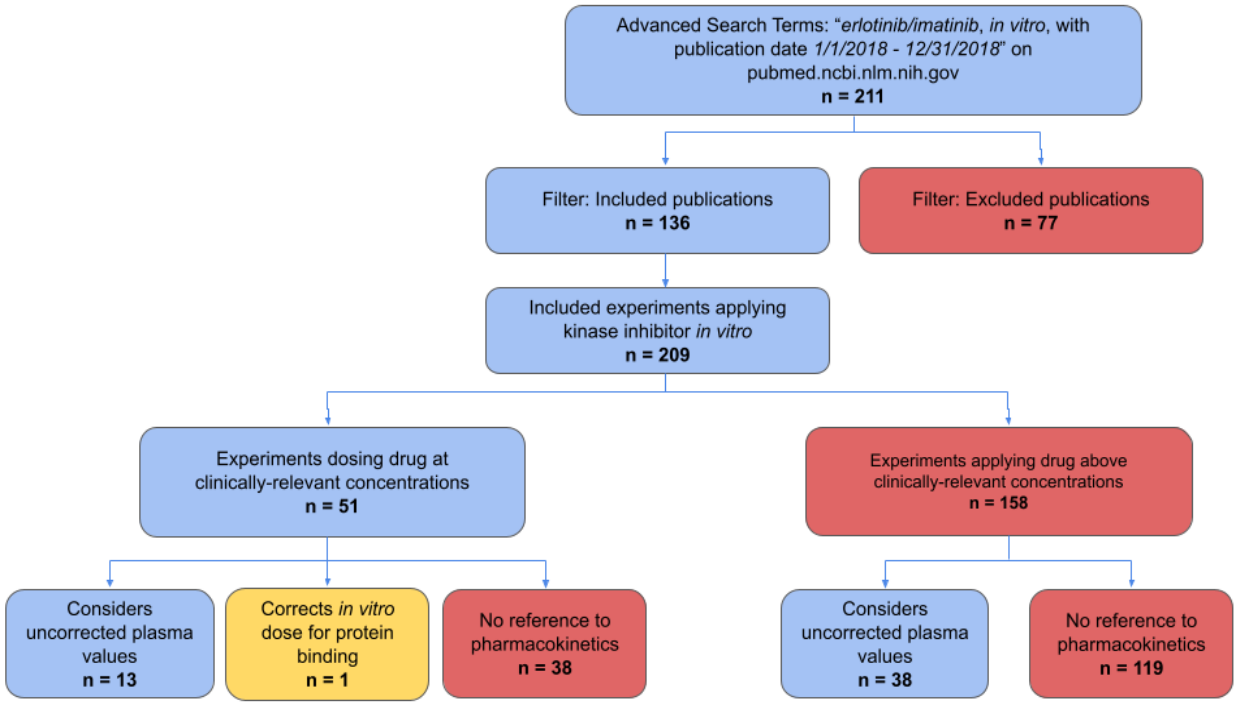

Figure 1. Flowchart of systematic literature review methodology. Results of two PubMed advanced searches were pooled, returning a total of 211 publications. After filtering for inclusion criteria, we analyzed 136 publications which included 209 kinase inhibitor experimental concentrations. Blue boxes indicate review workflow with included publications and experiments applying physiologically relevant doses or referencing pharmacokinetic parameters. Red boxes indicate publications and experiments meeting exclusion criteria, using *in vitro* doses above clinically relevant concentrations, or lacking references to pharmacokinetics in discussion. The yellow box represents the single publication found to incorporate protein binding effects into discussion of its *in vitro* drug concentration.

Considering a drug's pharmacokinetic profile, a highly influential factor on physiological drug activity, in preclinical models permits legitimate translation from *in vitro* studies to clinical trials and beyond. We were interested in finding the prevalence of references to protein binding and pharmacokinetic profiles in studies reporting *in vitro* efficacy, providing insight to the clinical justification that may accompany their experimental drug concentrations. Although the PubMed search yielded *in vitro* experiments applying six different ABL1 inhibitors and eight EGFR inhibitors, we pooled experiments using the top three inhibitors of each target kinase, for which we have reliable validation of mean serum concentrations (SML). These six inhibitors,

listed in Table 1, were analyzed for clinical pharmacokinetic references justifying their experimental *in vitro* concentrations (Figure 2). These experiments were sorted by target kinase, showing a slight disparity in rates of reference to pharmacokinetics accompanying discussion of *in vitro* efficacy by ABL1 (22%) and EGFR (19%) inhibitors. Though they represent only a subset of all the experimental doses gathered, studies of these six kinase inhibitors display the astounding lack of attention paid to applying clinically achievable drug concentrations in *in vitro* models.

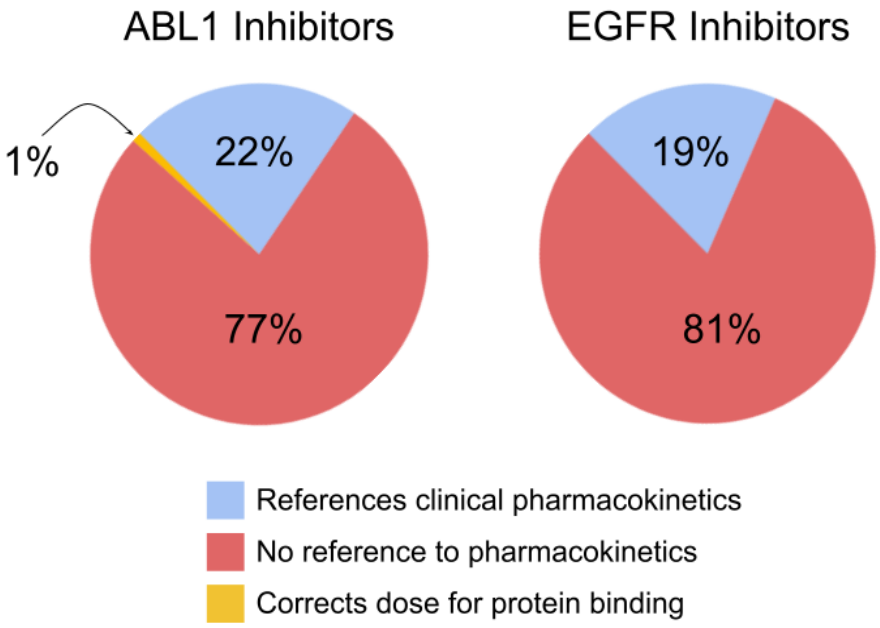

Figure 2. Proportions of ABL1 and EGFR inhibitor *in vitro* experiments referencing clinical pharmacokinetic parameters. Proportions were calculated for experiments applying the search's three most prevalent ABL1 and EGFR inhibitors.

Clinical pharmacokinetic analysis of novel inhibitors provides valuable instruction to further translational studies, facilitating a shift from drug efficacy seen *in vitro* to improving patient outcomes. Once established in the clinic, the clinical literature reports key pharmacokinetic parameters upon which further preclinical experiments may base drug

concentrations, though these guidelines are not followed in all cases. We continued to focus on concentrations applied for the three most-prevalent ABL1 and EGFR inhibitors in our analysis, identifying select *in vitro* concentrations for each drug without references to clinical pharmacokinetics.

Focusing on studies published in journals with impact factor > 3, examples of extravagantly high *in vitro* concentrations were reported for each of the six notable kinase inhibitors (Table 1). Similar to methods applied by Björkhem-Bergman et al., publications in higher-impact journals were chosen for focus, under the assumption that these reports are likely to gain more exposure in the research field. With the lowest ratio between experimental *in vitro* concentration and the inhibitor's effective mean serum concentration of studies reported here, a publication was found to employ erlotinib at a concentration 70-fold higher than is clinically achievable. All six inhibitors, shown to exist physiologically at least 90% plasma protein-bound, displayed substantial shifts in pharmacologically-active fractions after correcting C_{ave} values for protein binding (SML). These publications reporting efficacy by kinases well above clinically achievable values may lead to unwarranted drug development for repurposing, creating excitement behind clinical trials destined for failure.

Table 1. Pharmacokinetic parameters for select experimental *in vitro* concentrations lacking clinical pharmacokinetic dose justification.

Kinase Inhibitor	**Dose**	**C_{ave} in human serum (nM)**† [22, 28-32]	**Effective C_{ave} in human serum (nM)** [27]	**Protein binding** [33]	**Concentration used *in vitro* (nM)**
Afatinib	40 mg	54	7.4	95%	1830 [34]
Dasatinib	100 mg	34	11	96%	20000 [35]
Erlotinib	150 mg	3286	424.7	93%	29730 [36]
Gefitinib	250 mg	456	100.7	90%	12900 [37]
Imatinib	400 mg	3385	444	95%	8060000 [38]
Nilotinib	400 mg*	2149	131	98%	20000 [35]

*Dose administered twice daily, all others administered once daily
†C_{ave} values calculated by dividing reported $AUC_{0\text{-}t}$ by time of observation (t)
Citations included in superscript for individual publications and pharmacokinetics data sources

To attain a macroscale view of *in vitro* concentrations in the preclinical literature, all experiments applying these top-three ABL1 and EGFR inhibitors were sorted by inclusion of references to pharmacokinetics and analyzed for their applied concentrations (Figure 3). Median *in vitro* concentrations for experiments justifying their dose with clinical pharmacokinetic values were lower than those lacking references to pharmacokinetics for five of the six inhibitors.

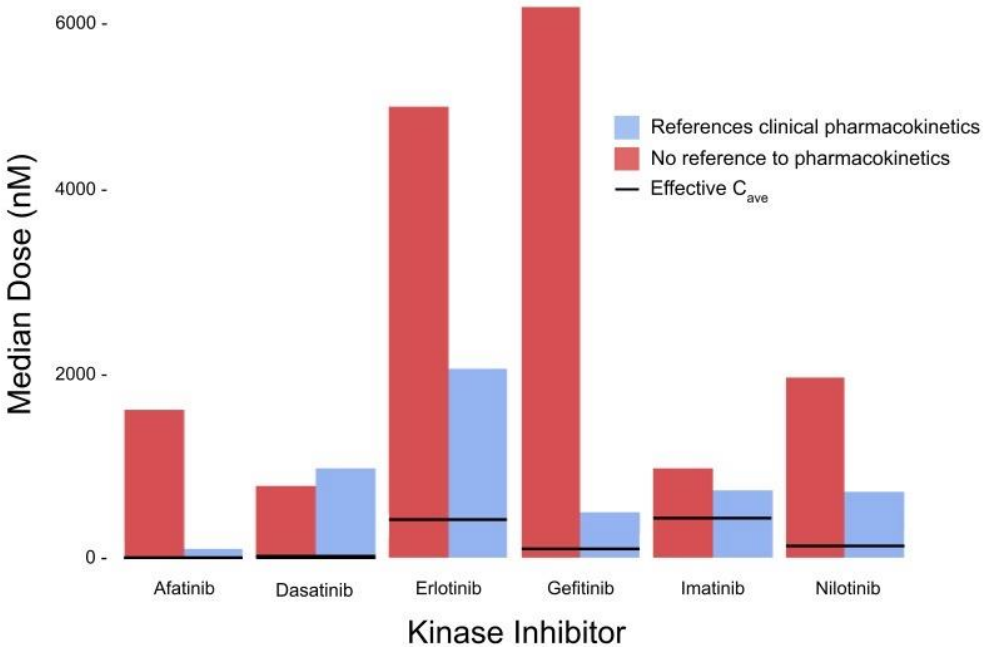

Figure 3. Median experimental *in vitro* concentrations of the three most-cited ABL1 and EGFR inhibitors. Publications correcting for plasma protein binding are considered here to reference clinical pharmacokinetics.

Disparities in median *in vitro* concentrations were observed in all three EGFR inhibitors, while experiments with dasatinib, an ABL1 inhibitor, were the only group found to have a higher median concentration when referencing clinical pharmacokinetics than not. Regardless of clinical pharmacokinetic justification for *in vitro* dosing, all twelve median concentrations were above their respective inhibitor's effective mean serum concentration — providing further evidence of insufficient consideration for pharmacokinetics when conducting *in vitro* studies.

Chapter 4

Discussion

While incredibly valuable to the process of drug development, *in vitro* models may overstate the efficacy of prospective kinase inhibitors when applied at concentrations greater than those clinically achievable. *In vitro* studies using FDA-approved kinase inhibitors have shown convincing activity against a wide range of conditions[34-38], though such reports are inconsequential if the reported drug activity cannot be translated effectively to patients. Low success rates in cancer drug clinical trials direct us to investigate the landscape of kinase inhibitor use in preclinical models; to our knowledge, this work has not been previously documented. Little consideration for physiological phenomena impacting drug concentrations, such as plasma protein binding, was expected in the preclinical literature, as well as the use of inhibitor concentrations that fail to match those cited in clinical pharmacokinetic studies.

In this study, *in vitro* experiments measuring inhibition of ABL1 and EGFR protein kinases showed little regard for pharmacokinetic differences between cell culture models and true physiological effects on serum levels. Our systematic review of the 2018 literature revealed a clear lack of consideration for clinical pharmacokinetics to justify *in vitro* concentrations used, as well as a disparity in concentrations applied by publications referencing or lacking discussion of pharmacokinetic parameters. Such a disparity highlights the value of reciprocity between clinical and preclinical reports, as application of unrealistically high *in vitro* concentrations works directly against the goal of productive translation to clinical use.

While it is important to highlight such a disparity between concentrations in studies including and lacking references to pharmacokinetics, we must consider these concentrations with regards to effective C_{ave} values, a measure of pharmacologically-active dose fractions.

Numerically presented in Table 1, these effective C_{ave} values provide physiologic context to *in vitro* dosing, factoring for the shift in cytotoxicity when experimenting with plasma protein concentrations closer to those in human serum. All of the six most-prevalent kinase inhibitors' median *in vitro* concentrations were found to exceed their effective C_{ave} values, regardless of the studies' inclusion of references to clinical pharmacokinetics (Figure 3). Reporting median values, we show that over half of the studies publish significant activity at concentrations above those reported here, some orders of magnitude above the calculated medians and entirely out of the scope of what could conceivably be obtained clinically.

These results align quite directly with arguments proposed by Smith and Houghton, which brought light to publications reporting anticancer activity by unrealistic *in vitro* concentrations of various compounds[25]. Specifically, publications displaying *in vitro* efficacy of sorafenib, a multi-kinase inhibitor, are disputed as clinically irrelevant due to their reporting IC_{50} values in the 1-10 μM range[39-42]. Though these concentrations align directly with those physiologically achievable, sorafenib exists in a 99.7% plasma protein-bound state[43], substantially reducing the fraction of bioavailable drug far below the micromolar range at which excitement is generated through *in vitro* models. The Cancer Cell Line Encyclopedia, a next-generation predictive *in vitro* model of drug efficacy, reports sorafenib IC_{50} values in 98% of cell lines tested greater than 1 μM[44] — further declaration of kinase inhibitor efficacy without consideration for binding effects that leave only 0.3% of the clinical dose active. Disparities between clinically achievable concentrations and those applied both in single cases (Table 1) and widespread (Figure 3) provide further evidence for Smith and Houghton's assertions.

Focusing on kinase inhibitors established as anticancer compounds, our results address the reality of *in vitro* experimentation on drugs past the cancer treatment realm. The

aforementioned proposal outlining flawed claims on sorafenib efficacy includes lengthy discussion pinpointing examples of *in vitro* vorinostat and metformin experimentation at concentrations above those achievable in steady-state plasma. Pharmacokinetic analysis reveals a relatively short clinical half-life for vorinostat[45], creating a disconnect between physiological drug action and *in vitro* conditions. Similarly, metformin is dependent on organic ion transporter activity to displace the drug into target cells[46], a function impaired in several malignancies[47]. Elimination, membrane transport, and plasma protein binding represent only a fraction of pharmacokinetic parameters affecting drug activity, along with incredibly complex interactions that vary physiological conditions. Consideration for these effects may require widespread discussion on the pharmacokinetics and pharmacodynamics of compounds applied in preclinical studies, a standard likely to shift *in vitro* concentrations closer to those more clinically relevant.

Although the literature review returned 209 *in vitro* inhibitor experiments with translational implications, a key limitation of this study is the restriction to PubMed searches based on only two kinase inhibitors. Our analysis focused primarily on the six most-commonly applied inhibitors, although imatinib and erlotinib, the drugs for which search terms were specified, combined for 121 out of the total 177 *in vitro* concentrations analyzed. To gain a more complete representation of the *in vitro* kinase inhibitor literature, individual searches for all six drugs may be necessary in future studies. Performing a specific search for dasatinib studies, for example, may direct focus toward works by authors who have greater familiarity with clinically relevant dasatinib concentrations. Many studies in our analysis included *in vitro* screens with several drugs, often with focus on imatinib or erlotinib; individual drug searches would permit targeting for studies solely applying the other four inhibitors and likely considering their clinical pharmacokinetics.

Of the six most commonly used drugs in our analysis, five were found to have higher median concentrations in the *in vitro* literature when lacking pharmacokinetic or clinical justification for the applied dose than those referencing such pharmacokinetic parameters (Figure 3). We believe this disparity in median concentrations demonstrates two possible cases, each immensely critical to effective preclinical drug validation. First, publications communicating inhibitor efficacy may report significant drug activity at a range of concentrations, the lowest of which then being analyzed in this study. Of these publications, those discussing pharmacokinetics generally do so to prospectively justify and provide clinical context for the concentrations applied, while those lacking references to pharmacokinetics are believed to have more often baselessly tested relatively high concentrations that showed significant *in vitro* effects. Alternatively, publications experimenting with a range of drug concentrations often report IC_{50} values, the drug concentration at which a biological process is inhibited by 50%. It is hypothesized here that a disparity between these *in vitro* concentrations may have arisen from reporting efficacy at irrelevant concentrations omitting references to pharmacokinetics, while those in the range of clinical relevance truly bolster their validity by including pharmacokinetic parameter consideration. In line with these views, visual inspection of the top-six inhibitors' *in vitro* concentrations reveals high, round concentrations (e.g. 5 or 10 μM) clustered in publications lacking references to pharmacokinetics, while lower, exact number concentrations (e.g. 9.9 or 733 nM) appear more concentrated in studies referencing clinical pharmacokinetic data (Supplementary File 2).

In summary, developing a drug from bench-to-bedside requires several evaluations for efficacy, with *in vitro* experimentation taking place quite early in the process. Low success rates for oncology drugs that reach Phase I clinical trials bring attention to the need for systemic

reform in preclinical models[48], as well as for journals communicating unconvincing preclinical data to the science community. The present study exhibits a key source of these unconvincing arguments, a lack of consideration for clinical pharmacokinetics to inform kinase inhibitor concentrations applied to *in vitro* assays. Though claiming efficacy to promote translational development, approximately four-fifths of kinase inhibitor studies with *in vitro* experiments here fail to reference clinical pharmacokinetics or justify concentrations with pharmacokinetic parameters. This analysis revealed only one study considering its *in vitro* concentration with respect to that factoring for plasma protein binding, a pharmacokinetic phenomenon highly influential to drug activity. Across all six inhibitors in our focus, median *in vitro* concentrations exceeded those clinically achievable, with the widest gap observed in those studies failing to justify concentrations with pharmacokinetic data. Fostering an agreement between clinical and preclinical conditions may spark the transparency and data-driven experimentation necessary to most efficiently translate groundbreaking therapies.

Appendix A Supplementary Files

Supplementary File 1. Raw systematic review data.

Supplementary File 2. *In vitro* concentrations for the six most-used inhibitors sorted by drug and inclusion of pharmacokinetic reference.

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ACADEMIC VITA

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Education

B.S. in Biology (Genetics and Developmental Biology)
Minor in Sociology

Thesis Title: *Evaluating In Vitro Dosing of Tyrosine Kinase Inhibitors for Clinical Repurposing: A Systematic Review*
Thesis Supervisor: Dr. Justin Pritchard

Relevant Experience

Summer Undergraduate Researcher — Philadelphia, PA
Perelman School of Medicine at the University of Pennsylvania — Summer 2020
Mentor: Dr. Saar Gill; Center for Cellular Immunotherapies
Description: Created a proof-of-concept experimental plan to introduce a novel horizontal gene transfer system, based on the bacterial conjugation system, to chimeric antigen receptor-expressing T cells.

Amgen Scholar — Pasadena, CA
California Institute of Technology — Summer 2019
Mentor: Dr. Kaihang Wang; The Division of Biology and Biological Engineering
Description: Generated a novel translationally-active synthetic RNA replicon based on the coliphage Qβ viral replication system.

Summer Undergraduate Researcher — Nashville, TN
Vanderbilt University Medical Center — Summer 2018
Mentor: Dr. Borden Lacy; Department of Pathology, Microbiology, and Immunology
Description: Applied techniques in protein purification and crystallization in an effort to determine crystal and molecular structures of HemA and HssR, heme-regulating proteins expressed by *Staphylococcus aureus*.

Awards

Dean's Science Scholarship — 2019 - present
Penn State Dean's List (all semesters) — 2017 - present
ABRCMS Presentation Award in Biochemistry — November 2018
Lou De Felice Summer Student Travel Award — August 2018
President's Freshman Award — April 2018
Karen Bruno Ganter Award for Sacrifice and Commitment — June 2017

Community Service and Leadership

Centre Volunteers in Medicine, Front Desk Volunteer	January 2020 - present
Millennium Society, Marathon Dancer and Fundraising Chair	August 2018 – May 2020
World in Conversation, Facilitator and Dialogue Assistant	January 2018 – May 2020
Millennium Scholars Program, Peer Mentor and Tutor	August 2019 - Present