This is the **submitted version** of an article published in:

Uranga, Jon, Lukas Hasecke, Jonny Proppe, Jan Fingerhut, and Ricardo A. Mata. "Theoretical Studies of the Acid–Base Equilibria in a Model Active Site of the Human 20S Proteasome." Journal of Chemical Information and Modeling 61, no. 4 (2021): 1942-1953. https://doi.org/10.1021/acs.jcim.0c01459.

Theoretical Studies of the Acid-Base Equilibria in a Model Active Site of the Human 20S Proteasome

Jon Uranga,* Lukas Hasecke, Jonny Proppe, Jan Fingerhut, and Ricardo A. Mata*

Institute of Physical Chemistry, University of Göttingen, Tammannstrasse 6, 37077, Göttingen, Germany

E-mail: juranga@gwdg.de; rmata@gwdg.de

Abstract

The 20S proteasome is a macromolecule responsible for the chemical step in the ubiquitin-proteasome system of degrading unnecessary and unused proteins of the cell. It plays a central role both in the rapid growth of cancer cells as well as in viral infection cycles. Herein, we present a computational study of the acid-base equilibria in an active site of the human proteasome (Caspase-Like), an aspect which is often neglected despite the crucial role protons play in the catalysis. As example substrates, we take the inhibition by epoxy and boronic acid containing warheads. We have combined cluster quantum mechanical calculations, replica exchange molecular dynamics and Bayesian optimization of non-bonded potential terms in the inhibitors. In relation to the latter, we propose an easily scalable approach to the reevaluation of non-bonded potentials making use of the hybrid Quantum Mechanics Molecular Mechanics (QM/MM) dynamics information. Our results show that coupled acid-base equilibria need to be considered when modeling the inhibition mechanism. The coupling between a neighboring lysine and the reacting threonine is not affected by the presence of the studied inhibitors.

Introduction

The human body operates through a series of interconnected cycles. Proteins are created and destroyed by a sensitive and meticulous chain of regulation processes which we only have come to grasp in the last few decades. One of the key players in this cycle is the proteasome, an enzymatic complex responsible for the degradation of unneeded or damaged proteins.[1] In case the unused or damaged proteins are not efficiently removed from the cell, it is known that the cell will commit apoptosis.[2–4] In cancer, abnormal cells exhibit uncontrolled growth, requiring a speeding up of the cycle and the proteasomes to work at very high rates.[5] Thereby, its inhibition emerges as an effective road to treatment.[6,7] It is also a pharmaceutical application of interest in the fight against coronaviruses, with the ubiquitin-proteasome system being identified as a central aspect of the infection cycle.[8] Despite best efforts, the development of reliable descriptors for inhibition capabilities has been sluggish,[9] while *in silico* simulations are still heavily dependent on empirical data of small candidate inhibitor libraries.

Different kinds of proteasome inhibitors have been proposed, those that interact with the active site by non-bonded interactions and the ones that covalently bind to an active residue in the pocket.[10] The inhibitors that cova-

lently bind to the active pocket are commonly seen as the ones with largest pharmaceutical potential, showing higher efficiency at lower dosages, and so lowering the side effects.[11] On the other hand, inhibitors which do not form any covalent bond can be also of great interest in long term therapies. The only covalent binding inhibitors available on the market are epoxyketone (Carfilzomib) and boronic acid based (Bortezomib and Ixazomib) compounds. Other inhibitors under these two families are being considered or currently under clinical trial. The mechanism of inhibition is discussed later in the text. We have focused on four different molecules chemically similar to the most promising drugs available or on trial for the proteasome, i.e. Ixazomib and Bortezomib (containing boronic acid warheads, the only market inhibitors in this study) and Oprozomib and Dihydroeponemycin (containing epoxy warheads), which are presented in Figure **??**.[12–17]

The 20S proteasome contains three different active sites: Caspase-Like (β1), Chymotrypsin-Like (β5), Trypsin-Like (β2), see Figure **??**.[18–20] It has been postulated that each site is specific to particular substrates and so it has been accepted that Chymotryptsin-Like performs cleavage after hydrophobic residues, Trypsin-Like after basic residues and Caspase-Like after acidic residues. However, one should bear in mind that this selectivity has also been placed in doubt. Studies on real proteins showed that the active sites are less specific than expected.[21]

All of these sites contain a terminal Threonine (Thr1) residue, which reacts with the substrates or the inhibitors by a nucleophilic attack through the alcohol at the side chain of the mentioned residue. Fig. **??** shows tentative mechanisms for both boronates and epoxyketone inhibitors. The chemistry at hand is quite clearly acid-base catalysis and as such the understanding of solvation within the site, protonation states before and during the reaction should be carefully assessed. Acid-base equilibria could offer a sensible explanation to the selectivity observed, and why it might change between *in vivo* and *in vitro*. Despite all this, the role of protons in enzyme communication and catalysis is often times neglected. Protons are extremely labile and mostly invisible in crystal structure data. NMR can be better used to monitor these particles, but the time resolution of such experiments is limited. Also in computations the role of protons in different mechanisms tend to be undervalued. Usual set up protocols in theoretical biophysics involve the estimation of pK_a values through fast (partly) empirical methods[22,23] and keeping such configurations fixed. There is an inherent risk to this approach, specially, if the pK_a values are estimated for the end chain, in close proximity to drug molecules or in residue groups which might share proton charges among them. One should also be careful about only evaluating protonation states for one step of the mechanism, since as a reaction proceeds, shifts in the electronic density at the pocket might induce also significant pK_a shifts. Several cases can be found in the literature where proton shifts are an integral part of the reaction mechanism and the catalytic machinery. Significant pK_a shifts of residues in the interior of enzymes have been reported, which would come to explain the *low* optimal pH observed for several enzymes.[24–26] Through the process of adding and/or removing protons, enzymes may form suitable environments for reactions to take place, and modulate different sites.[27] Indeed, there are examples of extreme electric fields being caused by shifting protons, leading to the catalysis.[28] Some of the authors have recently shown how two different thiamine diphosphate dependent enzymes can make use of hydrogen bond networks to communicate local changes in potential.[29]

In the reaction at hand, the initial nucleophilic attack requires the deprotonation of the alcohol in Thr1. Given that the optimal pH value of the proteasome is around 7,[30,31] it is counter intuitive how such a deprotonation could be linked to the optimal pH, as the pK_a value of Thr OH groups are much higher and the deprotonated states should not be populated. Under this assumption, at around neutral pH, the activity of the site should not be modulated unless it is coupled to other protonation changes in nearby residues. Previous studies have suggested a natural zwitterionic

state for the Thr1 residue, which would partly solve the conundrum.[32] At the same time, it has been argued that the residues concomitant to Thr1, i.e. Lys33 and Asp17, do not participate in the reaction.[32] This seems counterintuitive given that the latter are highly preserved across different proteasomes and the acid-base character of the reaction. Previous computational simulations actually hinted at an active role of the two residues in the nucleophilic attack of Thr1.[20,33]

In this work, the mechanism of the reaction of different inhibitor (or inhibitor-like) drugs with the Caspase-Like active center of the proteasome is investigated with theoretical calculations. We focus on computational pK_a estimations and the modelling of protons in the nucleophilic attack. This will constitute the only chemical step in boronic acid inhibitors. We show that our computational findings fall in accordance with the experimentally observed optimal pH at which the sites work if one considers a coupling between a neighboring lysine and the reacting threonine.

Computational methods

Cluster calculations. Our cluster models include only up to Thr1, Asp17 and Lys33 residues, which were capped at the backbone N and C atoms, and saturated with H atoms and the warhead of the inhibitor (Figure S2). Microsolvation has been applied whenever mentioned. We have performed geometry and frequency calculations, in the native (PDB code 5LE5), bortezomib (PDB code 5LF3) and dihydroeponemycin containing structures (PDB code 5LF1), constraining the α-carbons to their crystallographic positions. The Gaussian16 software package (RevA.03)[34] was employed for all density functional theory (DFT) calculations, using the B3LYP functional[35,36] and dispersion corrections,[37] together with the def2-SVPD basis sets for geometry optimizations.[38,39] Harmonic vibrational frequencies were computed by analytical differentiation of gradients. The frequencies were used to evaluate the Gibbs free energy in the harmonic oscillator approximation. Single point calculations have been carried out with the def2-TZVPD basis set. All calculations were done employing the SMD continuum model with the permitivity of water at 298 K.[40]

Bayesian Optimization (BO) is a machine learning technique tailored for efficient multidimensional optimizations. It has found previous application in force field optimizations, but to the best of our knowledge this is the first time it is applied to refit potential parameters according to a target QM/MM quantity. The BO procedure was performed with GPyOpt.[41,42] The underlying model was a Gaussian Process Regression with a Matérn52 kernel.

We have employed radial distribution functions of the inhibitor molecules in water as the target for the fit, computed at the PM6/TIP3P level of theory. The purpose of the BO procedure was to scale the original GAFF parameters to obtain the best possible similarity between the mentioned QM/MM radial distribution functions and the one from the BO optimized MM parameters. The actual target function was the standard deviation of the radial distribution function given by the difference between MM and QM/MM results.

The non-bonded parameters have been optimized with this procedure, departing from the Lennard-Jones parameters given by GAFF and the point charges obtained through RESP. This corresponds to the default parameterisation of the drug. Given the importance of hydrogen bond interactions of the warhead with the residues in the active site, the object of optimization were the non-bonded parameters of the polar atoms in the warhead. In order to preserve the overall neutral charge of the molecules the charges were scaled under the constraint:

$$\sum_i \lambda_q q_i = 0 \tag{1}$$

in the same way, the Lennard-Jones parameters were scaled as

$$V_{\mathrm{LJ}}(r) = 4\lambda_\epsilon \epsilon \left[\left(\frac{\lambda_\sigma \sigma}{r} \right)^{12} - \left(\frac{\lambda_\sigma \sigma}{r} \right)^{6} \right]. \tag{2}$$

The procedure is iterative, see Figure **??**.

One starts by collecting MM radial distribution functions in a grid for different scaling factors. The first ten points per optimization dimension are given by the Latin Hypercube sampling in order to gain insight of the surface that we are exploring.[43] Then, the expected improvement acquisition function is the one responsible to select the following point, by which the standard deviation is minimised.

Reference QM/MM dynamics. The studied drugs were simulated in a periodic box which extends 20 Å away from the drug with TIP3P water molecules. The parameters were assigned by GAFF and the warheads were included in the QM region employing PM6. We have employed a cutoff of 8 Å for the non-bonded interactions, using particle-mesh Ewald summation with a fourth-order B-spline interpolation and a tolerance of 10^{-5}. The non-bonded list was updated every 50 fs and the time step was set up to 2 fs, employing SHAKE to constrain bonds involving hydrogen atoms.

The system was minimised for 2000 cycles, 1000 with steepest descendent and 1000 with conjugate gradient. Then, the system was heated for 400 ps using a NVT ensemble, increasing the temperature from 0 to 300 K in the first 300 ps with the equal intervals, using Langevin dynamics with a collision frequency of 2.0 ps^{-1}. The system was then equilibrated for 400 ps using a NPT ensemble with isotropic position scaling and a relaxation time of 5.0 ps. The production phase was then performed for 5 ns saving snapshots every 50 steps.

MM dynamics. The molecular dynamics were performed in a periodic box which extends 20 Å away from the drug with TIP3P water molecules. The initial parameters were assigned by GAFF. We have employed a cut off of 8 Å for the non-bonded interactions, using particle-mesh Ewald summation with a fourth-order B-spline interpolation and a tolerance of 10^{-5}. The non-bonded list was updated every 50 fs and the MD time step was set up to 2 fs, employing SHAKE algorithm to constrain bonds involving hydrogen atoms.

The system was minimised for 2000 cycles, 1000 with steepest descendent and 1000 with conjugate gradient. Then, the system was heated up for 1 ns using a NVT ensemble, increasing the temperature from 0 to 300 K in the first 800 ps with equal intervals, using Langevin dynamics with a collision frequency of 2.0 ps^{-1}. The system was then equilibrated for 1 ns using a NPT ensemble with isotropic position scaling and a relaxation time of 5.0 ps. The production phase was then performed for 3 ns saving snapshots every 10 steps.

For the following points given by the Bayesian Optimization, the previous last structure was taken, heating it up for 400 ps from 0 to 300 K in the last 340 ps with equal intervals, using Langevin dynamics with a collision frequency of 2.0 ps^{-1}. The system was then equilibrated for 100 ps using a NPT ensemble with isotropic position scaling and a relaxation time of 5.0 ps. It is then, when the production phase was set to run.

Replica-Exchange set up. The performed simulations correspond to a single monomer (Caspase-Like monomer, chain N of the referenced PDB) which was initially protonated according to the computed pK_a values by the PROPKA algorithm and taking into account the crystalization pH of 6.7. Different protonation states of Thr1 were parametrized using the Gaussian09 software package (RevD.01).[44] We have selected the Thr1 residue capping at C atom and substituting the following N atom by a H atom. Different protonation states were optimized at B3LYP/def2-SVP and the point charges were computed within RESP at HF/6-31G* level, using the AMBER atom types.

The inhibitors were optimized constraining all the heavy atoms only allowing the H atoms and the warhead to move (Figure S1). The point charges were computed within RESP at HF/6-31G* level, using the GAFF atom types. The B atom was assigned with Lennard-Jones parameters of sp^2 c atom type. The bonded parameters of the boronic containing warheads, bond and angle, were obtained through the Seminario approach.[45] For this purpose we have optimized the inhibitor without any constraints and per-

formed a frequency calculation at B3LYP/def2-SVP, which is then used to obtain the mentioned bonded parameters.

Replica-Exchange simulations. All replica exchange constant pH simulations were carried with the AMBER software package,[46,47] using sander or pmemd, employing the FF14SB force field[48] for the protein and the GAFF force field for the ligand,[49,50] whose point charges were estimated with RESP. The protein-ligand complex was set in a periodic cuboid box of 8 Å, between the protein and the periodic box wall, of TIP3P water molecules and neutralized with Na^+ and Cl^- counterions. The cut off for non-bonded interactions was set to 8 Å, employing particle-mesh Ewald summation with a fourth-order B-spline interpolation and a tolerance of 10^{-5}. The non-bonded list was updated every 50 fs and the MD time step was set up to 2 fs, employing the SHAKE algorithm[51] to constrain bonds involving hydrogen atoms.

The hydrogen atoms of the system were first minimized for 2000 cycles (1000 with steepest descendent and 1000 with conjugate gradient), by restraining the rest of the atoms with a 10.0 kcal/mol/Å^2 force constant. Then, the system was minimized for 3000 cycles (1000 with steepest descendent and 2000 with conjugate gradient), restraining the backbone atoms of the protein with a 10.0 kcal/mol/Å^2 force constant. Finally, the system was minimized for 10000 cycles (2000 with steepest descendent and 8000 with conjugate gradient) without restraints (substrate included).

The system was then heated up from 0 to 300 K for 800 ps using a NVT ensemble using Langevin dynamics with a collision frequency of 5.0 ps^{-1}. Followed by another 200 ps at 300 K in the same ensemble. We have then equilibrated the system for 1000 ps in NPT ensemble at 300 K and with isotropic position scaling and a relaxation time of 5.0 ps. The production phase is done using 16 replicas employing the same ensemble and parameters as in the equilibration. The production is carried for 128 ns, attempting to change the protonation every 20 fs and attempting replica exchanges every 8 ps.[47,52] The heating and production phase were performed using GPUs.[53]

Results and Discussion

Cluster calculations of the active site

In a first set of calculations, we have applied electronic structure methods in the modeling of the active site. Our intent was to make use of a cluster approach in order to compute the pK_a values of a few selected residues. The usefulness of such cluster approaches in the study of reaction mechanisms has been previously highlighted.[54]

pK_a shift. In order to compute pK_a values we have made use of two model systems: the first one containing only the Thr1 residue and two explicit water molecules and the second one including Thr1, Asp17 and Lys33 residues, plus the two aforementioned water molecules, see Figure **??**. The last model system is a minimal representation of the active site, including only three residues which are preserved along the the three different active sites, whose electrostatics may play a crucial role in the activation mechanism.[55] For the estimation of pK_a values we have used a linear regression with two empirical parameters,[56] C_0 and C_1:

$$pK_a = C_0 + C_1 \cdot \frac{\Delta G^0}{\ln(10)RT} \qquad (3)$$

using the Gibbs free energy value of -271.9 kcal/mol for the proton in solvent.[57–59] The training set and linear regression used in this work can be found in the Supporting Information (Table S1 and Table S2).

The pK_a of the terminal Thr1N atom is documented to range from 6 to 9. We have computed the pK_a only accounting for the Thr1 residue, obtaining a value of 7.4 ± 1.2. This value is very close to the aforementioned optimal proteasome pH, which is said to be around 7, at which point there would be a close to even distribution of protonated and deprotonated states. The same procedure was ap-

plied to calculate the pK_a value of the Thr1Oγ, known to be around 15.[56] As it should be expected, the value will strongly depend on the protonation of the amine moiety. A value of 15.5 ± 2.6 is obtained for deprotonated NH_2 and a lowering of the pK_a is achieved by setting it as NH_3^+, ($pK_a = 11.3 \pm 1.8$). The results are shown schematically in Figure **??**a.

The pK_a values for the Thr1Oγ will not only be connected to the amine protonation state but will, also be modulated through the protonation states of the residues nearby. Therefore, we extended to a minimal model of the environment, including the neighboring residues (Asp17 and Lys33). These are the (potentially) charged closest residues to the Thr1Oγ, and could in turn impact the activation step.

Setting Thr1 and Lys33 as NH_2 we obtain a pK_a of 17.2 for the deprotonation of the alcohol. Setting any of the two sites as NH_3^+ the pK_a of the alcohol is lowered roughly by 3-5 units. Finally, if both N atoms are set as NH_3^+, the pK_a of the alcohol is lowered even more, down to $pK_a = 10.8$. The discussed combination of protonation states and pK_a can be seen in Figure **??**b. It is interesting to observe that the protonation of the Lys33 residue has a slightly stronger impact in the pK_a of the alcohol than the amine in Thr1 (difference of 2 units). This should be taken with some caution, due to the size of the model employed, but is in line with the physics of the problem. The Lys33 residue, due to its location, will interact with the alcohol moiety. Therefore, any charge placed there will be in closer contact than even a charge in the same molecule.

The pK_a values of residue Lys33 are also analysed. These values are presented later on for a contrast analysis with replica exchange simulations with the purpose of estimating the trustworthiness of the presented methodology for the pK_a estimation. The results again show that the pK_a value shifts up (about 1 unit) upon the removal of the proton from the terminal N atom, see Figure **??**c.

Inhibition reaction pathway. The cluster models predict that the pK_a value of the terminal N atom is very close to the observed optimal pH. Now we will analyse the pK_a values along the inhibition reaction coordinate, which will expectedly also couple with the energetics of the different protonation states. The results of cluster models have to be considered with caution, as they may lack some important interactions at the active site. This is particularly relevant in reactions with a large shift/transfer in molecular charge (a clear example can be found in sulfite oxidation).[60,61]

The reaction pathways have been modeled for both a boronic and epoxyketone warheads, see Figures **??** and **??**. In the former case, there is only one reaction step, the Thr1Oγ nucleophilic attack at the Boron atom, leading to the covalently bound tetrahedral intermediate. In the case of the epoxyketone, this is followed by the ring closure reaction, which we had also previously modeled in another study of the system.[20]

The pK_a values of the products are observed to be shifted. In both cases, boronic acid and epoxyketone containing warheads, the pK_a shift is upwards (about 3 units). Hence, we expect that the amount of protons along the reaction coordinate will not be necessarily constant, as the system's proton affinity increases respective to the inhibitor present at the pocket.

Combining the information from the cluster pK_a calculations and the reaction paths, we observe that the optimal pH is connected to both the Lys33 and the amine in Thr1. By protonating the amine groups, it is easier for the nucleophilic oxygen to be deprotonated. However, below the optimal pH, both groups would be protonated, effectively blocking the reaction.

In the work by Saha et al.[62] it is suggested that both Lys33 and the amino group of Thr1 are uncharged, based on a much lower pK_a for Lys33. We have also calculated the barriers in this case, which change only slightly (2.2 kcal/mol for epoxy containing warhead inhibitors and 0.7 kcal/mol for boronic acid containing inhibitors). Given the long-range nature of electrostatic interactions, the convergence of the computed pK_a in dependence of the simulated system size can be difficult to achieve. Cluster models in many cases will prove insufficient, since they lack important interactions around the active site. However,

these effects do tend to be additive in nature, and the relative shifts are less model-dependent (as the MD results later in the text confirm). In this work, we have focused on the relative shift of pK_a in the titratable sites, not so much on their absolute values. For a more conclusive assignment of the Lys protonation state, one would require an extension of the models used.

MD simulations of inhibitors and active site

The model calculations performed in the previous section provided some important insight, which requires further confirmation. The results hint at how the activation step could be facilitated by a pK_a shift of the Thr1 residue. However, it would be important to validate the pK_a values obtained including more residues, as well as verify if the observed low value for Lys33 is kept when one includes the inhibitor. In the following sections, we move away from cluster models to the inclusion of the enzyme environment.

In order to study the impact of inhibitor(-like) molecules, one should be aware that the quantities we are analysing will react very sensitively to the parameterization of the compounds in question. Therefore, to assert the robustness of the simulations, we constructed two sets of parameters for each inhibitor molecule. In one series of calculations, we apply a standard GAFF parameterization. In a second series, we make use of refitted potential terms based on QM/MM simulations of the compounds in water. The latter fitting procedure is carried out through Bayesian Optimization. We describe in the next section the procedure used and later compare the two parameterizations in determining pK_a values for the active site in the presence of the non-covalently bound inhibitors.

Bayesian Optimization

First of all, we test the proposed method for epoxyketone containing inhibitors, whose force field parameters are present in the GAFF library, with the aim of validating it. Then, the missing parameters for boronic acid containing warheads are obtained. All the procedures can be seen in the Supporting Information (Table S3 and Figures S3-S10).

BO parameters for epoxyketones Dihydroeponemycin and Oprozomib are the two compounds included in this study containing an epoxyketone warhead. In this case, the activated Thr1 alkoxy group attacks the ketone carbon atom. The starting parameters are provided by the standard GAFF protocol. Five parameters were used in total: a scaling factor for all point charges in the molecule, and four parameters for the vdW terms of the carbonyl and the epoxy moieties' O atoms. There are two reasons why the whole molecular charge is scaled and not just a local refitting of the warhead atoms. First of all, it guarantees charge conservation by construction. Secondly, it keeps the molecular dipole orientation as obtained by RESP, albeit changing the magnitude. It is thereby possible to alter the local interactions while maintaining the pattern of longer range interactions (to which the radial distribution function is less sensitive). We tested two different approaches to the BO. In a first optimization, we refitted the parameters iteratively (it.). This procedure is discussed below. In another optimization, we optimized all 5 parameters simultaneously. It is the composite (com.) approach, which has the potential to deliver best fitted curves, but is only achievable by a multivariate approach such as BO.

We start by describing the step-wise BO (it.). The point charges were first scaled following ten points given by the latin hypercube sampling. Based on the results of these first MD runs, the BO algorithm is used with the purpose of obtaining the best scaling factors for the charge which minimises the radial distribution obtained by MM, see Figure **??**. It is important to note that the Lennard-Jones parameters are untouched at this stage of the procedure.

Once the best scaling factor for the charge is obtained we have used the same strategy in order to scale the Lennard-Jones parameters. O35 corresponds to the O atom in the epoxy moiety. The evaluated points and the

surface are shown in Figure **??**. If one moves away from that point, the standard deviation increases, obtaining a worse representation of the radial distribution function by MM parameters. It is interesting to note that the σ parameter has greater influence on the radial distributions than the ϵ parameter.

The obtained scaling factors for the charge and Lennard-Jones are close to unity which indicate that the parameters given by GAFF are reliable. The comparison between radial distribution functions is shown in Figure **??**.

Boronic acid containing inhibitors

Bortezomib and Ixazomib are inhibitors containing this type of warhead, that upon the activated Thr1 is being attacked by the formed alkoxy group. Our goal is to study the non covalent interactions preliminar to the covalent bond formation. While performing the parametrization of the drugs, it is common some parameters not to be available. This is the case of the boron atom herein, for which no data can be found in GAFF. In this particular case, we have used the Lennard-Jones parameters of sp^2 C atom for the boron atom, and Bayesian Optimizations come as an ideal method in order to obtain reliable parameters.

The results for the multidimensional optimization procedure lead to better results of the radial distribution function.

Replica exchange constant pH simulations

In order to verify some of the findings made in the cluster calculations, we performed molecular dynamics (MD) simulations of a single chain (Caspase-Like), as a more realistic model of the proteasome active site. This is still far from a description of the whole proteasome which weights about 750 kDa, but a necessary approximation given the cost of the constant pH simulations. The simulations were performed with X-ray structure of the 20S proteasome.

First, we wish to address the effect of different protonation states to the activation process of Thr1. It is not possible to conduct constant pH simulations for the Thr1Oγ, since there is no paramaterisation for the group. Therefore, we focus on the protonation state of the Lys33, and if the picture obtained in the cluster calculations is the same when simulating the larger system.

We have set up different protonation states for Thr1, computing the pK_a value of the neighboring Lys33 residue. The obtained results, see Table 1, show that upon the addition of a proton to residue Thr1, the pK_a value of residue Lys33 drops to a value of 7.79, while the regular is estimated to be 10.4.[25]

The value of 10.76, obtained when the Thr1 amine is deprotonated, is consistent with the cluster calculations. However, we observe a much larger drop in the pK_a when this amine group is charged. This would hint at an even stronger coupling. One of the two residues will be protonated. If we were to believe that the nucleophilic attack happens with the Thr1 in a zwitterionic state, this would only be possible by keeping the Lys33 protonated.

Table 1: pK_a and Hill coefficient (n) values of Lys33 in the Caspase pocket varying the protonation state of Thr1.

	NH_2 OH	NH_3^+ O^-	NH_3^+ OH
pK_a	10.76 ±0.01	10.50 ±0.05	7.79 ±0.03
n	0.84 ±0.01	0.62 ±0.04	0.68 ±0.03

Replica exchange constant pH with the drug in the Caspase pocket

In order to test the effect of the drug in the pK_a values we have conducted replica exchange simulations with four different drugs in the Caspase-Like pocket. The dynamics were performed employing the non optimized parameters and also the parameters optimized by the multidimensional Bayesian Optimizations (com.). The results, presented in Figure **??**, show little differences in the pK_a values of Lys33 upon the presence of the inhibitors. It should be noted, however, that this would not be quite clear with the default GAFF parameters. It seems advisable to carry out such robustness tests when modeling novel inhibitors.

The results show that the pK_a values of the Lys33 are largely unaffected by the inhibitors. This would in turn mean that the acid-base coupling between Thr1 and Lys33 is likely not disrupted by the studied inhibitors. Similar acid-base equilibria should be observed before and after the inhibitor binds.

Conclusions

Theoretical investigations carried out in the proteasome have made different suggestions regarding the chemical bottleneck for inhibition or proteolysis in the proteasome.[32,63,64] Some of these studies were based on assumptions which have in the meantime been discarded. One such assumption relates to the inhibition mechanism of epoxyketones.[65] Recent high-resolution crystal data and computational studies have shown that instead of the expected 6-membered ring final product, the covalently bound inhibitor forms a 7-membered ring with Thr1.[20,33] On the other hand, the Thr1 residue has often been assumed to be deprotonated (amine terminal Thr1) based on rough model pK_a estimation.[22,23,66,67] Surprisingly, the obtained pK_a value is close to the optimal pH, and so it is not straightforward to set this residue as deprotonated. Indeed, it is known that the pK_a value of the terminal N atom ranges from 6.8 to 9.1.[68] The modulation of the enzymatic activity through the shift of protons could also enable communication processes between active pockets[69] based on protonation state changes of bridging residues, similar to the one observed in ThDP-dependent enzymes.[29]

In this work we show that the activation of Thr1 residue to form an alkoxy is strongly connected to the protonation state of a neighboring lysine (Lys33), part of the catalytic triad. The positively charged Lys33 significantly increases the population of charged groups in Thr1. The coupling has been evidenced by cluster quantum mechanical calculations with a confirmation of the Lys33 pK_a values through replica exchange simulations.

The inhibition reaction pathway is presented showing that the amount of protons could change along the reaction pathway. This needs to be highlighted as it is a common procedure in current biophysical simulations. This also opens up the possibility for active site communication, by pulling/pushing protons from the catalytic triad.

We have employed a machine learning technique in order to obtain force field parameters, which we have then been used in molecular dynamic simulations of inhibitors. We have obtained parameters for the epoxy-ketone and boronic acid containing inhibitors, namely Dihydroeponemycin and Oprozomib and Bortezomib and Ixazomib. Our results agree with the parameters given by the GAFF library but also allows more flexibility, providing parameters for atoms which are not present in such data bank, i.e. boronic acid containing warheads. This method, minimises the standard deviation of the radial distribution obtained by MM with respect to a reference, in our case PM6.

The molecular dynamics simulations show that the studied inhibitors should have little impact on the pocket acid-base equilibria. We propose that the activation of Thr1Oγ occurs through acid-base equilibria strongly influenced by the pK_a of Lys33. Although this does not seem to be a discriminating factor for different inhibitors, it could weigh on two important mechanisms: signaling mechanisms in the proteasome and active site specificity. Further calculations in this direction are currently being pursued in our lab.

Acknowledgement We would like to thank Dr. Ashwin Chari and Dr. Fabian Hennenberg for fruitful discussions. J. U. thank Fundación Ramón Areces for funding through a postdoctoral fellowship (BEVP30A5832). J.P. acknowledges funding of this research by the German Research Foundation (DFG) via project 389479699/GRK2455.

Table 2: pK_a and Hill coefficient (n) values of Lys33 at with four studied drugs in the Caspase pocket with and without Bayesian Optimized parameters.

	GAFF		BO	
	pK_a	n	pK_a	n
Bortezomib	8.24 ±0.04	0.82 ±0.05	7.21 ±0.04	0.72 ±0.05
Ixazomib	6.33 ±0.06	0.57 ±0.04	7.83 ±0.04	0.64 ±0.03
Dihydroeponemycin	7.74 ±0.07	0.43 ±0.03	7.12 ±0.03	0.72 ±0.03
Oprozomib	7.18 ±0.06	0.56 ±0.04	7.85 ±0.08	0.65 ±0.07

Supporting Information Available

- Figure S1: constrained atoms of the studied inhibitors.
- Figure S2: employed cluster model.
- Table S1: linear regression of the pK_a training data set.
- Table S2: parameters of the linear regression.
- Table S3: BO scaling parameters.
- Figure S3: Normalized standard deviation versus the scaling factor for the point charges of Oprozomib.
- Figure S4: scaling factor for Lennard-Jones parameters, σ and ϵ, uncertainty of the obtained points and the following point to be evaluated given by the acquisition function. The upper panel shows the carbonyl moiety oxygen (O10) of Oprozomib and the lower panel shows the epoxy oxygen (O35).
- Figure S5: Normalized standard deviation versus scaling factor for the point charges of Dihydroeponemycin.
- Figure S6: scaling factor for Lennard-Jones parameters, σ and ϵ, uncertainty of the obtained points and the following point to be evaluated given by the acquisition function. The upper panel shows the carbonyl moiety oxygen (O26) of Dihydroeponemycin and the lower panel shows the epoxy oxygen (O6).
- Figure S7: Normalized standard deviation versus scaling factor for the point charges of Bortezomib.
- Figure S8: Scaling factor for Lennard-Jones parameters, σ and ϵ, uncertainty of the obtained points and the following point to be evaluated given by the acquisition function. The upper panel shows the B atom of Bortezomib and the lower panel shows the O atoms.
- Figure S9: Normalized standard deviation versus scaling factor for the point charges of Ixazomib.
- Figure S10: Scaling factor for Lennard-Jones parameters, σ and ϵ, uncertainty of the obtained points and the following point to be evaluated given by the acquisition function. The upper panel shows the B atom of Ixazomib and the lower panel shows the O atoms.
- GitHub repository for pK_a calculations: `https://github.com/jproppe/pka`
- QM cluster data, inputs and outputs, are available at: `https://doi.org/10.25625/LC8LUY`
- Data of the Replica Exchange simulations, inputs, outputs and employed parameter files are available at: `https://doi.org/10.25625/ZDXSCP`
- Data of the Bayesian Optimizations is available at: `https://doi.org/10.25625/CKPBVK`

- QM data for the calibration of the pK_a values is available at: `https://doi.org/10.25625/WRZO07`

References

(1) Glickman, M. H.; Ciechanover, A. The Ubiquitin-Proteasome Proteolytic Pathway: Destruction for the Sake of Construction. *Physiol. Rev.* **2002**, *82*, 373–428.

(2) Adams, J.; Palombella, V. J.; Sausville, E. A.; Johnson, J.; Destree, A.; Lazarus, D. D.; Maas, J.; Pien, C. S.; Prakash, S.; Elliott, P. J. Proteasome Inhibitors: A Novel Class of Potent and Effective Antitumor Agents. *Cancer Res.* **1999**, *59*, 2615–2622.

(3) Adams, J.; Palombella, V. J.; Elliott, P. J. Proteasome Inhibition: a New Strategy in Cancer Treatment. *Invest. New Drugs* **2000**, *18*, 109–121.

(4) Goldberg, A. L. Protein Degradation and Protection Against Misfolded or Damaged Proteins. *Nature* **2003**, *426*, 895–899.

(5) Chen, L.; Madura, K. Increased Proteasome Activity, Ubiquitin-Conjugating Enzymes, and eEF1A Translation Factor Detected in Breast Cancer Tissue. *Cancer Res.* **2005**, *65*, 5599–5606.

(6) Spatarao, V.; Norbury, C.; Harris, A. L. The Ubiquitin-Proteasome Pathway in Cancer. *Brit. J. Cancer* **1998**, *77*, 448–455.

(7) D'Arcy, P.; Brnjic, S.; Olofsson, M. H.; Fryknäs, M.; Lindsten, K.; De Cesare, M.; Perego, P.; Sadeghi, B.; Hassan, M.; Larsson, R.; Linder, S. Inhibition of Proteasome Deubiquitinating Activity as a New Cancer Therapy. *Nat. Med.* **2011**, *17*, 1636–1640.

(8) Raaben, M.; Posthuma, C. C.; Verheije, M. H.; te Lintelo, E. G.; Kikkert, M.; Drijfhout, J. W.; Snijder, E. J.; Rottier, P. J. M.; de Haan, C. A. M. The Ubiquitin-Proteasome System Plays an Important Role during Various Stages of the Coronavirus Infection Cycle. *J. Virol.* **2010**, *84*, 7869–7879.

(9) Guedes, R. A.; Aniceto, N.; Andrade, M. A. P.; Salvador, J. A. R.; Guedes, R. C. Chemical Patterns of Proteasome Inhibitors: Lessons Learned from Two Decades of Drug Design. *Int. J. Mol. Sci.* **2019**, *20*, 5326.

(10) Beck, P.; Dubiella, C.; Groll, M. Covalent and Non-Covalent Reversible Proteasome Inhibition. *Biol. Chem.* **2012**, *393*, 1101–1120.

(11) Ray, S.; Murgin, A. S. New Electrophiles and Strategies for Mechanism-Based and Targeted Covalent Inhibitor Design. *Biochemistry* **2019**, *58*, 5234–5244.

(12) Adams, J.; Kauffman, M. Development of the Proteasome Inhibitor Velcade™ (Bortezomib). *Cancer Invest.* **2004**, *22*, 304–311.

(13) Moreau, P. et al. Ixazomib, an Investigational Oral Proteasome Inhibitor (PI), in Combination with Lenalidomide and Dexamethasone (IRd), Significantly Extends Progression-Free Survival (PFS) for Patients (Pts) with Relapsed and/or Refractory Multiple Myeloma (RRMM): The Phase 3 Tourmaline-MM1 Study (NCT01564537). *Blood* **2015**, *126*, 727–727.

(14) Richardson, P. G. et al. A Phase 2 Study of Bortezomib in Relapsed, Refractory Myeloma. *New Engl. J. Med.* **2003**, *348*, 2609–2617.

(15) Infante, J. R.; Mendelson, D. S.; Burris, H. A.; Bendell, J. C.; Tolcher, A. W.; Gordon, M. S.; Gillenwater, H. H.; Arastu-Kapur, S.; Wong, H. L.; Papadopoulos, K. P. A First-in-Human Dose-Escalation Study of the Oral Proteasome Inhibitor Oprozomib in Patients

with Advanced Solid Tumors. *Invest. New Drug.* **2016**, *34*, 216–224.

(16) Shirley, M. Ixazomib: First Global Approval. *Drugs* **2016**, *76*, 405–411.

(17) Sugawara, K.; Hatori, M.; Nishiyama, Y.; Tomita, K.; Kamei, H.; Konishi, M.; Oki, T. Eponemycin, a New Antibiotic Active Against B16 Melanoma. I. Production, Isolation, Structure and Biological Activity. *J. Antibiot.* **1990**, *43*, 8–18.

(18) Unno, M.; Mizushima, T.; Morimoto, Y.; Tomisugi, Y.; Tanaka, K.; Yasuoka, N.; Tsukihara, T. The Structure of the Mammalian 20S Proteasome at 2.75 ÅResolution. *Structure* **2002**, *10*, 609–618.

(19) Harshbarger, W.; Miller, C.; Diedrich, C.; Sacchettini, J. Crystal Structure of the Human 20S Proteasome in Complex with Carfilzomib. *Structure* **2015**, *23*, 418–424.

(20) Schrader, J.; Henneberg, F.; Mata, R. A.; Tittmann, K.; Schneider, T. R.; Stark, H.; Bourenkov, G.; Chari, A. The Inhibition Mechanism of Human 20S Proteasomes Enables Next-Generation Inhibitor Design. *Science* **2016**, *353*, 594–598.

(21) Marques, A. J.; Palanimurugan, R.; Matias, A. C.; Ramos, P. C.; Dohmen, R. J. Catalytic Mechanism and Assembly of the Proteasome. *Chem. Rev.* **2009**, *109*, 1509–1536.

(22) Søndergaard, C. R.; Olsson, M. H. M.; Rostkowski, M.; Jensen, J. H. Improved Treatment of Ligands and Coupling Effects in Empirical Calculation and Rationalization of pKa Values. *J. Chem. Theory and Comput.* **2011**, *7*, 2284–2295.

(23) Olsson, M. H. M.; Søndergaard, C. R.; Rostkowski, M.; Jensen, J. H. PROPKA3: Consistent Treatment of Internal and Surface Residues in Empirical pKa Predictions. *J. Chem. Theory Comput.* **2011**, *7*, 525–537.

(24) Ho, M. C.; Ménétret, J. F.; Tsuruta, H.; Allen, K. N. The Origin of the Electrostatic Perturbation in Acetoacetate Decarboxylase. *Nature* **2009**, *459*, 393.

(25) Isom, D. G.; Castañeda, C. A.; Cannon, B. R.; García-Moreno E., B. Large Shifts in pKa Values of Lysine Residues Buried Inside a Protein. *P. Natl. Acad. Sci. USAs* **2011**, *108*, 5260–5265.

(26) Ishikita, H. Origin of the pKa Shift of the Catalytic Lysine in Acetoacetate Decarboxylase. *FEBS Lett.* **2010**, *584*, 3464–3468.

(27) Huang, Y.-m. M.; You, W.; Caulkins, B. G.; Dunn, M. F.; Mueller, L. J.; Chang, C.-e. A. Protonation States and Catalysis: Molecular Dynamics Studies of Intermediates in Tryptophan Synthase. *Protein Sci.* **2016**, *25*, 166–183.

(28) Fried, S. D.; Bagchi, S.; Boxer, S. G. Extreme Electric Fields Power Catalysis in the Active Site of Ketosteroid Isomerase. *Science* **2014**, *346*, 1510–1514.

(29) Dai, S.; Funk, L.-M.; Rabe von Pappenheim, F.; Sautner, V.; Paulikat, M.; Schröder, B.; Uranga, J.; Mata, R. A.; Tittmann, K. Low-Barrier Hydrogen Bonds in Enzyme Cooperativity. *Nature* **2019**, *573*, 609–613.

(30) Liepe, J.; Mishto, M.; Textoris-Taube, K.; Janek, K.; Keller, C.; Henklein, P.; Kloetzel, P. M.; Zaikin, A. The 20S Proteasome Splicing Activity Discovered by SpliceMet. *PLOS Comput. Biol.* **2010**, *6*, 1–11.

(31) Tang, G.; Perng, M. D.; Wilk, S.; Quinlan, R.; Goldman, J. E. Oligomers of Mutant Glial Fibrillary Acidic Protein (GFAP) Inhibit the Proteasome System in Alexander Disease Astrocytes, and the Small Heat Shock Protein αB-Crystallin Reverses the Inhibition. *J. Biol. Chem.* **2010**, *285*, 10527–10537.

(32) Wei, D.; Tang, M.; Zhan, C.-G. Fundamental Reaction Pathway and Free Energy Profile of Proteasome Inhibition by Syringolin A (SylA). *Org. Biomol. Chem.* **2015**, *13*, 6857–6865.

(33) Serrano-Aparicio, N.; Świderek, K.; Moliner, V. Theoretical Study of the Inhibition Mechanism of Human 20S proteasome by dihydroeponemycin. *Eur. J. Med. Chem.* **2019**, *164*, 399–407.

(34) Frisch, M. J. et al. Gaussian16 Revision A.03. 2016; Gaussian Inc. Wallingford CT.

(35) Becke, A. D. Density-Functional Thermochemistry. I. The Effect of the Exchange-Only Gradient Correction. *J. Chem. Phys.* **1992**, *96*, 2155–2160.

(36) Lee, C.; Yang, W.; Parr, R. G. Development of the Colle-Salvetti Correlation-Energy Formula into a Functional of the Electron Density. *Phys. Rev. B* **1988**, *37*, 785–789.

(37) Grimme, S.; Ehrlich, S.; Goerigk, L. Effect of the Damping Function in Dispersion Corrected Density Functional Theory. *J. Comput. Chem.* **2011**, *32*, 1456–1465.

(38) Weigend, F.; Ahlrichs, R. Balanced Basis Sets of Split Valence, Triple Zeta Valence and Quadruple Zeta Valence Quality for H to Rn: Design and Assessment of Accuracy. *Phys. Chem. Chem. Phys.* **2005**, *7*, 3297–3305.

(39) Weigend, F. Accurate Coulomb-Fitting Basis Sets for H to Rn. *Phys. Chem. Chem. Phys.* **2006**, *8*, 1057–1065.

(40) Marenich, A. V.; Cramer, C. J.; Truhlar, D. G. Universal Solvation Model Based on Solute Electron Density and on a Continuum Model of the Solvent Defined by the Bulk Dielectric Constant and Atomic Surface Tensions. *J. Phys. Chem. B* **2009**, *113*, 6378–6396.

(41) The GPyOpt authors, GPyOpt: A Bayesian Optimization framework in Python. `http://github.com/SheffieldML/GPyOpt`, 2016.

(42) Frazier, P. I. A Tutorial on Bayesian Optimization. `http://arxiv.org/abs/1807.02811`, 2018.

(43) S., M. D.; Z., J. The Generalization of Latin Hypercube Sampling. *Reliab. Eng. Syst. Safe.* **2016**, *148*, 96 – 108.

(44) Frisch, M. J. et al. Gaussian09 Revision D.01. Gaussian Inc. Wallingford CT 2009.

(45) Seminario, J. M. Calculation of Intramolecular Force Fields from Second-Derivative Tensors. *Int. J. Quantum Chem.* **1996**, *60*, 1271–1277.

(46) Case, D. et al. AMBER, University of California, San Francisco. 2018.

(47) Swails, J. M.; York, D. M.; Roitberg, A. E. Constant pH Replica Exchange Molecular Dynamics in Explicit Solvent Using Discrete Protonation States: Implementation, Testing, and Validation. *J. Chem. Theory Comput.* **2014**, *10*, 1341–1352.

(48) Maier, J. A.; Martinez, C.; Kasavajhala, K.; Wickstrom, L.; Hauser, K. E.; Simmerling, C. ff14SB: Improving the Accuracy of Protein Side Chain and Backbone Parameters from ff99SB. *J. Chem. Theory and Comput.* **2015**, *11*, 3696–3713.

(49) Wang, J.; Wolf, R. M.; Caldwell, J. W.; Kollman, P. A.; Case, D. A. Development and Testing of a General Amber Force Field. *J. Comput. Chem.* **2004**, *25*, 1157–1174.

(50) Wang, J.; Wang, W.; Kollman, P. A.; Case, D. A. Automatic Atom Type and Bond Type Perception in Molecular Mechanical Calculations. *J. Mol. Graph. Model.* **2006**, *25*, 247 – 260.

(51) Ryckaert, J. P.; Ciccotti, G.; Berendsen, H. J. C. Numerical Integration of the

Cartesian Equations of Motion of a System with Constraints: Molecular Dynamics of n-alkanes. *J. Comput. Phys.* **1977**, *23*, 327–341.

(52) Götz, A. W.; Williamson, M. J.; Xu, D.; Poole, D.; Le Grand, S.; Walker, R. C. Routine Microsecond Molecular Dynamics Simulations with AMBER on GPUs. 1. Generalized Born. *J. Chem. Theory Comput.* **2012**, *8*, 1542–1555.

(53) Mermelstein, D. J.; McCammon, J. A.; Walker, R. C. pH-Dependent Conformational Dynamics of Beta-Secretase 1: a Molecular Dynamics Study. *J. Mol. Recognit.* **2019**, *32*, e2765.

(54) Siegbahn, P. E.; Himo, F. The Quantum Chemical Cluster Approach for Modeling Enzyme Reactions. *WIREs Comput. Mol. Sci.* **2011**, *1*, 323–336.

(55) Demchuk, E.; Genick, U. K.; Woo, T. T.; Getzoff, E. D.; Bashford, D. Protonation States and pH Titration in the Photocycle of Photoactive Yellow Protein. *Biochemistry-US* **2000**, *39*, 1100–1113.

(56) Klicić, J. J.; Friesner, R. A.; Liu, S.-Y.; Guida, W. C. Accurate Prediction of Acidity Constants in Aqueous Solution via Density Functional Theory and Self-Consistent Reaction Field Methods. *J. Phys. Chem. A* **2002**, *106*, 1327–1335.

(57) McQuarrie, D. A. *Statistical Mechanics*; Harper's Chemistry Series; HarperCollins Publishing, Inc.: New York, 1976.

(58) Shields, G.; Seybold, P. *Computational Approaches to Predict pKa Values*; CRC Press, 2014.

(59) Bootsma, A. N.; Wheeler, S. Popular Integration Grids Can Result in Large Errors in DFT-Computed Free Energies. 2019; `https://chemrxiv.org/articles/Popular_Integration_Grids_Can_Result_in_Large_Errors_in_DFT-Computed_Free_Energies/8864204/5`.

(60) Andrejić, M.; Mata, R. A. Local Hybrid QM/QM Calculations of Reaction Pathways in Metallobiosites. *J. Chem. Theory Comput.* **2014**, *10*, 5397–5404.

(61) Caldararu, O.; Feldt, M.; Cioloboc, D.; van Severen, M.-C.; Starke, K.; Mata, R. A.; Nordlander, E.; Ryde, U. QM/MM Study of the Reaction Mechanism of Sulfite Oxidase. *Sci. Rep.-UK* **2018**, *8*, 4684.

(62) Saha, A.; Oanca, G.; Mondal, D.; Warshel, A. Exploring the Proteolysis Mechanism of the Proteasomes. *J. Phys. Chem. B* **2020**, *124*, 5626–5635.

(63) Wei, D.; Lei, B.; Tang, M.; Zhan, C.-G. Fundamental Reaction Pathway and Free Energy Profile for Inhibition of Proteasome by Epoxomicin. *J. Am. Chem. Soc.* **2012**, *134*, 10436–10450.

(64) Wei, D.; Fang, L.; Tang, M.; Zhan, C.-G. Fundamental Reaction Pathway for Peptide Metabolism by Proteasome: Insights from First-Principles Quantum Mechanical/Molecular Mechanical Free Energy Calculations. *J. Phys. Chem. B* **2013**, *117*, 13418–13434.

(65) Groll, M.; Kim, K. B.; Kairies, N.; Huber, R.; Crews, C. M. Crystal Structure of Epoxomicin: 20S Proteasome Reveals a Molecular Basis for Selectivity of α',β'-Epoxyketone Proteasome Inhibitors. *J. Am. Chem. Soc.* **2000**, *122*, 1237–1238.

(66) Li, H.; Robertson, A. D.; Jensen, J. H. Very Fast Empirical Prediction and Rationalization of Protein pKa Values. *Proteins* **2005**, *61*, 704–721.

(67) Bas, D. C.; Rogers, D. M.; Jensen, J. H. Very Fast Prediction and Rationalization of pKa Values for Protein–Ligand Complexes. *Proteins* **2008**, *73*, 765–783.

(68) Oregioni, A.; Stieglitz, B.; Kelly, G.; Rittinger, K.; Frenkiel, T. Determination of the pKa of the N-terminal Amino

Group of Ubiquitin by NMR. *Sci. Rep.-UK* **2017**, *7*, 43748.

(69) Kumawat, A.; Chakrabarty, S. Hidden Electrostatic Basis of Dynamic Allostery in a PDZ Domain. *P. Natl. Acad. Sci. USA* **2017**, *114*, E5825–E5834.