Rational engineering of L-Asparaginase reveals importance of dual activity for cancer cell toxicity

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Abbreviations
AEP, asparagine endopeptidase; ALL, acute lymphoblastic leukemia; AS, active site; GA, genetic algorithm; HB, hydrogen bonding; IC50, half-maximal inhibitory concentration; L-ASN, Escherichia coli L-asparaginase; MD, molecular dynamics; PSSM, Position Specific Scoring Matrix

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Abstract

Using proteins in a therapeutic context often requires engineering to modify functionality and enhance efficacy. We have previously reported that the therapeutic anti-leukemic protein macromolecule, *Escherichia coli* L-Asparaginase, is degraded by leukemic lysosomal cysteine proteases. Here, we successfully engineered L-Asparaginase, to resist proteolytic cleavage and, at the same time, improve activity. We employed a novel combination of mutant sampling using a genetic algorithm in tandem with flexibility studies using Molecular Dynamics to investigate the impact of lid-loop and active site mutations on drug activity. Applying these methods, we successfully predicted the more active L-Asparaginase mutants N24T and N24A. For the latter, a unique hydrogen bond network contributes to higher activity. Furthermore, interface mutations controlling secondary glutaminase activity demonstrated the importance of this enzymatic activity for drug cytotoxicity. All selected mutants were expressed, purified and tested for activity and ability to form the active tetrameric form. By introducing the N24A and N24A|R195S mutations to the drug L-Asparaginase, we are a step closer to individualized drug design.


Introduction

Proteins can be used as efficient drugs with high specificity and limited side effects. However, compared to the total number of known small molecule drugs, the number of protein drugs is relatively low\(^1\). Nevertheless, recent advances in protein engineering and design\(^2\)-\(^5\) have facilitated the development of new protein drugs, useful where small molecules do not provide sufficient specificity, or cause significant side effects\(^6\).

Here, we focus on the engineering of *Escherichia coli* L-Asparaginase (L-ASN), a protein drug, widely used in the hematological malignancy, acute lymphoblastic leukemia (ALL)\(^7\). The bacterial enzyme L-ASN, which is active as a homotetramer with four non-allosteric active sites, primarily hydrolyses the amino acid asparagine to aspartic acid and ammonia\(^8\)-\(^10\). Additionally L-ASN hydrolyses glutamine\(^11\), though this activity varies according to the source of the enzyme\(^12\). With one exception\(^8\), a steep drop in both serum asparagine and glutamine amino acid levels has been observed two weeks after treatment with L-ASN preparations\(^13\)-\(^15\). When asparagine is depleted in the blood, protein synthesis in malignant lymphoblasts is compromised, leading to the failure of cellular functions and eventually, apoptosis\(^16\).

L-ASN is a key component of chemotherapeutic regimens for childhood ALL. Unlike most other cytotoxics, side effects from L-ASN treatment are relatively mild. Nevertheless, since L-ASN is a bacterial protein, a major side effect is the development of hypersensitivity ranging from mild allergic reactions to potentially fatal anaphylaxis\(^17\). The underlying development of antibodies is also known to be responsible for the so-called ‘silent inactivation’, which leads to a rapid decline in L-ASN activity due to the presence of neutralizing antibodies\(^17\). In such cases, the enzyme Erwinase, derived from the bacterium *Erwinia chrysanthemi*, is often used as a substitute. Nevertheless, Erwinase has different pharmacokinetic and immunogenic properties, showing a shorter half-life than L-ASN\(^18\). L-ASN use is also associated with neurotoxicity\(^19,20\), attributed to its secondary glutaminase activity\(^11,21,22\).

We have previously reported that the lysosomal proteases Cathepsin B and Asparagine Endopeptidase (AEP) produced by lymphoblasts hydrolyze L-ASN, resulting in inactivation and
exposure of epitopes implicated in the immune response. We identified that the stepwise cleavage of L-ASN by AEP, originates at Asn 24 (N24). An N24G point mutant resisted AEP-mediated cleavage but showed decreased asparaginase activity\textsuperscript{23}.

The aim of this work was to create an AEP-resistant L-ASN mutant with enzymatic activity comparable to the native compound. We additionally examined the glutaminase/asparaginase activity ratio of the enzyme and explored mutations to modify this property. To achieve this we used a unique combination of computational protein engineering tools. High scoring mutants from the \textit{in silico} analysis were then experimentally verified.
Materials and methods

Genetic Algorithm: mutant sampling and selection
A genetic algorithm (GA) previously used for protein modeling\(^{24}\) was adapted in order to sample the sequence space of the lid-loop and interfaces of the protein tetramer complex. All operators within our original GA software were kept and an additional sequence point mutation operator, plus the DCOMPLEX\(^{25}\) energy score were added. Molecular Dynamics (MD) simulations were performed on the wild type (WT) complex, providing several different starting conformations for the interface residues to be sampled using the GA. In total 24,000 mutated complexes were created and a subset was defined for further investigation. To show a stable distribution of mutants, the complete GA sampling protocol was repeated multiple times (details in SI Materials and Methods).

Molecular Dynamics preparation and simulation
In addition to the negative reference N24G and the WT, the four highest scoring mutations were selected from GA sampling, in order to study the proteins’ dynamical and conformational features by MD simulations. These four mutants include N24A, N24T, N24H and N24S\text|\text{D281S}. The crystal structure of L-ASN was taken from the Protein Data Bank\(^{26}\) (PDB entry 3eca). Each system was energy-minimized and equilibrated for 50 ps at constant volume, and consequently at constant pressure, with backbone atoms restrained to their original positions in both cases. After the equilibration each system was simulated for 10ns at room temperature (T=298K) (for details see SI Materials and Methods).

Analysis of MD trajectories
All MD simulations and analyses were performed with the AMBER suite\(^{27}\). The root mean square fluctuation (RMSF) was calculated for residues 6-38 which contain the flexible lid-loop. The $\chi^1$ angle of T12 was monitored to map the orientation of the nucleophilic hydroxyl group of T12 within the AS. The T12 orientation along each MD trajectory was analyzed and a time series plotted, see Figure 2B. In order to generate Figure 2B we averaged T12 orientations over complete trajectories and for all four monomers. In addition to standard MD analysis methods,
we used internal coordinate fluctuations as a measure of flexibility. Furthermore, key distances and hydrogen bond counts within the active site (AS) were calculated. The free energy of binding was calculated using the MM-PBSA protocol\textsuperscript{28} (for details see SI Materials and Methods). The AS cavity volume of all four monomers was measured along each trajectory using the software suite CAVER\textsuperscript{29}. Additionally, the compactness of the tetramer was calculated for each snapshot of the trajectory using the method described in the context of the GA\textsuperscript{24}.

**Creation and purification of L-ASN recombinants**

L-ASN recombinants were created as described previously\textsuperscript{23}. Preparation of purified recombinant proteins and estimation of protein molecular weight and aggregation by SEC-MALLS (size exclusion chromatography coupled with multi angle laser light scattering) were carried out by the Protein Production Division of the Technology Facility in the Department of Biology at the University of York, UK.

**Estimation of asparaginase and glutaminase enzyme activity**

Asparaginase enzyme activity of the L-ASN recombinants was determined using a quantitative chromogenic assay (Medac Asparaginase Activity kit [MAAT], Medac GmbH, Hamburg) as reported previously\textsuperscript{30}. Glutaminase enzyme activity of the L-ASN recombinants was assessed by continuous coupled spectrophotometry based on L-glutamine hydrolysis and L-glutamate oxidation, with L-glutamate dehydrogenase as auxiliary enzyme and β-nicotinamide adenine dinucleotide as redox reagent.

**Cell viability-proliferation assays**

Cell viability was analyzed by using the CellTiter96 Aqueous One Solution Cell proliferation Assay kit (MTS tetrazolium assay, Promega, Madison, WI, USA) following the manufacturer’s protocol. The half-maximal inhibitory concentrations (IC\textsubscript{50}) of WT and mutant L-ASN recombinants were tested at equivalent asparaginase activities of the compounds in the ALL cell lines, REH, SupB15 and MV4:11. IC\textsubscript{50} values were calculated using the ED50 PLUS V1.0 online software. All experiments were performed in triplicate (for details see SI Materials and Methods).
Statistical analysis

To demonstrate that the combined usage of the X-ray and five key structures derived from MD, enriches the structural search space for possible point mutations, we compared several subsets of mutations generated by the GA, and investigated their frequency distributions with a t-distribution test. For each of two possible scenarios, X-ray only and X-ray + MD, three independent subsets, each consisting of 120 parallel GA runs, were generated. By comparing these subsets in pairs for each scenario, we were able to ascertain whether these were significantly different to each other, thus pointing to the depth of the search space (Table S4). Correlations for the 12···25 and 25···283 bonds were examined using Pearson $\chi^2$-square tests with Yates correction. The statistical significance of differences in asparaginase and glutaminase activities and IC50 values of WT and mutant L-ASN compounds were evaluated using a one-sided independent samples Student’s t-test. A p-value < 0.05 was considered statistically significant. The Pearson correlation coefficient was calculated to examine the correlation between glutaminase activity and drug cytotoxicity, as well as the correlation between computational predictors and asparaginase/glutaminase activity.
Results

Sampling of mutation space

A novel GA-based protocol, described in the SI Materials and Methods, was used in order to preselect potential amino acid substitutions for residue 24 and its main interacting partner D281 (Figure 1A). A combination of different molecular force fields and conformational search techniques was used to find mutants maintaining the fragile hydrogen-bond (HB) network of the AS, as observed in the WT X-ray structure. Conformations exhibiting a sufficiently stable HB network are shown in Figure 1B. 2,000 new conformations/mutations were created in total. Due to limited computational resources we chose the top four mutants (N24A, N24T, N24H and N24S|D281E), together with the WT and the N24G mutant for extensive MD analysis.

The GA was also used to sample potential mutations at the interfaces of the monomers, which form the active tetramer. For this interface sampling, a total of 24,000 new structures were produced. The mutant distribution of these structures can be seen in Figure S1, where the most populated residue can be identified as Y250. For this position the most acceptable mutant is Y250L; Y250N and Y250Q are not applicable, as they would create new potential AEP-cleavage sites. For further analysis we chose mutant Y250L and the next most frequent non-tyrosine substituting mutant, R195S.

In Table S1, the calculated Position Specific Scoring Matrix (PSSM), derived from a standard PSI-BLAST\textsuperscript{31} run, using the WT L-ASN sequence, is shown. Using the PSSM as a reference for the naturally occurring amino acid substitutions, the likelihood of the mutants selected by our GA approach can be estimated. For example, it can be seen that for residue N24, the most conserved mutation is N24G with a score of 49% in the calculated PSSM; N24A the mutation selected by the GA, accounts for only 8% and N24T for only 3%. For D281, the second residue in the double mutant, D281G has the highest conservation with 39%; D281E accounts for 13%. R195 has the highest conservation for itself, R (26%), and the score for R195S is only 4%. Finally, for Y250 the most common mutant according to the PSSM would be Y250P, with 42%; Y250L is not observed in homologous sequences at all (0%).
Molecular dynamics simulations

In order to understand the effect of these mutations on the stability of the lid-loop and the conformation of the AS, we performed MD simulations on WT L-ASN and five mutant proteins with variations in position 24 (N24G, N24A, N24T, N24H and N24S|D281E). The influence of interface mutants on the glutaminase activity was studied, simulating the WT, N24A|Y250L and N24A|R195S. The stability of the AS loop was measured by RMSF of the loop residues. Furthermore, conformations of key AS residues and HB networks in the region of the loop and AS were analyzed in the course of the MD simulations. For the interface mutants we analyzed the AS volume and the compactness of the tetramers. These analyses helped to identify structural features, which are important for enzyme activity; details are given below.

Flexibility of the lid-loop

Residue N24 is part of a lid-loop sitting above the AS and serving as a closing lid. This loop is found in an open, helical and closed, strand-like conformation. Furthermore, the region directly preceding the flexible loop includes residue T12, which is a member of the AS and initiates the nucleophilic attack on the substrate. The stable, closed conformation of the lid-loop is observed when a substrate molecule is present in the AS of the enzyme; T12 is only positioned correctly in this conformation. In the absence of substrate molecule in the AS, the lid-loop is highly mobile and is observed only rarely in crystal structures. Therefore, the correct balance between stability and flexibility must be maintained for correct functioning of the enzyme. If the lid-loop is too flexible/unstable it may not be able to close properly and would disrupt the orientation of T12, causing significant loss of enzymatic activity. In contrast, an excessively rigid lid-loop may hinder substrate entry into the AS.

Calculating RMSF profiles from the MD simulations allowed the analysis of the lid-loop in WT L-ASN and the five N24 mutants. These RMSF profiles, averaged over the four monomers of each protein, are shown in Figure 2A. The N24G mutant has a much higher loop flexibility compared to those of WT and the other mutants, which clearly is one of the reasons for its decreased catalytic activity (Figure 3). Contrarily, N24A and N24T have very stable lid-loops,
resulting in a tightly locked substrate molecule in the AS, stabilized for the catalytic reaction. The N24H mutant also displays low flexibility in the central part of the loop; however, the C-terminal region of the loop shows high RMSF values, likely to cause stability problems. The double mutant (N24S|D281E) has a similar RMSF profile to WT, with a slight increase in flexibility for residues 20-24. These observations are further confirmed by the analysis of the φ/φ angle deviations of the lid-loop (Figure S2).

**Orientation of T12**

MD simulations also show that mutations in position 24 change the HB network in the area around the AS and influence the conformation of Y25, which plays an important function stabilizing the conformation of T12. Visual analysis of the T12···Y25 pair revealed that, for the WT and the N24A mutant, the correct orientation of T12 is maintained with the hydroxyl group pointing to the centre of the AS. In contrast, for the N24G mutant (and to some extent N24T) Y25 is shifted and is not able to form HBs with T12. As a result, the hydroxyl group of T12 is often replaced by the methyl group in the AS, which cannot perform a nucleophilic attack.

In order to describe the orientation of the T12 hydroxyl and methyl groups in the AS, we have monitored the value of the χ¹ dihedral angle along the MD trajectory, for each mutant. The resulting time series, converted to binary plots (see Methods) are shown in Figure 2B (details in Figure S2C). Our results show that WT and the N24A mutant have the hydroxyl group present in the AS for the majority of the simulations. Contrarily, the N24G and N24T mutants show a lower occupancy of the correct hydroxyl group orientation, suggesting a lower stability of the AS. This explains why the N24T mutant, although having the most stable lid-loop among all the studied mutants, has a lower activity compared to the N24A mutant. For the N24G mutant, the low occupancy of the hydroxyl group and the decreased stability of the lid-loop, explain its substantial reduction of activity. Interestingly, with the substrate bound, N24T is able to recover some of the correct T12 orientation, whereas this is not the case for N24G. The T12 occupancy of N24H is intermediate between N24T and WT. However, given the fact that N24H displays significant lid-loop instability and that N24S|D281E also shows suboptimal results, we decided to exclude these lid-loop mutants from further analysis. We therefore decided to only further
focus on WT, the N24G mutant, as a negative reference, and the N24T and N24A mutant proteins.

**Distance and hydrogen bond analysis of active site residues**

The stability of the AS is monitored by calculating the number of HBs, and the distances between several key residues in and around the AS along each MD trajectory. These residues include T12 and Y25, where Y25 is important for the correct orientation of T12\(^32\). Distances between residues A20/T21 and Q367 describe the openness of the lid-loop. Interactions between N24···D281, N24···E283 and Y25···E283 stabilize the AS. Figure 2C shows the shortest side-chain distances for all these pairs and the donor-acceptor distances for T12···Y25 and Y25···E283 (details in Figure S3), in all studied proteins. The HB counts for T12···Y25, N24···Y25, N24···D281, N24···E283 and Y25···E283 are indicated in Table S2 and a graphical illustration is provided in Figure 1B. Calculating both the distances and HB counts, allows the analysis of weak interactions, which do not fall within the HB distance and angle cutoff (see Materials and Methods).

Regarding the distance analysis for the amino acid pair T12 and Y25, the lowest distance is found for N24A, followed by N24T and then the others. WT shows a less stable T12···Y25 interaction and for N24G the largest distance and lowest HB count (Table S2) is observed. These observations are further confirmed by the results shown in Figure S3C. Lid-loop openness monitoring distances, A20···Q367 and T21···Q367, are smallest for N24A, and show the highest average value and deviation for N24T; WT and N24G behave similarly.

As shown in Figure 2C and Table S2, the HB between residues N24 and D281 is maintained for WT and N24T only. N24G and N24A lack the HB donors and acceptors in the side-chain of residue 24. In absolute numbers, compared to WT, N24T shows an increase of ~37% in the hydrogen-bonding capacity of the N24···D281 distance to maintain a formal HB.

When using the HB counts to calculate the correlation between T12···Y25, the important bond for correct orientation of T12, and Y25···E283, the bond compensating the loss of N24···D281 in N24A, we made several interesting observations. For WT and N24T we observe a significant anti-correlation (WT: p < 0.002, N24T: p < 0.006), where it is more likely that the bonds are
observed separately. There is no significant level of correlation associated with the two bonds found for N24G. Interestingly, a significant correlation is found for N24A (p < 0.001), which means that the two bonds are more likely to occur together. This analysis reveals a special HB configuration in N24A, which is locally more stable than in any of the other three cases, and is likely to contribute to the increased activity. This stabilizing HB network between T12, Y25 and E283 can be seen in Figure 1C.

Active site cavity volume, tetramer compactness and free energy of binding as a measure of glutaminase activity

Using the GA, two interface mutants were selected to increase the stability of the active tetramer: Y250L and R195S. These two mutants were combined with the lid-loop mutant N24A. The AS of L-ASN lies partly at the monomer-monomer interface, and therefore, changes in the interface packing will likely affect the activity of the enzyme. As each active site is composed of two monomers and the entrance to the binding pocket lies within the interface, we assumed that tighter packing might decrease the active site cavity size, and thus specifically prevent the entering of the larger Gln molecule, whereas access for the smaller Asn is not interrupted. Therefore, this optimization should specifically reduce glutaminase activity, while asparaginase activity may be affected to a lesser degree. Increased tetramer stability was assessed by monitoring the free energy of binding (Figure S4A) and compactness of the tetramer (Figure S4B) while altered properties of the AS, likely to influence glutaminase/asparaginase activity ratio, were assessed by monitoring cavity size and flexibility (standard deviation) (Figure S4C). N24T results in the least compact tetramer; however, the AS cavity volume is comparable to WT, providing an explanation as to why N24T is predicted to have a higher glutaminase activity. In contrast, N24A|Y250L has the smallest AS cavity with the least flexibility and the lowest tetramer compactness. This result is also partly confirmed by the free energy of binding, where N24A|Y250L has the lowest value of the two interface mutants, indicating that this mutant is likely to have substantially decreased glutaminase activity (Figure S4A). The cavity volume of the N24A|R195S mutant is also smaller than in WT, indicating that this mutant might also have a decreased glutaminase activity, although to a lesser extent than seen for the N24A|Y250L mutant. These computational predictions were experimentally confirmed (see below).
Experimental confirmation

To confirm the computationally derived findings, the WT and mutant L-ASN proteins were expressed, and the state of oligomerisation and activity measured (Table S3, Figure 3). As previously reported, we showed that N24G activity is reduced to approximately 40% of the WT; however, the oligomerisation ability is not compromised in this mutant, with 95% of the compound occurring as tetramers, similar to that in the N24A mutant. The activity of N24A is higher than that of the WT, with a ratio of 1:1.17. However, N24T shows no significant change in activity compared to WT. Regarding glutaminase activity, as shown in Figure 3, only N24T has a significantly increased glutaminase activity while the double mutants N24A|Y250L and N24T|Y250L have barely detectable glutaminase activity. These double mutants also have reduced asparaginase activity (N24A|Y250L, 72% and N24T|Y250L, 65% compared to WT). In contrast, the N24A|R195S double mutant has close to half of the WT glutaminase activity but a similar asparaginase activity. These experimental findings correlate well with modelling predictions; a correlation coefficient of 0.96 is observed between measured asparaginase activity and T12 hydroxyl group positioning, and 0.83 between the glutaminase activity and our compactness measure.

In order to investigate the effect of altered glutaminase activity on drug cytotoxicity, IC50 concentrations for WT and mutant L-ASN were estimated in leukemia cell lines (Figure 3). These IC50 values were determined using equivalent asparaginase activities of the compounds (Figure 4). Therefore, all IC50 values have been weighted by the measured asparaginase activities. Three leukemia cell lines were studied – SupB15, MV4:11 and REH – with respectively, high, intermediate and low sensitivity to L-ASN. The N24A mutant was the compound with the lowest average and most consistent IC50 values (on average 79% of WT with ratios of 75%, 88% and 75% in SupB15, MV4:11 and REH respectively), and therefore the best cell-kill properties. Overall, the N24T and N24A|R195S mutants were equivalent to WT L-ASN (averaged estimated IC50 compared to WT, 93% and 103% respectively); however both show inconsistent results between the different cell lines and higher standard deviations than seen for N24A. N24T, with the highest glutaminase activity, had lower IC50 values compared to WT but the average value was still higher than the one of N24A. Halving the glutaminase
activity did not adversely affect cytotoxicity, with IC50 values of the N24A|R195S mutant similar to that of the WT compound. Importantly, the N24A|Y250L mutant, with minimal glutaminase activity, is also the least potent cytotoxic L-ASN compound, with an overall IC50 of 243% compared to WT L-ASN. Accordingly near-complete elimination of glutaminase activity is associated with reduced cytotoxicity. An overall Pearson correlation coefficient of 0.73 was observed between the modification in glutaminase activity and drug cytotoxicity, with the highest correlation observed in the intermediate-sensitive MV4:11 cells (MV4:11, 0.99; SupB15, 0.7; REH, 0.5). Thus, in MV4:11 cells, L-ASN mutants that vary significantly in glutaminase activity (N24T, N24A|R195S and N24A|Y250L) also demonstrate significantly different cytotoxic efficacy.
Discussion

We previously showed that L-ASN is degraded by two proteases, one of which, AEP, is predominantly expressed in lymphoblasts of high-risk patients. Furthermore, we were able to show that the stepwise cleavage of L-ASN by AEP originates at N24, in the L-ASN lid-loop, and can be blocked by a single amino acid substitution in this position\textsuperscript{23}. Here, we identified the most suitable amino acid substitution in the L-ASN lid-loop that maintains resistance to AEP-cleavage while preserving, and even improving L-ASN activity. Combining this substitution with a second mutation at the monomer interface of the active tetrameric L-ASN molecule, allows control over hydrolysis of the secondary substrate, glutamine. All mutants were identified using a GA to sample possible amino acid substitutions; none of the described mutants could have been identified using an approach based on evolutionary amino acid conservation. Flexibility and structural properties of WT and mutant L-ASN were investigated using MD simulations. Applying a combination of several structural descriptors to interpret the results of MD simulations helped link the impact of mutations to asparaginase/glutaminase activity. Based on the descriptors, our predictions of mutant activities correlated well with experimental measurements of asparaginase and glutaminase activities and also to a large extent, with results of cytotoxicity assays. From these observations, we propose that the L-ASN mutants N24A and N24A|R195S show the best potential for further drug development.

A clear correlation between the flexibility of the lid-loop and the activity of different mutants is observed. For example, N24G has the highest flexibility observed amongst the mutations, but presents the lowest asparaginase activity. The two mutants with the most stable lid-loop, N24A and N24T, show the highest asparaginase and glutaminase activities compared to the WT. Measuring the independent flexibility of the lid-loop in internal coordinate space, averaged over the four monomeric subunits, revealed that N24A has the lowest standard deviation. Experiments showed that N24A is more active than N24T, although N24T is more stable in the lid-loop region than N24A. As an additional, complementing measure, we used the correct orientation of the nucleophile in the active site helping to predict which of these two mutants has the higher asparaginase activity. This measure revealed that indeed for N24A the hydroxyl group of T12 is
in the correct substrate-attack conformation most of the time, similar to WT – a trend not observed for N24T or the other lid-loop mutants.

In the context of the HB analysis, the N24A mutant has certain distinctive characteristics. N24A has the most stable HB for the pair 25···283, and unique to the N24A mutation, a significant direct correlation is observed between the HBs of 25···283 and 12···25. These features markedly rearrange the AS in N24A. By contrast, there is a significant negative correlation between the occurrences of these HB pairs in WT and N24T, probably caused by a very stable 24···281 HB. Indeed, the HB network associated with the AS mechanism can be described as a double lever, where HBs between residues 24 and 281 on top of the AS slightly pull out the residues below, which include Y25 and E283, and thus prevent a HB between these residues. Consequently, N24T and WT can rarely simultaneously form 25···283 and 12···25 HBs. N24A on the other hand forms both bonds, which impart an improved, stabilizing network in the most crucial part of the AS. In support of the above findings, we note the importance of the interaction between Y25 and T12, also demonstrated by Aung et al.32 where any mutation of Y25 leads to a complete loss of enzyme activity.

The second objective of this study was to identify mutants in the interface of the L-ASN monomers that rearrange the packing close to the AS, thereby affecting the relative selectivity of asparagine/glutamine hydrolysis. Instead of random, or evolutionary-based selection of potential mutants, we used a GA to identify energetically favorable mutations. For this we created several thousand mutants with lower or equal energy to the WT and chose the ones with the highest occurrences, a principle successfully used in protein design before33. Surprisingly, by far the residue with the highest count during all GA simulations was residue Y250. In this position, the mutant Y250L was most common, a mutation which is not likely to be observed in a PSSM for a stable homotetramer interface, due to the importance of tyrosine for solvation34. It is important to note that Y250 lies next to N248, a residue where mutations have been shown to cause a significant reduction of glutaminase and, to a lesser degree, asparaginase activity35. N248 forms a HB with the catalytic residue D90 across the monomer-monomer interface (Figure S5A) and is involved in the stabilization of the transition state during catalysis. The mutation N248A has been shown to destabilize the catalysis of the weaker glutamine substrate to a much higher
degree than asparagine. However, there is no direct interaction between N248 and Y250 and no major conformational change in the vicinity of N248 can be seen with the Y250L mutation. Consequently, we suspect that the decreased glutaminase activity of the N24A|Y250L mutant comes from small conformational changes at the interface, which lead to tighter monomer-monomer binding and a subsequent decrease in active site volume. The second interface mutant selected was R195S. In L-ASN two monomers form a tightly packed dimer; two of these dimers form the final tetrameric structure. Four R195 residues, one from each monomer, are situated at the interface of the two dimers, each interacting with a double strand on their opposite monomer. These large residues appear to act as spacers, preventing tight packing between monomers (Figure S5B). Therefore, once the amino acid Arg is replaced by the smaller Ser this effect is reduced. Indeed, MD simulations revealed that both Y250L and R195S show increased tetramer compactness and a decreased AS cavity, correlating well with the measured reductions in glutaminase activities.

Calculations for the free energy of binding indicated that the Y250L mutant has tighter monomer-monomer binding. However, it is important to stress that the sole use of free energy changes would neither have correlated particularly strongly with the prediction of mutation effects nor given us useful insights into the structural mechanisms of this protein drug. Thus, we believe that multiple structural descriptors are needed for a clear understanding of the effect of interface mutants in L-ASN and the descriptors listed above provide a firm base for further improvements.

Though the glutaminase activity of L-ASN has been linked to therapeutic toxicity, its effect on the leukemic cell has been debated. Our data shows that N24A, with stable glutaminase activity, appears to be the most effective drug. N24A|R195S which has half the glutaminase activity of the WT nevertheless has comparable asparaginase activity and cytotoxicity. N24A|Y250L, which has no glutaminase activity, has significantly reduced cytotoxicity when compared to WT. Thus, some glutaminase activity is required to induce apoptosis in cells already affected by a substantial lack of asparagine. Though glutamine is a non-essential amino acid, it appears to be essential to cancer cells as a major source of energy, nitrogen and carbon. The suggestion is that glutamine metabolism links with the mitochondrial Tricarboxylic acid cycle to maintain the
availability of metabolic intermediates critical for cancer cell survival and proliferation. Indeed, glutamine addiction in cancer cells represents a potential therapeutic target. This may explain why, at least in vitro, L-ASN depletion of exogenous glutamine enhances cytotoxicity. As glutaminase activity is also associated with therapeutic toxicity, a compound with a lower enzymatic activity but comparable cytotoxicity has potential clinical application.

We altered L-ASN properties to go beyond evolution and optimize this drug for different treatment scenarios. Firstly the drugs have been engineered to resist degradation and inactivation by AEP produced by leukemic blast cells. We have designed two mutants that can be exchanged during cancer therapy, according to the needs and observed side effects of the patient, either reducing the dosage and thus antigenicity (N24A), or reducing the glutaminase activity, thus potentially reducing toxicity (N24A|R195S). Also, we show for the first time that secondary glutaminase activity is required for the action of the drug. Importantly, this has been achieved only looking at one species of L-asparaginase, as opposed to previous studies that compare the activities and cancer cell-kill ability of homologous asparaginase proteins. Our newly introduced L-ASN mutants N24A and N24A|R195S show potential for therapeutic use and should form a sound basis for future clinical trials.

Future directions include further molecular optimization of the drug, such as humanization of the antigenic epitopes to reduce allergic reactions and modification of subunit aggregation to enable increased drug access in solid tumors. A combination of these different aims will enable us to create a tailored cancer drug with maximum effectiveness and minimum side effects. Ultimately, to target solid tumors, the aim is to combine the above enhancements of the drug with tissue specific antibodies.
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Authorship

Contribution: The manuscript was written by M.N.O., M.K., V.S. and P.A.B.; The work was designed and devised by M.N.O., M.K., V.S. and P.A.B.; All authors read and edited the manuscript; M.N.O. and M.K. performed and analyzed the computational experiments; N.P., S.K., J.L. and V.S. designed and performed the experimental work; M.N.O. and M.K. made the figures.

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Figure 1 Close-up of the AS and high occupancy hydrogen bonds during MD simulations. (A) Overall view of the L-ASN tetramer with focus on a single monomer (green). In the close-up the AS (orange sticks) and lid-loop (blue) are shown, as well as important residues from the interacting second monomer (light pink). An aspartic acid molecule, in spacefill, is shown within the AS. (B) Residues of the AS are shown in different conformations and hydrogen bond configurations, found throughout the MD simulation. Simulations i-iv are ordered in a time dependent fashion; denoted by arrows (i) Here a more closed conformation for the AS lid-loop is shown where a hydrogen bond (red) between N24 and E283 is found. (ii) One of the main hydrogen bonds in the AS of the WT enzyme is found between D281 and N24. Furthermore, the very important stabilization of T12 by Y25 can be seen. (iii) The hydrogen bond between D281 and N24 fluctuates and can be found in different conformations. (iv) Y25 can also form a stabilizing hydrogen bond to residue E283. (C) The AS and surrounding is shown for mutant N24A. The lid-loop is colored blue. D281 and E283 are colored light pink. Y25 (orange) makes a hydrogen bond (red dashed line) to E283 and the nucleophile T12 (orange). This conformation is found more often for the mutant N24A.

Figure 2 Lid-loop flexibility (RMSF), THR12 side-chain orientation, and distance analysis for important residues of the AS. (A) The RMSF for L-ASN WT (black) and 5 mutants are depicted in different colors; calculated for the residue range, 6-38. The lowest RMSF is found for N24T (blue) and N24A (green). N24H (yellow) shows a very high c-terminal RMSF. N24G (red) shows the highest RMSF of all L-ASN mutants. The double mutant N24S|D281E (light brown) has a slightly increased RMSF compared to the WT. (B) Here the fraction of correctly placed hydroxyl group towards the AS compared to the incorrect conformation involving the methyl group is shown. The highest fraction can be seen for N24A, the lowest for N24T and N24G. (C) Keys distances measured in Å. The average and standard deviations are calculated for all snapshots found in the MD simulations of the WT (black) and the mutants N24G (red), N24A (green) and N24T (blue). Hydrogen bonds can be formed for distances around 2.8Å.
Figure 3 L-ASN activity and cell-kill assays. All experiments have been conducted three times (n = 3). Standard deviations are calculated for each protein. (A) Asparaginase activity measured for WT and mutant L-ASN. (B) Glutaminase activity for WT and mutant L-ASN. IC50 values were estimated for WT and mutants at asparaginase-equivalent doses of the compounds, thus reflecting the sole impact of modification of glutaminase activity on drug cytotoxicity. (C) Cytotoxicity assays on drug-sensitive SupB15 cells. (D) Cytotoxicity assays on intermediate drug sensitive MV4:11 cells (E) Cytotoxicity assays on drug-resistant REH cells. * p<0.05; ** p<0.01.

Figure 4 Relative glutaminase activity in the context of leukaemic cell kill. In these cell-kill assays the asparaginase activity was kept constant. For N24A, N24T, N24A R195S and N24A Y250L the relative glutaminase activity (x-axis) weighted by the asparaginase activity, and relative IC50 (y-axis) values are shown separately. The cytotoxic activity of each mutant L-ASN is indicated for the leukemic cell lines SupB15 (very drug sensitive, grey squares), MV 4:11 (intermediate sensitivity, black squares) and REH (resistant, white squares). Values > 1 (dashed line) indicate IC50 values lower than WT compound, values equal to 1 indicate cytotoxicity equivalent to WT L-ASN and values <1 indicate a higher IC50 than WT. For each mutant, an asterisk above the identifier indicates significant differences in glutaminase activity. Significant relative differences in cytotoxicity of mutant L-ASN (compared to WT) are indicated by an asterisk on the right side of the data point. * p<0.05; ** p<0.01.
Figures

Figure 1
Figure 2
Figure 3
Figure 4
Rational engineering of L-Asparaginase reveals importance of dual activity for cancer cell toxicity

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