DESNASEs, a group of RNASE-based safety switches for Controlled Cell Elimination.

Author: **Rojo**pitiavana Solofondrazaintsoanirina*
**Correspondence:* solofo.rojo@gmail.com
DOI: 10.5281/zenodo.10464874, *published online:* January 6, 2024

Table of Contents

Disclaimer

Introduction

Safety switch systems represent gene expression units capable of transcription and translation, resulting in the production of polypeptides that induce cell death upon exposure to specific activating ligands. Illustrative examples of safety switches include Herpes viruses-derived thymidine kinases, which convert pyrimidine and guanosine analogs into cytotoxic nucleoside triphosphates [1]; proapoptotic polypeptides fused with a mutant FK506 binding protein (FKBP), inducing apoptosis upon dimerization in the presence of small molecule ligands such as AP1510, AP1903, or AP21967 [2]; microbial nitroreductases [3], catalyzing the conversion of prodrugs like CB1954 (5-aziridinyl-2,4-dinitrobenzamide) or metronidazole into cytotoxic nitroso radicals; and cell surface molecules like CD20 [4], truncated EGFR [5], or RQR8 [6], which sensitize expressing cells to antibody-mediated lysis.

This discourse introduces RNAse-based safety switches, collectively known as DESNase (a combination of the words '**des**tabilized' and 'R**NAse**'), capable of activation through small molecule ligands such as Shield-1, Trimethoprim, Methotrexate, or 4-hydroxytamoxifen. The DESNase system comprises a fusion of a cytotoxic domain (an RNAse polypeptide) and a destabilizing domain (refer to FIGURE 1). The latter prompts rapid degradation of the entire fusion polypeptide. In the presence of a small molecule ligand, also referred to as a trigger or activator, the DESNAse is stabilized, enabling the cytotoxic domain to initiate the degradation of its RNA substrates. This action block protein synthesis, ultimately culminating in cell death.

Design

General design of DESNAse

The cytotoxic domain can be any RNAses proven to induce cytotoxicity upon introduction into the cytosol of cells. Exemplary candidates include recombinant human pancreatic ribonucleases with the capability to circumvent endogenous ribonuclease inhibitors [7–11]. The destabilizing domain can be selected from a recombinant human or *Escherichia coli* dihydrofolate reductase polypeptide, recombinant human FK506 binding protein (FKBP), and recombinant ligand binding domain of the human estrogen receptor (refer to Table 1).

Table 1: Destabilization domains and their corresponding ligands.

Destabilization domain	Ligand	References
Recombinant *E.coli* dihydrofolate reductase	Trimethoprim, Methotrexate, MCC8529	[12–14]
Recombinant human dihydrofolate reductase	Trimethoprim, Methotrexate	WO2021046451A1
Recombinant FK506 binding protein (FKBP)	Shield-1	[15]
LBD of estrogen receptor	CMP8, 4-hydroxytamoxifen	[16]

The cytotoxic domain can be fused at either the N- or C-terminus of the destabilizing domain. Both domain can be separated by a peptide linker (FIGURE 2), which can be flexible [17] (ex: $(G_4S)_n$) or rigid. A PEST or degron sequence [18] can be inserted at the C- or N-terminus of the fusion construct to further reduce its half-life (FIGURE 3). One or more Nuclear Localization Sequence (NLS) [19] can be incorporated into the fusion construct to facilitate its translocation into the nucleus (see FIGURE 4). A mitochondrial targeting sequence (MTS) can be incorporated into the fusion construct to shuttle it to the mitochondria.

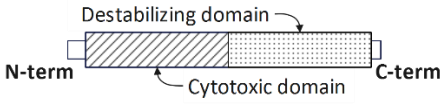

FIGURE 1: General organization of a DESNAse fusion polypeptide.

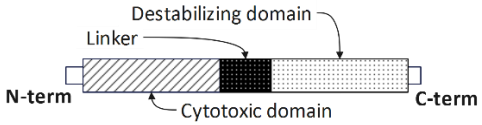

FIGURE 2: A linker polypeptide connects the cytotoxic domain and the destabilizing domain of DESNAse.

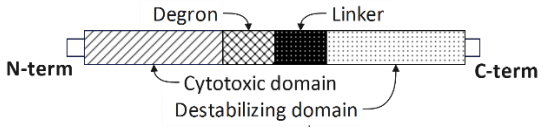

FIGURE 3: A degron sequence is inserted at the C-terminus of the cytotoxic domain of DESNAse.

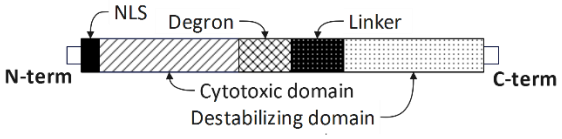

FIGURE 4: A nuclear localization sequence (NLS) is inserted at the N-terminus of DESNAse. THe NLS can be replaced with an adequate signal peptide.

Destabilizing domains can be fused at both the N- and C-termini of the cytotoxic domain to further diminish its basal activities (FIGURE 5). The incorporation of one or more protease-cleavage sites between the cytotoxic domain and the destabilizing domain [20,21] facilitates the release of the cytotoxic domain in its near-native form when the system is stabilized upon interaction with a suitable ligand (FIGURE 6).

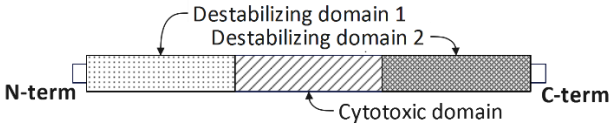

FIGURE 5: A first and a second destabilizing domains are inserted at the N- and C-terminus of the cytotoxic domain, respectively.

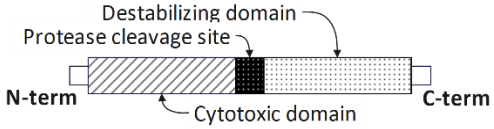

FIGURE 6: A protease cleavage site is inserted between the cytotoxic domain and the destabilizing domain of DESNAse.

While polypeptides derived from bacteria may exhibit immunogenicity, a preferred strategy to mitigate this response involves using recombinant polypeptides derived from human genes. To achieve this, a humanized DESNAse can be generated by fusing a recombinant human-derived pancreatic ribonuclease with a recombinant human-derived dihydrofolate reductase, FKBP, or the ligand-binding domain of the estrogen receptor.

Cytotoxic domains are not restricted to RNAses; alternative options include the full-length or active fragment of plants or bacteria-derived ribosome-inactivating polypeptides (ex: diphtheria toxin, Pseudomonas exotoxin A, ricin), granzyme B (with its Gly-Glu dipeptide removed or replaced with a protease-cleavable N-terminal inhibitory peptide [22]), and DNAses (actin-resistant variants [23–25]), can also serve as effective cytotoxic domains.

Human and bovine pancreatic RNAses are examples of ribonucleases that are amenable to mutation for reducing their overall affinity for cytosolic ribonuclease inhibitors (RI). The lower the affinity of RI for a given RNAse variant and the more toxic is the resulting DESNAse. This can translate into a lower transfection efficiency, but the surviving cells and their clones become highly susceptible to DESNAse-mediated killing upon the application of the appropriate small molecule ligand.

Gene expression unit

A gene expression unit or cassette is a DNA sequence that comprises, from 5' to 3': a promoter, a gene of interest or transgene (which encode DESNase), and a polyadenylation sequence (see FIGURE 7).

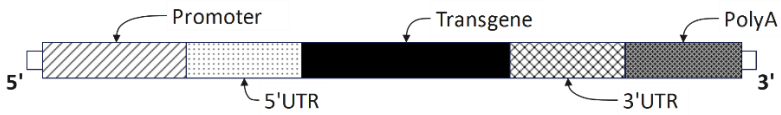

FIGURE 7: A gene expression unit. 5'UTR: 5' untranslated region; 3'UTR: 3' untranslated region; PolyA: Ppolyadenylation sequence.

The DESNase polypeptide can be computationally reverse-translated and reverse-transcribed to yield a DNA sequence, subsequently codon-optimized to permit efficient translation within the desired host organism (ex: *Mus musculus*, *Homo sapiens*). A promoter is required to drive transgene's expression. The promoter can be strong, weak, constitutive, tissue-specific [26], or inducible (ex: promoters that comprise one or more NFAT, NF-κB, NR4A, or any well-known response elements). A polyadenylation (polyA) sequence can be inserted at the 3' end of the fusion construct coding sequence, serving to both terminate transcription and polyadenylate the cleaved messenger RNA.

The gene of interest or can be directly cloned into a plasmid comprising all requisite cis-acting elements for proper transgene expression, or assembled into a functional gene expression unit before being cloned into an appropriate vector.

The DESNAse-encoding gene can be co-expressed with another gene, with both genes separated by a cis-separator—a sequence encoding a 2A peptide [27,28] or an internal ribosome entry site (IRES) . Their joint expression is driven by a single promoter (FIGURE 8).

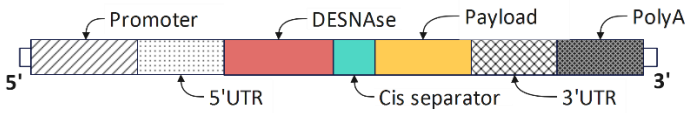

FIGURE 8: One promoter drives the joint-expression expression of DESNAse and a second gene (Payload). The cis-separator can be a 2A peptide sequence or an internal ribosome entry site (IRES).

Sequences encoding DESNAse, a payload, and a positive marker can be joint-expressed (FIGURE 9). Each gene is separated from the others by a cis-separator. DESNAse is useful for negative selection. The positive marker can be a coding sequence for a fluorescent protein, a cell-surface marker, or an antibiotic or drug resistance. Cis-separator 1 can be a 2A peptide or an IRES (for example, if the payload encodes a secreted polypeptide). Cis-separator 2 is preferably a 2A peptide, but it may also be an IRES. The promoter is preferably inducible. In the induced state, expression of the positive maker permits the selection of cells also expressing the payload. The DESNAse is useful to selectively eliminate cells that still express the payload when the promoter is supposedly in the uninduced state. If the positive marker is a cell-surface marker, then it can be used alone (the DESNAse and cis separator 2 sequences can be removed) or in combination with DESNAse to select and discard cells still expressing the payload when the promoter is supposedly in the uninduced state.

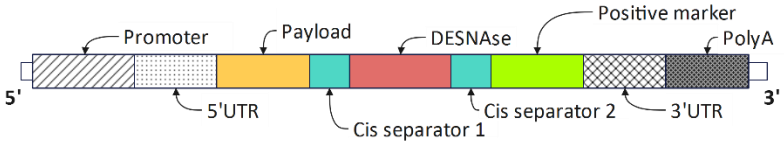

FIGURE 9: A gene expression unit wherein a payload sequence, DESNAse, and a positive marker sequence are joint-expressed.

Two or more gene expression units, each consisting of a promoter, a transgene, and a polyadenylation sequence, can be combined to create a chain or sequence of gene expression units. An example of a chain of gene expression units

is shown in FIGURE 10. For instance, transgene 1 can encode any polypeptide, such as a chimeric antigen receptor (CAR), an engineered T-cell receptor (eTCR), a synthetic notch receptor (SynNotch), or a synthetic receptor well known in the art [30]. Transgene 2 can either be a DESNAse coding sequence (CDS) or a polycistron comprising a DESNAse CDS. If Promoter 1 is constitutive and Promoter 2 is inducible, the basal expression of the latter can be further reduced by opting for a weaker polyadenylation sequence (PolyA1 and/or PolyA2). PolyA1 and PolyA2 can also be substituted with a bidirectional polyadenylation sequence like SV40 polyA.

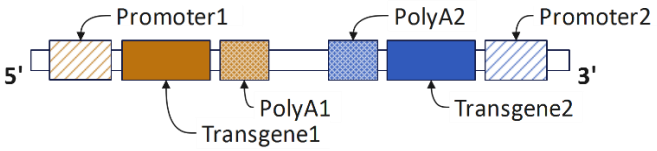

FIGURE 10: a chain composed of two gene expression units, with the first in the forward and the second in the reverse orientation.

FIGURE 11 illustrates another example of a chain comprising two gene expression units. Promoter 1 can be strong constitutive, for example the human EF1-alpha promoter. Transgene 1a can encode any polypeptide, including a chimeric antigen receptor (CAR) or an engineered T-cell receptor (eTCR). Cis-separator 1 can be an internal ribosome entry site or a 2A peptide sequence. Transgene 1b can encode any polypeptide, for example iCASP9. PolyA can be a bidirectional polyadenylation sequence, for example SV40 polyA. Promoter 2 can be an inducible promoter, for example NFAT-inducible promoter. Transgene 2a can encode a DESNAse. Cis-separator 2 is preferably a 2A peptide sequence or an IRES. Transgene 2b can encode a cell-surface marker, for example RQR8 [6]. This chain of gene expression units can be cloned into a suitable vector, for example a PiggyBac transposon plasmid [31], and then used to transform or engineer various cell types, including T lymphocytes. The cell-surface marker facilitates the selection of transfected cells (positive selection), followed by a period of rest lasting hours or days, after which constitutively activated cells can be selected and discarded (negative selection). It can also synergize with DESNAse to enhance negative selection. iCASP9, DESNAse, and the cell-surface receptor can be employed in combination to effectively eliminate engineered cells.

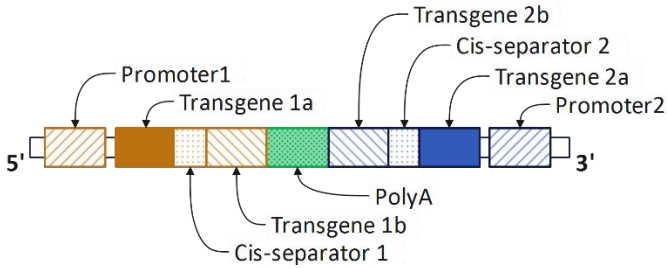

FIGURE 11: a chain of two gene expression units with a shared polyadenylation (PolyA) sequence.

A bidirectional polyadenylation (polyA) sequence can be obtained by joining the 3' end of a forward-oriented first polyA sequence with the 5' end of a reverse-oriented second polyA sequence. The strength of each polyA sequence can vary; for example, the first polyA can be very weak (ex: HSV thymidine kinase polyA), while the second can be very strong (ex: bovine growth hormone polyA). Such a construct is useful for further reducing the basal expression of a downstream transgene driven by an inducible promoter.

Implementation

Polypeptide sequence of exemplary recombinant RNAses, cytotoxic domains, and destabilizing domains are available in Table S1, Table S2, and Table S3, respectively.

Herein are exemplary DESNAse polypeptides comprising (from N- to C-terminus):

- **Construct 1:** a recombinant human RNAse 1 (ERDD_hRNase1) cytotoxic domain, and a recombinant *E. coli* DHFR variant C12 (C12_ecDHFR) destabilizing domain:
MKESRAKKFQRQHMDSDSSPSSSSTYCNQMMRRRNMTQGRCKPVNTFVHEPLVDVQNVCFQEKVTCKNGQGNCYKSNSSMHITDCRETRDSDYP
NCAYRTSPKERHIIVACEGSPYVPVHFDASVEDSTISLIAALAVDRVIGMENAMPWNLPADLAWFKRNTLNKPVIMGRHTWESIGRPLPGRKNIILSSQP
GTDDRVTRVKSVDEAIAACGDVPEIMVIGGGRVYEQFLPKAQKLYLSHIDAEVDGDTHFPDYEPDDWESVFSEFHDADALNSHSYCFEILERR
- **Construct 2:** a recombinant human RNAse 1 (ERDD_hRNase1) cytotoxic domain, a $(G_4S)_3$ linker, and a recombinant human FKBP12 (VPSS_hFKBP12) destabilizing domain:
MKESRAKKFQRQHMDSDSSPSSSSTYCNQMMRRRNMTQGRCKPVNTFVHEPLVDVQNVCFQEKVTCKNGQGNCYKSNSSMHITDCRETRDSDYP
NCAYRTSPKERHIIVACEGSPYVPVHFDASVEDSTGGGGSGGGGSGGGGSGVQVETISPGDGRTFPKRGQTCVVHYTGMLSSGKKVDSSRDRNKPFKF
MLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKPE
- **Construct 3:** a recombinant *E. coli* DHFR variant C12 (C12_ecDHFR) destabilizing domain, a recombinant ubiquitin (which comprises a protease cleavable sequence), and a recombinant *Bos taurus* RNAse 1 (ARDRR_btRNase1) cytotoxic domain:
MISLIAALAVDRVIGMENAMPWNLPADLAWFKRNTLNKPVIMGRHTWESIGRPLPGRKNIILSSQPGTDDRVTRVKSVDEAIAACGDVPEIMVIGGGR
VYEQFLPKAQKLYLSHIDAEVDGDTHFPDYEPDDWESVFSEFHDADALNSHSYCFEILERRQIFVRTLTGRTITLEVEPSDTIENVRARIQDREGIPPDQQR
LIFAGRQLEDGRTLSDYNIQRESTLHLVLRLRSGKETAAAKFERQHMDSSTSAASSSNYCNQMMASRNLTKRDCKPVNTFVHESLADVQAVCSQKNVA
CKRGQTNCYQSYSTMSITDCRETRSSKYPNCAYKTTQANKHIIVACEGNPYVPVHFDASV

Herein are exemplary gene expression units comprising (from 5' to 3'):

- **Construct 4:** an *Homo sapiens*-derived EF1-alpha promoter, an *Homo sapiens* codon-optimized DNA sequence encoding **Construct 1**, and an SV40 polyadenylation sequence:
GGCTCCGGTGCCCGTCAGTGGGCAGAGCGCACATCGCCCACAGTCCCCGAGAAGTTGGGGGGAGGGGTCGGCAATTGAACCGGTGCCTAGAGA
AGGTGGCGCGGGGTAAACTGGGAAAGTGATGTCGTGTACTGGCTCCGCCTTTTTCCCGAGGGTGGGGGAGAACCGTATATAAGTGCAGTAGTC
GCCGTGAACGTTCTTTTTCGCAACGGGTTTGCCGCCAGAACACAGGTAAGTGCCGTGTGTGGTTCCCGCGGGCCTGGCCTCTTTACGGGTTATGG
CCCTTGCGTGCCTTGAATTACTTCCACCTGGCTGCAGTACGTGATTCTTGATCCCGAGCTTCGGGTTGGAAGTGGGTGGGAGAGTTCGAGGCCTT
GCGCTTAAGGAGCCCCTTCGCCTCGTGCTTGAGTTGAGGCCTGGCCTGGGCGCTGGGGCCGCCGCGTGCGAATCTGGTGGCACCTTCGCGCCTGT
CTCGCTGCTTTCGATAAGTCTCTAGCCATTTAAAATTTTTGATGACCTGCTGCGACGCTTTTTTTCTGGCAAGATAGTCTTGTAAATGCGGGCCAAG
ATCTGCACACTGGTATTTCGGTTTTTGGGGCCGCGGGCGGCGACGGGGCCCGTGCGTCCCAGCGCACATGTTCGGCGAGGCGGGGCCTGCGAGC
GCGGCCACCGAGAATCGGACGGGGGTAGTCTCAAGCTGGCCGGCCTGCTCTGGTGCCTGGTCTCGCGCCGCCGTGTATCGCCCCGCCCTGGGCG
GCAAGGCTGGCCCGGTCGGCACCAGTTGCGTGAGCGGAAAGATGGCCGCTTCCCGGCCCTGCTGCAGGGAGCTCAAAATGGAGGACGCGGCGC
TCGGGAGAGCGGGCGGGTGAGTCACCCACACAAAGGAAAAGGGCCTTTCCGTCCTCAGCCGTCGCTTCATGTGACTCCACGGAGTACCGGGCGC
CGTCCAGGCACCTCGATTAGTTCTCGAGCTTTTGGAGTACGTCGTCTTTAGGTTGGGGGGAGGGGTTTTATGCGATGGAGTTTCCCCACACTGAG
TGGGTGGAGACTGAAGTTAGGCCAGCTTGGCACTTGATGTAATTCTCCTTGGAATTTGCCCTTTTTGAGTTTGGATCTTGGTTCATTCTCAAGCCTC
AGACAGTGGTTCAAAGTTTTTTTCTTCCATTTCAGGTGTCGTGAGCTCGTTGTCGGTACTC**GCCGCCACC**ATGAAGGAGAGCAGGGCCAAGAAGT
TCCAGAGGCAGCACATGGACAGCGACAGCAGCCCTAGCAGCAGCAGCACCTACTGCAACCAGATGATGAGGAGGAGGAACATGACCCAGGGCA
GGTGCAAGCCCGTGAACACCTTCGTTCACGAGCCCTTGGTGGACGTGCAGAACGTGTGCTTCCAGGAGAAGGTGACCTGCAAGAACGGCCAGGG
CAACTGCTACAAGAGCAACAGCAGCATGCACATCACCGACTGCAGGGAGACCAGGGACAGCGACTACCCTAACTGCGCTTACAGGACCAGCCCT
AAGGAGAGGCACATCATCGTGGCCTGCGAGGGCTCTCCTTACGTGCCCGTTCACTTCGATGCCTCTGTGGAGGACAGCACCATCAGCCTGATCGC
CGCCTTGGCTGTGGATAGGGTGATCGGCATGGAGAACGCCATGCCCTGGAACCTGCCCGCTGACTTGGCTTGGTTCAAGAGGAACACCCTGAAC
AAGCCCGTGATCATGGGCAGGCACACCTGGGAGAGCATCGGCAGGCCTTTACCCGGAAGGAAGAACATCATCCTGAGCAGCCAGCCCGGCACC
GACGATAGGGTGACCAGGGTGAAATCTGTGGACGAGGCCATCGCCGCCTGCGGAGATGTGCCTGAGATCATGGTGATCGGAGGAGGAAGGGT
GTACGAGCAGTTCCTGCCCAAGGCCCAGAAGCTGTACCTGAGCCACATCGACGCCGAGGTTGACGGCGACACCCACTTCCCTGACTACGAGCCTG
ATGACTGGGAGAGCGTGTTCAGCGAGTTCCACGACGCCGACGCCTTGAACAGCCACAGCTACTGCTTCGAGATCCTGGAGAGGAGG**TGATAA**GA
ACGGGCTTTATTTGTGAAATTTGTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGCAATAAACAAGTTAACAACAACAATTGCATTCATTTTAT
GTTTCAGGTTCAGGGGGAGGTGTGGGAGGTTTTTTAAAGC
- **Construct 5:** Four **NFAT/AP-1 response elements**; an **YB_TATA** core promoter; a Multiple Cloning site (MCS); an EMCV internal ribosome entry site (IRES); a *Homo sapiens* codon-optimized DNA sequence encoding **Construct 2**, a 2A peptide sequence, and a truncated NGFR; and a bGH polyA:
AGCTTGGATCC**AAGAGGAAAATTTGTTTCATACAGAAGGCGTTAAGAGGAAAATTTGTTTCATACAGAAGGCGTTAAGAGGAAAATTTGTTTC**
ATACAGAAGGCGTTAAGAGGAAAATTTGTTTCATACAGAAGGCGTTCAAGCTTGTCGACTCGAGATCCGG**TCTAGAGGGTATATAATGGGGGC**
CACTAGTCTACTACCAGAGCTCATCTGCCTGCAGGTCGACTCTAGAGGATCCCGGGTACCGAGCTCGAATTCGGATATCCTCGAGACTAGTGGGC
CCGTTTAAACACATGAATTGCTCGAGGTTATTTTCCACCATATTGCCGTCTTTTGGCAATGTGAGGGCCCGGAAACCTGGCCCTGTCTTCTTGACGA
GCATTCCTAGGGGTCTTTCCCCTCTCGCCAAAGGAATGCAAGGTCTGTTGAATGTCGTGAAGGAAGCAGTTCCTCTGGAAGCTTCTTGAAGACAA
ACAACGTCTGTAGCGACCCTTTGCAGGCAGCGGAACCCCCCACCTGGCGACAGGTGCCTCTGCGGCCAAAAGCCACGTGTATAAGATACACCTGC
AAAGGCGGCACAACCCCAGTGCCACGTTGTGAGTTGGATAGTTGTGGAAAGAGTCAAATGGCTCTCCTCAAGCGTATTCAACAAGGGGCTGAAG

GATGCCCAGAAGGTACCCCATTGTATGGGATCTGATCTGGGGCCTCGGTGCACATGCTTTACATGTGTTTAGTCGAGGTTAAAAAAACGTCTAGG
CCCCCCGAACCACGGGGACGTGGTTTTCCTTTGAAAAACACGATGATAATATGAAGGAGAGCAGGGCCAAGAAGTTCCAGAGACAGCACATGGA
CAGCGACAGCAGCCCTAGCAGCAGCAGCACCTACTGCAACCAGATGATGAGGAGGAGGAACATGACCCAGGGCAGGTGCAAGCCCGTGAACAC
CTTCGTGCACGAGCCCTTAGTGGACGTGCAGAACGTGTGCTTCCAGGAGAAGGTGACCTGCAAGAACGGCCAGGGCAACTGCTACAAGAGCAAC
AGCAGCATGCACATCACCGACTGCAGGGAGACCAGGGACAGCGACTACCCTAACTGCGCCTACAGGACCAGCCCTAAGGAGAGGCACATCATCG
TGGCCTGCGAGGGCTCTCCTTACGTGCCCGTTCACTTCGACGCTTCTGTGGAGGACAGCACCGGAGGAGGAGGATCTGGAGGAGGAGGATCTG
GAGGAGGAGGCTCTGGAGTGCAGGTGGAGACCATCAGCCCTGGAGATGGAAGGACCTTCCCTAAGAGAGGCCAGACCTGCGTGGTGCACTACA
CCGGCATGCTGAGCAGCGGAAAGAAGGTGGACAGCAGCAGGGACAGGAACAAGCCCTTCAAGTTCATGCTGGGCAAGCAGGAGGTGATCAGA
GGCTGGGAGGAGGGAGTGGCTCAGATGAGCGTGGGACAGAGGGCTAAGCTGACCATCAGCCCTGACTACGCCTACGGCGCCACAGGACACCCT
GGAATCATCCCTCCTCATGCCACACTGGTGTTCGACGTGGAGCTGCTGAAGCCCGAGGGCAGCGGCGCCACCAACTTCAGCCTGCTGAAGCAGG
CCGGAGATGTTGAGGAGAACCCTGGACCTATGTACGACGGCCCTAGGCTGCTGCTGCTGCTGCTGCTGGGAGTTAGCTTAGGAGGAGCCAAGGA
GGCCTGCCCTACCGGCTTGTACACCCACTCTGGAGAGTGCTGCAAGGCCTGCAACCTGGGCGAGGGAGTTGCTCAGCCTTGTGGAGCTAACCAG
ACCGTGTGCGAGCCCTGCCTGGACAGCGTGACCTTCAGCGATGTTGTGAGCGCTACCGAGCCCTGCAAGCCCTGCACCGAGTGTGTGGGCTTACA
GTCTATGAGCGCTCCCTGCGTGGAGGCCGACGACGCTGTGTGTAGGTGTGCTTATGGATACTACCAGGACGAGACCACCGGCAGGTGCGAAGCT
TGTAGGGTTTGTGAGGCCGGCTCTGGCTTGGTGTTCAGCTGCCAGGACAAGCAGAACACCGTGTGCGAGGAGTGCCCTGACGGCACATACAGCG
ACGAAGCCAACCACGTGGACCCTTGCCTGCCCTGCACCGTTTGTGAGGACACCGAAAGGCAGCTGAGGGAGTGCACCAGGTGGGCCGATGCTGA
GTGTGAGGAGATCCCTGGAAGGTGGATCACCAGGAGCACACCTCCCGAGGGCTCTGACAGCACAGCTCCTAGCACACAGGAGCCTGAAGCTCCT
CCCGAGCAGGACCTGATCGCCAGCACAGTTGCTGGAGTGGTGACCACCGTGATGGGCAGCAGCCAACCCGTGGTTACACGAGGCACCACAGACA
ACCTGATCCCTGTGTACTGCAGCATCCTGGCCGCCGTGGTGGTGGGCTTAGTTGCCTACATCGCCTTCAAGAGGTGGAACTGATAACTGTGCCTTC
TAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATT
GCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCAT
GCTGGGGATGCGGTGGGCTCTATGG

Applications

A cell expressing DESNAse undergoes cell death when cultured in the presence of an effective amount of an appropriate small molecule ligand. Consequently, DESNAse can replace other systems, including iCASP9 [2], RapaCaspase-9 [32], thymidine kinases [1,33,34], and nitroreductases [3,35].

When DESNAse expression is controlled by an inducible promoter, only induced cells are killed when an effective amount of an appropriate small molecule ligand is added to the culture. For example, T cells can be transfected with a transposase expression vector and a transposon plasmid comprising a payload and a DESNAse, both of which are under the control of an IL-2-derived NFAT promoter. Following the step of antigen stimulation, positive selection, and clonal expansion, the cells are allowed to rest for hours or days. Treating the supposedly resting cells with an effective amount of an appropriate small molecule ligand will lead to the selective elimination of engineered T cells that still constitutively co-express the payload and DESNAse. A similar approach—where only the DESNAse is retained and the payload is removed—can be employed to eliminate constitutively activated engineered T cells form a mixed population.

DESNase can serve as an inducible and highly potent therapeutic payload suitable for loading into gene therapy systems, such as oncolytic adenoviruses and replication-proficient or defective retroviral vectors. It does not interfere with viral replication or induce cell death in transduced cells unless the animal model or the patient treated with the gene therapy is administered with an effective amount of an appropriate small molecule ligand, leading to the targeted elimination of infected or transduced cells.

DESNase can synergize with other safety switches in enhancing the overall safety of cell therapy. For example, T lymphocytes or immortalized cell lines established from malignant tissue can be engineered with a DNA sequence comprising one or more DESNAse and iCASP9 CDS integrated in their genome to ensure elimination if safety concerns arise. In another example, bi-allelic loss of the gene encoding or depletion of 5-methylcytosine dioxygenase TET2 can improve the persistence of CAR T cells [36], but such alteration can lead to the acquisition of genomic instability and enable antigen-independent cell proliferation [37]. A combination of DESNAse, iCASP9, and a cell-surface marker [6] (FIGURE 11) will considerably improve the safety of such therapeutic and accelerate its adoption as a treatment modality against hematological and solid malignancies.

A DESNAse comprises a cytotoxic domain and a destabilization domain. The term “an appropriate small molecule ligand” refers to the stabilizing ligand associated with the destabilization domain (see Table 1). The term “an effective amount of an appropriate small molecule ligand” means that the concentration of the small molecule ligand is such that it leads to the death of at least 1% of DESNAse expressing cells. The concentration can be expressed in pM (picomolar), nM (nanomolar), µM (micromolar), mM (millimolar), or M (molar) units, ranging between 1pM and 500mM. An effective amount of an appropriate small molecule ligand can be determined through simple routine experiments involving mock controls and DESNAse-expressing cells, both expressing a reporter gene. These cells can be cultured in vitro or injected into one or multiple animal models or human subjects, which are treated at various concentrations of the small molecule ligand, or a fixed amount of placebo or saline, for minutes, hours, or days. The “an effective amount of an appropriate small molecule ligand” can also be expressed in ng/Kg (nanograms of small molecule ligand per kilogram of body weight) or in mg/Kg (milligrams of small molecule ligand per kilogram of body weight), ranging between 0.1ng/kg and 500 mg/Kg.

If the cells are immune cells, and the promoter driving DESNAse expression is induced in the presence of an antigen, then the term “the cells are allowed to rest for hours or days” means culturing the cells, for at least one hour, in the presence of cytokines (ex: IL2/IL15/IL21 and accessorily IL7), in the absence of stimulating ligand (ex: CD3 and/or CD28 antibodies), and in the absence of small molecule ligand that might activate DESNAse. In cases where the promoter comprises one or more hypoxia-response elements, then the cells are cultured in normoxic condition. The overarching goal is to culture the cells for at least one hour and in such a way that the promoter driving DESNAse expression returns and remains (or is maintained) in the uninduced state.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest statement

The author declares no conflict of interest.

References

[1] C. N. Barese, A. E. Krouse, M. E. Metzger, C. A. King, C. Traversari, F. C. Marini, R. E. Donahue, and C. E. Dunbar, “Thymidine kinase suicide gene-mediated ganciclovir ablation of autologous gene-modified rhesus hematopoiesis,” *Mol Ther* **20**, 1932–1943 (2012).

[2] K. C. Straathof, M. A. Pulè, P. Yotnda, G. Dotti, E. F. Vanin, M. K. Brenner, H. E. Heslop, D. M. Spencer, and C. M. Rooney, “An inducible caspase 9 safety switch for T-cell therapy,” *Blood* **105**, 4247–4254 (2005).

[3] A. V. Sharrock, T. S. Mulligan, K. R. Hall, E. M. Williams, D. T. White, L. Zhang, K. Emmerich, F. Matthews, S. Nimmagadda, et al., “NTR 2.0: a rationally engineered prodrug-converting enzyme with substantially enhanced efficacy for targeted cell ablation,” *Nat Methods* **19**, 205–215 (2022).

[4] T. van Meerten, M.-J. Claessen, A. Hagenbeek, and S. B. Ebeling, “The CD20/αCD20 ‘suicide’ system: novel vectors with improved safety and expression profiles and efficient elimination of CD20-transgenic T cells,” *Gene Ther* **13**, 789–797 (2006).

[5] T. B. Shabaneh, H. F. Moffett, S. M. Stull, T. Derezes, L. J. Tait, S. Park, S. R. Riddell, and M. J. Lajoie, “Safety switch optimization enhances antibody-mediated elimination of CAR T cells,” *Front. Mol. Med* **2**, 1026474 (2022).

[6] B. Philip, E. Kokalaki, L. Mekkaoui, S. Thomas, K. Straathof, B. Flutter, V. Marin, T. Marafioti, R. Chakraverty, et al., “A highly compact epitope-based marker/suicide gene for easier and safer T-cell therapy,” *Blood* **124**, 1277–1287 (2014).

[7] H. A. Erickson, M. D. Jund, and C. A. Pennell, “Cytotoxicity of human RNase-based immunotoxins requires cytosolic access and resistance to ribonuclease inhibition,” *Protein Engineering, Design and Selection* **19**, 37–45 (2006).

[8] G. Gotte and M. Menegazzi, “Biological Activities of Secretory RNases: Focus on Their Oligomerization to Design Antitumor Drugs,” *Front. Immunol.* **10**, 2626 (2019).
[9] A. Attery, P. Dey, P. Tripathi, and J. K. Batra, “A ribonuclease inhibitor resistant dimer of human pancreatic ribonuclease displays specific antitumor activity,” *International Journal of Biological Macromolecules* **107**, 1965–1970 (2018).
[10] T. J. Rutkoski and R. T. Raines, “Evasion of ribonuclease inhibitor as a determinant of ribonuclease cytotoxicity,” *Curr Pharm Biotechnol* **9**, 185–189 (2008).
[11] P. A. Leland, K. E. Staniszewski, B.-M. Kim, and R. T. Raines, “Endowing Human Pancreatic Ribonuclease with Toxicity for Cancer Cells,” *Journal of Biological Chemistry* **276**, 43095–43102 (2001).
[12] E. Nakahara, V. Mullapudi, G. E. Collier, L. A. Joachimiak, and J. D. Hulleman, “Development of a New DHFR-Based Destabilizing Domain with Enhanced Basal Turnover and Applicability in Mammalian Systems,” *ACS Chem. Biol.* **17**, 2877–2889 (2022).
[13] H. Peng, V. Q. Chau, W. Phetsang, R. M. Sebastian, M. R. L. Stone, S. Datta, M. Renwick, Y. T. Tamer, E. Toprak, et al., “Non-antibiotic Small-Molecule Regulation of DHFR-Based Destabilizing Domains In Vivo,” *Mol Ther Methods Clin Dev* **15**, 27–39 (2019).
[14] M. Iwamoto, T. Björklund, C. Lundberg, D. Kirik, and T. J. Wandless, “A general chemical method to regulate protein stability in the mammalian central nervous system,” *Chem Biol* **17**, 981–988 (2010).
[15] J. S. Grimley, D. A. Chen, L. A. Banaszynski, and T. J. Wandless, “Synthesis and analysis of stabilizing ligands for FKBP-derived destabilizing domains,” *Bioorg Med Chem Lett* **18**, 759–761 (2008).
[16] Y. Miyazaki, H. Imoto, L. Chen, and T. J. Wandless, “Destabilizing Domains Derived from the Human Estrogen Receptor,” *J. Am. Chem. Soc.* **134**, 3942–3945 (2012).
[17] G. Li, Z. Huang, C. Zhang, B.-J. Dong, R.-H. Guo, H.-W. Yue, L.-T. Yan, and X.-H. Xing, “Construction of a linker library with widely controllable flexibility for fusion protein design,” *Appl Microbiol Biotechnol* **100**, 215–225 (2016).
[18] H. Chassin, M. Müller, M. Tigges, L. Scheller, M. Lang, and M. Fussenegger, “A modular degron library for synthetic circuits in mammalian cells,” *Nat Commun* **10**, 2013 (2019).
[19] S. Kosugi, M. Hasebe, N. Matsumura, H. Takashima, E. Miyamoto-Sato, M. Tomita, and H. Yanagawa, “Six Classes of Nuclear Localization Signals Specific to Different Binding Grooves of Importin α,” *Journal of Biological Chemistry* **284**, 478–485 (2009).
[20] X. J. Gao, L. S. Chong, M. S. Kim, and M. B. Elowitz, “Programmable protein circuits in living cells,” *Science* **361**, 1252–1258 (2018).
[21] Y. Miyamae, L. Chen, Y. Utsugi, H. Farrants, and T. J. Wandless, “A Method for Conditional Regulation of Protein Stability in Native or Near-Native Form,” *Cell Chemical Biology* **27**, 1573-1581.e3 (2020).
[22] P. Ho, C. Ede, and Y. Y. Chen, “Modularly Constructed Synthetic Granzyme B Molecule Enables Interrogation of Intracellular Proteases for Targeted Cytotoxicity,” *ACS Synth Biol* **6**, 1484–1495 (2017).
[23] C. Q. Pan, D. V. Sinicropi, and R. A. Lazarus, “Engineered properties and assays for human DNase I mutants,” *Methods Mol Biol* **160**, 309–321 (2001).
[24] C. Q. Pan and R. A. Lazarus, “Hyperactivity of human DNase I variants. Dependence on the number of positively charged residues and concentration, length, and environment of DNA,” *J Biol Chem* **273**, 11701–11708 (1998).
[25] C. Lam, L. Santell, B. Wilson, M. Yim, S. Louie, D. Tang, D. Shaw, P. Chan, R. A. Lazarus, et al., “Taming hyperactive h DN ase I: Stable inducible expression of a hyperactive salt- and actin-resistant variant of human deoxyribonuclease I in CHO cells,” *Biotechnology Progress* **33**, 523–533 (2017).
[26] C. Zheng and B. J. Baum, “Evaluation of promoters for use in tissue-specific gene delivery,” *Methods Mol Biol* **434**, 205–219 (2008).
[27] Z. Liu, O. Chen, J. B. J. Wall, M. Zheng, Y. Zhou, L. Wang, H. Ruth Vaseghi, L. Qian, and J. Liu, “Systematic comparison of 2A peptides for cloning multi-genes in a polycistronic vector,” *Sci Rep* **7**, 2193 (2017).
[28] A. L. Szymczak-Workman, K. M. Vignali, and D. A. A. Vignali, “Design and Construction of 2A Peptide-Linked Multicistronic Vectors,” *Cold Spring Harb Protoc* **2012**, pdb.ip067876 (2012).
[29] J. Zhao, Y. Li, C. Wang, H. Zhang, H. Zhang, B. Jiang, X. Guo, and X. Song, “IRESbase: a Comprehensive Database of Experimentally Validated Internal Ribosome Entry Sites,” preprint, Bioinformatics, 2020.

[30] F. Teng, T. Cui, L. Zhou, Q. Gao, Q. Zhou, and W. Li, “Programmable synthetic receptors: the next-generation of cell and gene therapies,” *Sig Transduct Target Ther* **9**, 7 (2024).
[31] J. E. Doherty, L. E. Huye, K. Yusa, L. Zhou, N. L. Craig, and M. H. Wilson, “Hyperactive piggyBac gene transfer in human cells and in vivo,” *Hum Gene Ther* **23**, 311–320 (2012).
[32] L. Bouquet, E. Bôle-Richard, W. Warda, M. Neto Da Rocha, R. Trad, C. Nicod, R. Haderbache, D. Genin, C. Ferrand, et al., “RapaCaspase-9-based suicide gene applied to the safety of IL-1RAP CAR-T cells,” *Gene Ther* **30**, 706–713 (2023).
[33] M. Locatelli, F. Delhaes, O. Cherpin, M. E. Black, S. Carnesecchi, O. Preynat-Seauve, Y. Hibaoui, and K.-H. Krause, “Optimization of Thymidine Kinase-Based Safety Switch for Neural Cell Therapy,” *Cells* **11**, 502 (2022).
[34] C. Karreman, “New positive/negative selectable markers for mammalian cells on the basis of Blasticidin deaminase-thymidine kinase fusions,” *Nucleic Acids Res* **26**, 2508–2510 (1998).
[35] S. Curado, D. Y. R. Stainier, and R. M. Anderson, “Nitroreductase-mediated cell/tissue ablation in zebrafish: a spatially and temporally controlled ablation method with applications in developmental and regeneration studies,” *Nat Protoc* **3**, 948–954 (2008).
[36] J. A. Fraietta, C. L. Nobles, M. A. Sammons, S. Lundh, S. A. Carty, T. J. Reich, A. P. Cogdill, J. J. D. Morrissette, J. E. DeNizio, et al., “Disruption of TET2 promotes the therapeutic efficacy of CD19-targeted T cells,” *Nature* **558**, 307–312 (2018).
[37] N. Jain, Z. Zhao, J. Feucht, R. Koche, A. Iyer, A. Dobrin, J. Mansilla-Soto, J. Yang, Y. Zhan, et al., “TET2 guards against unchecked BATF3-induced CAR T cell expansion,” *Nature* **615**, 315–322 (2023).

Supplementary materials

Table S1: Polypeptide sequences of some wild-type and recombinant RNAses.

Name	Description	Sequence
Human pancreatic ribonuclease/hRNase1 /hRNaseA	Uniprot: P07998, AA:29-156	KESRAKKFQRQHMDSDSSPSSSSTYCNQMMRRRNMTQGRCKPVNTFV HEPLVDVQNVCFQEKVTCKNGQGNCYKSNSSMHITDCRLTNGSRYPNCA YRTSPKERHIIVACEGSPYVPVHFDASVEDST
ERDD_hRNase1	L86E/N88R/G89D/R91D	KESRAKKFQRQHMDSDSSPSSSSTYCNQMMRRRNMTQGRCKPVNTFV HEPLVDVQNVCFQEKVTCKNGQGNCYKSNSSMHITDCRETRDSDYPNCA YRTSPKERHIIVACEGSPYVPVHFDASVEDST
(*Bos taurus*) btRNase1/btRNaseA	Uniprot: P61823	KETAAAKFERQHMDSSTSAASSSNYCNQMMKSRNLTKDRCKPVNTFVH ESLADVQAVCSQKNVACKNGQTNCYQSYSTMSITDCRETGSSKYPNCAYK TTQANKHIIVACEGNPYVPVHFDASV
ARDRR_btRNase1	K31A/D38R/R39D/N67R/G8 8R	KETAAAKFERQHMDSSTSAASSSNYCNQMMASRNLTKRDCKPVNTFVH ESLADVQAVCSQKNVACKRGQTNCYQSYSTMSITDCRETRSSKYPNCAYK TTQANKHIIVACEGNPYVPVHFDASV

Table S2: Polypeptide sequences of some cytotoxic domains.

Name	Description	Sequence
Diphtheria toxin	Domain A and B of Diphtheria toxin (AA:1-389)	MSRKLFASILIGALLGIGAPPSAHAGADDVVDSSKSFVMENFSSYHGTKPG YVDSIQKGIQKPKSGTQGNYDDDWKGFYSTDNKYDAAGYSVDNENPLSG KAGGVVKVTYPGLTKVLALKVDNAETIKKELGLSLTEPLMEQVGTEEFIKRF GDGASRVVLSLPFAEGSSSVEYINNWEQAKALSVELEINFETRGKRGQDA MYEYMAQACAGNRVRRSVGSSLSCINLDWDVIRDKTKTKIESLKEHGPIK NKMSESPNKTVSEEKAKQYLEEFHQTALEHPELSELKTVTGTNPVFAGANY AAWAVNVAQVIDSETADNLEKTTAALSILPGIGSVMGIADGAVHHNTEEI VAQSIALSSLMVAQAIPLVGELVDIGFAAYNFVESI
Pseudomonas Exotoxin A	Domains Ib (AA:365–404) and III (AA:405–613)	SADVVSLTCPVAAGECAGPADSGDALLERNYPTGAEFLGDGGDISFSTRG TQNWTVERLLQAHRQLEERGYVFVGYHGTFLEAAQSIVFGGVRARSQDL DAIWRGFYIAGDPALAYGYAQDQEPDARGRIRNGALLRVYVPRSSLPGFY RTGLTLAAPEAAGEVERLIGHPLPLRLDAITGPEEEGGRLETILGWPLAERT VVIPSAIPTDPRNVGGDLDPSSIPDKEQAISALPDYASQPGKPPREDLK
Ricin toxin	Subunit A (AA:36-302)	IFPKQYPIINFTTAGATVQSYTNFIRAVRGRLTTGADVRHEIPVLPNRVGLPI NQRFILVELSNHAELSVTLALDVTNAYVVGYRAGNSAYFFHPDNQEDAEAI THLFTDVQNRYTFAFGGNYDRLEQLAGNLRENIELGNGPLEEAISALYYYST GGTQLPTLARSFIICIQMISEAARFQYIEGEMRTRIRYNRRSAPDPSVITLEN SWGRLSTAIQESNQGAFASPIQLQRRNGSKFSVYDVSILIPIIALMVYRCAP PPSSQF
Human Granzyme B	Uniprot: P10144 ; AA:21-247	IIGGHEAKPHSRPYMAYLMIWDQKSLKRCGGFLIRDDFVLTAAHCWGSSI NVTLGAHNIKEQEPTQQFIPVKRPIPHPAYNPKNFSNDIMLLQLERKAKRT RAVQPLRLPSNKAQVKPGQTCSVAGWGQTAPLGKHSHTLQEVKMTVQE DRKCESDLRHYYDSTIELCVGDPEIKKTSFKGDSGGPLVCNKVAQGIVSYGR NNGMPPRACTKVSSFVHWIKKTMKRY
Human DNAse I	Q9R/E13R/T14K/H44R/N74 K/T205K	LKIAAFNIRTFGRKKMSNATLVSYIVQILSRYDIALVQEVRDSRLTAVGKLLD NLNQDAPDTYHYVVSEPLGRKSYKERYLFVYRPDQVSAVDSYYYDDGCEP CGNDTFNREPAIVRFFSRFTEVREFAIVPLHAAPGDAVAEIDALYDVYLDV QEKWGLEDVMLMGDFNAGCSYVRPSQWSSIRLWTSPTFQWLIPDSADT TAKPTHCAYDRIVVAGMLLRGAVVPDSALPFNFQAAYGLSDQLAQAISDH YPVEVMLK

Table S3: Sequences of some wild-type polypeptides and the corresponding recombinant destabilizing domains.

Name	Description	Sequence
ecDHFR	Uniprot: P0ABQ4	MISLIAALAVDRVIGMENAMPWNLPADLAWFKRNTLNKPVIMGRHTWE SIGRPLPGRKNIILSSQPGTDDRVTWVKSVDEAIAACGDVPEIMVIGGGRV YEQFLPKAQKLYLTHIDAEVEGDTHFPDYEPDDWESVFSEFHDADAQNSH SYCFEILERR
C12_ecDHFR	W74R/T113S/E120D/Q146L	MISLIAALAVDRVIGMENAMPWNLPADLAWFKRNTLNKPVIMGRHTWE SIGRPLPGRKNIILSSQPGTDDRVTRVKSVDEAIAACGDVPEIMVIGGGRVY EQFLPKAQKLYLSHIDAEVDGDTHFPDYEPDDWESVFSEFHDADALNSHS YCFEILERR
hFKBP12	Uniprot: P62942	GVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFM LGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVF DVELLKLE
VPSS_hFKBP12	F36V/L106P/E31S/D32S	GVQVETISPGDGRTFPKRGQTCVVHYTGMLSSGKKVDSSRDRNKPFKFM LGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVF DVELLKPE
Ligand Binding Domain (LBD) of human Estrogen Receptor (LBD_hER)	Uniprot: P03372; AA: 305..549	SLALSLTADQMVSALLDAEPPILYSEYDPTRPFSEASMMGLLTNLADRELV HMINWAKRVPGFVDLTLHDQVHLLECAWLEILMIGLVWRSMEHPGKLLF APNLLLDRNQGKCVEGMVEIFDMLLATSSRFRMMNLQGEEFVCLKSIILL NSGVYTFLSSTLKSLEEKDHIHRVLDKITDTLIHLMAKAGLTLQQQHQRLAQ LLLILSHIRHMSNKGMEHLYSMKCKNVVPLYDLLLEMLDAHRL
LBD_hER_#50	T371A/L384M/M421G/N519S/G521R/Y537S	SLALSLTADQMVSALLDAEPPILYSEYDPTRPFSEASMMGLLTNLADRELV HMINWAKRVPGFVDLALHDQVHLLECAWMEILMIGLVWRSMEHPGKLL FAPNLLLDRNQGKCVEGGVEIFDMLLATSSRFRMMNLQGEEFVCLKSIILL NSGVYTFLSSTLKSLEEKDHIHRVLDKITDTLIHLMAKAGLTLQQQHQRLAQ LLLILSHIRHMSSKGMEHLYSMKCKNVVPLSDLLLEMLDAHRL
Human DHFR (hDHFR)	Uniprot: P00374	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQRMTTTSSVEGKQ NLVIMGKKTWFSIPEKNRPLKGRINLVLSRELKEPPQGAHFLSRSLDDALKL TEQPELANKVDMVWIVGGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFF PEIDLEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND
EHI_hDHFR	M1Δ/Q36E/Q103H/Y122I	~~M~~MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFERMTTTSSVEGKQ NLVIMGKKTWFSIPEKNRPLKGRINLVLSRELKEPPQGAHFLSRSLDDALKL TEHPELANKVDMVWIVGGSSVIKEAMNHPGHLKLFVTRIMQDFESDTFF PEIDLEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND