
Artificial miRNAs targeting multiple hydrolases

FIELD OF THE INVENTION

[001] The present invention relates to an artificial microRNA (artificial miRNA or amiRNA) comprising
5 an artificial miRNA guide strand sequence targeting two or more mammalian hydrolases acting on
ester bonds, wherein the two or more hydrolases are preferably endogenous hydrolases of a Chinese
hamster ovary (CHO) cell. The invention further relates to a mammalian expression vector comprising
a polynucleotide sequence encoding said artificial miRNA and to a mammalian cell (e.g., a CHO cell)
comprising said artificial miRNA. The invention further relates to the use of said mammalian cell for
10 producing a protein of interest and to the use of the artificial miRNA for preventing expression of at
least two endogenous hydrolases in the mammalian cell and/or hydrolase activity associated with a
recombinant protein of interest produced therein. Moreover, provided is a method for manufacturing
a protein of interest comprising introducing said artificial miRNA into a mammalian cell and to a
composition comprising a recombinant protein of interest and optionally polysorbate, wherein the
15 recombinant protein of interest is obtained by said method.

BACKGROUND

[002] A large number of companies observe polysorbate (PS) degradation and associated
(sub-)visible particle formation in biological drug formulations, which compromise the stability of the
20 drug product, ultimately posing a risk towards delivering innovative medicines to patients. The main
culprits of PS degradation are hydrolytic host cell proteins (HCPs) originating from the production cell
lines, which are mostly Chinese hamster ovary (CHO) cell derived. Here, a small portion of particularly
difficult-to-remove HCPs - mainly lipases - cause hydrolytic cleavage of PS resulting in the
accumulation of free fatty acid aggregates/particles.

25 [003] High product quality and stability are imperative for biopharmaceutical drug products. Particle
formation in formulated drug products (DP) like monoclonal antibody (mAb) solutions has been a
concern in the biopharmaceutical industry and enzymatic polysorbate (PS) degradation was identified
as one main root cause. Due to the structural similarity between PS, which is frequently used as a
surfactant in DP formulations, and triglycerides as natural substrates for hydrolases acting on ester
30 bonds, such as lipases, these enzymes are capable of hydrolyzing PS into polyoxyethylene (POE)
and fatty acids and thus can cause the formation of particles that are composed of fatty acids.

[004] Particle formation in biopharmaceutical DP formulations has a negative impact on long-term
stability of the DP and thus may impose a risk to cause immune reactions in patients. In addition,
some contaminating hydrolases/lipases (e.g. putative phospholipase B-like-2 (PLBL2)) represent
35 difficult to remove non-human host cell proteins (HCP) and were already reported to be immunogenic
in patients and should therefore be removed or avoided in pharmaceutical DP compositions. It has

been observed that the HCPs co-purified with the active pharmaceutical ingredient varies among different biopharmaceuticals and hence the contaminating co-purified hydrolases/lipases are believed to differ between biopharmaceutical products, cell lines and manufacturing processes.

[005] In recent years, several residual HCPs were identified in drug product formulations and reported as potentially being responsible for PS degradation. The identified enzymes were mainly assigned to the class of lipases, a subclass of esterases catalyzing the hydrolysis of lipids (Yunyu et al., American Pharmaceutical Review, 2020). Among others, group XV lysosomal phospholipase A2 isomer X1 (LPLA2), putative phospholipase B-like-2 (PLBL2), liver carboxylesterase, phospholipase A2 group VII (PLA2G7), lysophospholipase 2 (LYPLA2), lysosomal acid lipase A and lipoprotein lipase (LPL) were detected in activity-based protein profiling assays and final drug products indicating these enzymes as potentially critical towards PS degradation (Hall et al., Journal of Pharmaceutical sciences, 2016, 105(5): 1633-1642; Dixit et al., Journal of Pharmaceutical Sciences, 2016, 105(5):1657-1666; Zhang et al., Journal of Pharmaceutical Sciences, 2020, 109(11): 3300-3307; Chiu et al., Biotechnology and Bioengineering, 2017, 114(5): 1006-1015; Li et al., Analytical Chemistry, 2021, 93(23): 8161-8169). However, not only lipases but also other enzymes with PS degradation potential were identified.

[006] Li and colleagues reported the identification of 82 proteins using an activity-based protein profiling approach to enrich potential PS degrading enzymes. For instance, sialic acid acetyltransferase (SIAE) was identified in this study as the possible root cause for PS80 degradation (Li et al., Analytical Chemistry, 2021, 93(23): 8161-8169). Also, palmitoyl-protein thioesterase 1 (PPT1) has been described by Graf et al., 2021 (Graf et al., Journal of Pharmaceutical Sciences, 2021, 110(11): 3558-3567). Some of the difficult-to-remove HCPs showed PS degrading properties already in trace amounts being present in formulated drug products. The presence of HCP contaminants in formulated drug products in trace amounts represents a major problem in terms of detectability of low abundant HCPs and makes the identification of the lipase that are actually responsible for PS hydrolysis more challenging (Hall et al., Journal of pharmaceutical sciences, 2016, 105(5): 1633-1642; Zhang et al., Journal of Pharmaceutical Sciences, 2020, 109(11): 3300-3307). Furthermore, Aboulaich et al. observed variations in HCP profiles associated with different kind of mAb formats (Aboulaich et al., Biotechnology progress, 2014, 30(5): 1114-1124). Similar results were reported for difficult-to-remove lipases, whereby lipases could be identified in mAb 1 formulations but not in mAb 2 formulations. This can be explained by the binding behavior of the described lipase towards the produced mAb (Tran et al., Journal of chromatography A, 2016, 1438: 31-38; Li et al., Analytical Chemistry, 2021, 93(23): 8161-8169). Consequently, individual lipases resist the purification process and thus might be responsible for the degradation of PS. Furthermore, the choice of the final production cell line has an impact on the resulting HCP profile. Dependent on the HCP expression profile of the selected production cell line, different amounts and compositions of mAb-bound HCPs can be expected after the purification process and in the final drug product. Moreover, batch-to-batch variations regarding the final HCP content and composition are common. Thus, there is a need to reduce contaminating

lipase activity in biopharmaceuticals. Since the contaminating lipases seem to vary between products, there is a need to target simultaneously multiple lipases. In addition, some lipases (PLBL2) were reported to be immunogenic in patients and hence removal of such lipases would also be advantageous for this reason, preferably prior to purification, i.e., already during the cell line development and upstream process.

[007] One possible mitigation strategy is the removal of such critical HCPs in the production cell line. Engineering of mammalian cell lines and especially CHO cells has a long history. Therefore, cell lines are altered to obtain and/or lose a certain phenotype or characteristics by overexpression, downregulation or knockout (KO) of individual genes or combinations thereof, such as to improve productivity, controlling product quality, such as glycosylation and ensuring cell line stability. In addition to more traditional knock-out and knock-in approaches, more recently gene-editing technologies have become increasingly important. Another alternative approach is RNA interference, collectively describing small non-coding RNAs resulting in post-transcriptional control of mRNAs.

[008] Multi-gene regulation can be achieved via microRNAs (miRNAs). Various miRNA expression studies in CHO cells showed that endogenous miRNAs are differentially expressed during different cultivation conditions, such as nutrient-depletion, following temperature shifts or in different cultivation phases (Druz et al., *Biotechnology and Bioengineering*, 2011, 108(7): 1651-1661; Gammell et al., *Journal of Biotechnology*, 2007, 130(3): 213-218; Hernández Bort et al., *Biotechnology Journal*, 2012, 7(4): 500-515). Additionally, natural miRNAs impacting recombinant protein expression and secretion were identified by the comparison of expression profiles of high and low producing CHO cells (Lin et al., *Biotechnology Progress*, 2011, 27(4): 1163-1171; Hammond et al., *Biotechnology and Bioengineering*, 2012, 109(6): 1371-1375; Maccani et al., *Applied Microbiology and Biotechnology*, 2014, 98(17): 7535-7548). In recent years, various human miRNAs were proven as pro-productive and/or beneficial for cell growth in CHO cell lines. Therefore, comprehensive miRNA mimic libraries were transiently transfected into CHO cells and the respective change in phenotype was analyzed (Strothbek et al., *Metabolic Engineering*, 2013, 20:157-166; Fischer et al., *Biotechnol J.* 2014 Oct;9(10):1279-1292). Consequently, miRNA candidates with a positive impact on cell performance were stably co-expressed with different recombinant products.

[009] Cell line engineering resulted in a product-independent increase in productivity during fed-batch cultivation while no impact on product quality attributes was observed (Strothbek et al., *Metabolic Engineering*, 2013, 20:157-166; Fischer et al., *Biotechnology and Bioengineering*, 2017, 114(7): 1495-1510). These results demonstrate not only the feasibility, but also the high potential of miRNA-based cell line engineering in order to generate a genetically optimized CHO production cell line. However, to date, CHO cell line engineering strategies using miRNAs were exclusively conducted using naturally occurring miRNAs or modifications thereof including cross-species applications.

[010] The discovery of miRNA in the early 1990s (Lee et al., *Cell*, 1993, 75(5): 843-854) opened a new chapter in the research field of gene regulation, presumably half of the human transcriptome underlies the regulation of miRNAs. miRNAs direct the coordinated repression of multiple messenger

RNA (mRNA) transcripts, forming complex gene regulatory networks. Target recognition for miRNA is based on partial complementarity between the miRNA and the 3' untranslated region (3'UTR) of each target mRNA, although targeting is also observed in open reading frames (ORFs) at lower frequency. While miRNAs are naturally occurring as small RNAs repressors for gene expression, artificial short interfering RNAs (siRNAs) have been developed as powerful tools for experimental gene repression. In contrast to the partial complementarity observed with miRNA target interactions, siRNAs are designed to be perfectly complementary to a single mRNA target, enabling the repression of individual genes and are well established and can be designed and generated on demand.

[011] In order to enable a tailored gene regulation of multiple specific target lipases self-designed and non-naturally occurring artificial miRNAs (amiRNA) need to be designed. Such artificially designed miRNAs to target multiple desired mRNAs are rare and not established. *De novo* design of miRNAs is difficult and has not been shown for targets such as hydrolases (e.g., lipases and/or carboxylesterases). Moreover, while some natural miRNAs have been identified that improve e.g., productivity, no natural miRNAs seem to have been described or identified that would target and downregulate an enzyme class such as hydrolases. Thus, there is a need to target multiple hydrolases simultaneously involved in PS degradation and/or associated with immunogenicity (including lipases and/or carboxylesterases).

SUMMARY OF THE INVENTION

[012] Based on micro-conserved regions in the mRNA sequence of two sets of target HCPs, we provide a proof-of-concept for a simultaneous multi-lipase knockdown in CHO cells using single artificial miRNAs (amiRNAs). The designed amiRNAs were not only able to reduce PS degradation but lay the foundation to expand this tool to other areas of cell line phenotype engineering.

[013] The aim of the present invention is to provide a cell line engineering approach for simultaneously targeting multiple hydrolases acting on ester bonds, such as lipases, in mammalian cells to reduce hydrolase activity, particularly PS degrading activity and/or immunogenic activity of contaminating hydrolases in compositions comprising a recombinant protein of interest, such as mAbs, produced in said mammalian cells. Therefore, highly conserved transcripts of hydrolases acting on ester bonds, particularly lipases, were targeted by *de novo* designed artificial microRNAs (amiRNA). These amiRNAs simultaneously knock-down multiple hydrolases, such as lipases, via perfect and/or imperfect mRNA target binding and can be used for stable or transient expression of amiRNAs targeting multiple hydrolases (e.g., lipases) along with the transgene of interest. Methods of reducing hydrolase expression in a mammalian cells using said amiRNA(s) resulting in reduced hydrolytic activity and thus polysorbate degradation in a product produced in the mammalian cells relates to all unit operations of the manufacturing process including the production process using cell cultures (vial thaw until harvest process, including HCCF), downstream intermediates and composition comprising said intermediates, as well as more final compositions, such as bulk drug substance (DS) and formulated drug product (DP). Particularly, a proof-of-concept is provided that artificial miRNAs

comprising designed artificial miRNA guide strand sequences targeting multiple hydrolases (e.g., lipases or carboxylesterases) can be *de novo* designed and are effective in reducing and/or preventing PS degrading activity in the harvested cell culture fluid of a mammalian cell used for expression of a protein of interest (due to the presence of contaminating HCPs with hydrolase activity acting on ester bonds). Because these artificial miRNAs can target multiple targets simultaneously and can further be used as a mixture of different artificial miRNAs, this approach allows tackling contaminating PS degradation in compositions comprising a protein of interest already in the upstream process and without knowing the exact hydrolase responsible for the observed degradation.

[014] In one aspect the invention relates to an artificial microRNA (miRNA) comprising an artificial miRNA guide strand sequence targeting two or more mammalian hydrolases acting on ester bonds. The two or more hydrolases are endogenous hydrolases of a mammalian cell, preferably of a human or a rodent cell, more preferably of a CHO cell. The artificial miRNA guide strand sequence may have perfect or imperfect complementarity to target sequences of at least two mRNAs encoding the two or more mammalian hydrolases. Preferably, the artificial miRNA guide strand sequence has imperfect complementarity to all or all but one target sequences of the at least two mRNAs encoding the two or more mammalian hydrolases. The target sequences are located in the 3' untranslated regions (3'UTR) and/or in the coding regions of the at least two mRNAs encoding the two or more mammalian hydrolases, preferably in the coding regions of the at least two mRNAs encoding the two or more mammalian hydrolases. In certain embodiments, the artificial miRNA targeting two or more mammalian hydrolases reduces expression and/or activity of said two or more hydrolases in a mammalian cell, preferably a rodent or human cell, more preferably a CHO cell.

[015] The artificial miRNA guide strand sequence has about 19 to 25 nucleotides, preferably 20 to 24, more preferably 21 to 23 nucleotides. Further, the artificial miRNA guide strand sequence hybridizes to the target sequences of the at least two mRNAs encoding the two or more mammalian hydrolases independently, without the formation of a bulge loop and/or with formation of a maximum of two bulge loops, preferably without the formation of a bulge loop. Preferably, the artificial miRNA guide strand sequence has at least 55% sequence complementarity with the target sequence(s) of the at least two mRNAs encoding the two or more mammalian hydrolases; and/or wherein the miRNA guide strand sequence has at least 13 Watson-Crick base pairings with the target sequence(s) of the at least two mRNAs encoding the two or more mammalian hydrolases. Examples of suitable miRNA guide strand sequences are artificial miRNA guide strand sequence having the nucleotide sequence of SEQ ID NO: 1 (LP 8mer), SEQ ID NO: 2 (LP 6mer), SEQ ID NO: 3 (LH 8mer), SEQ ID NO: 4 (LH 9mer) or SEQ ID NO: 5 (LH Non-canonical). The artificial miRNA may be a pri-miRNA, a pre-miRNA, a double stranded mature miRNA (comprising the passenger strand and the guide strand) or a single stranded miRNA guide strand. The artificial miRNA may also be a single or double stranded miRNA mimic.

[016] In certain embodiments, one of the two or more mammalian hydrolases is lipoprotein lipase (LPL). The target sequence(s) in the mRNA encoding LPL is preferably within nucleotides 560-590,

700-745, 930-960 or 1330-1360 of SEQ ID NOs: 6 or 7, preferably within nucleotides 560-590, 705-745, or 930-960 of SEQ ID NOs: 6 or 7. In additional or alternative embodiments, the two or more mammalian hydrolases are selected from the group consisting of lipoprotein lipase (LPL), phospholipase B-like 2 (PLBL2), phospholipase A1 (Pla1a), inactive pancreatic lipase-related protein 1 (PNLIPRP1), inactive pancreatic lipase-related protein 2 (PNLIPRP2), pancreatic triacylglycerol lipase precursor (PNLIP), lipase member I precursor (LipI), lipase member H precursor (LipH), lipase member G precursor (LipG), lipase member C precursor (LipC) and isoforms thereof.

[017] In certain embodiments, the artificial miRNA guide strand sequence has imperfect complementarity to the target sequence(s) of at least one of the at least two mRNAs encoding the two or more mammalian hydrolases and wherein said miRNA guide strand sequence comprises a 5' seed region starting at nucleotide 2 (nt 2) and a 3' complementary region that undergo base pairing with said target sequence(s), wherein the 5' seed region comprises (i) a sequence of 8 nucleotides (nt 2-9) complementary to the target sequence(s) and having 0, 1, 2 or 3 mismatches with the target sequence(s), preferably 0, 1 or 2 mismatches, and (ii) a GC-content of 55-70% wherein all or all but one or all but two guanine (G) or cytosine (C) are complementary to the target sequence(s); and wherein the 3' complementary region comprises (i) a sequence of the last 6 nucleotides complementary to the target sequence(s) and having 1, 2 or 3 mismatches with the target sequence(s), preferably wherein the mismatches are not consecutive mismatches, and (ii) a GC-content of $\leq 50\%$ in the last 6 nucleotides, wherein 1 or 2 of the complementary nucleotides are G or C. Examples of such artificial miRNA guide strand sequences are artificial miRNA guide strand sequences having the nucleotide sequence of SEQ ID NO: 2 (LP 6mer), SEQ ID NO: 4 (LH 9mer) or SEQ ID NO: 5 (LH Non-canonical).

[018] In other embodiments, the artificial miRNA guide strand sequence has imperfect complementarity to the target sequence(s) of at least one of the at least two mRNAs encoding the two or more mammalian hydrolases and wherein said miRNA guide strand sequence comprises a 5' seed region starting at nucleotide 2 (nt 2) and a 3' complementary region that undergoes base pairing with said target sequence(s), wherein the 5' seed region comprises (i) a sequence of 8 nucleotides (nt 2-9), wherein at least the first 4 consecutive nucleotides (at least nt 2-5) are complementary to the target sequence(s), wherein the sequence of 8 nucleotides comprises at least one mismatch with the target sequence(s), and (ii) a GC-content of 55-75%, wherein at least 4 of the consecutive nucleotides are complementary to the target sequence(s) are G or C; and wherein the 3' complementary region comprises (i) a sequence of the last 6 nucleotides complementary to the target sequence(s) and having 0 or 1 mismatches with the target sequence(s), preferably wherein the last nucleotide is complementary to the target sequence(s) and/or the penultimate nucleotide is a mismatch, and (ii) a GC-content of 30-50% in the last 6 nucleotides, preferably wherein at least 2 of the complementary nucleotides are G or C. Examples of such artificial miRNA guide strand sequences are artificial miRNA guide strand sequence having the nucleotide sequence of SEQ ID NO: 1 (LP 8mer) or SEQ ID NO: 2 (LP 6mer).

[019] In another aspect, the invention provides a mammalian expression vector comprising a polynucleotide sequence encoding the artificial miRNA according to the invention. The mammalian expression vector may further comprise at least one gene of interest encoding a recombinant protein of interest. In certain embodiments, the mammalian expression vector comprises a polynucleotide sequence encoding two or more of the artificial miRNAs according to the invention, wherein the artificial miRNAs are identical or different.

[020] In yet another aspect, the invention relates to a mammalian cell comprising the artificial miRNAs according to the invention or the mammalian expression vector according to the invention. The mammalian cell may be a human or a rodent cell, preferably a rodent cell, more preferably a CHO cell. In a preferred embodiment, the mammalian cell further expresses a recombinant protein of interest.

[021] In yet another aspect, the invention relates to a use of the artificial miRNA according to the invention in a mammalian cell for preventing expression of at least two endogenous hydrolases in the mammalian cell and/or preventing hydrolase activity and/or polysorbate degrading activity associated with a recombinant protein of interest produced in the mammalian cell.

[022] In yet another aspect, provided is a method for manufacturing a protein of interest comprising the steps of: (a) introducing an expression vector comprising a gene of interest encoding a recombinant protein of interest into a mammalian cell; (b) introducing an artificial miRNA comprising an artificial miRNA guide strand sequence targeting two or more mammalian hydrolases acting on ester bonds into the mammalian cell, wherein the artificial miRNA is introduced before, simultaneously or after step (a) into the mammalian cell, wherein the artificial miRNA is introduced as RNA or as a mammalian expression vector comprising a polynucleotide sequence encoding said artificial miRNA; (c) cultivating said mammalian cell under conditions that allow expression of the recombinant protein of interest; and (d) harvesting the recombinant protein of interest in the harvested cell culture fluid. The method according to the invention may further comprise the following steps: (e) purifying the recombinant protein of interest; and (f) formulating the purified recombinant protein of interest into a composition. The harvested cell culture fluid has reduced hydrolase activity and/or reduced PS degrading activity. Preferably the harvested cell culture fluid has reduced hydrolase activity and/or reduced PS degrading activity compared to harvested cell culture fluid comprising the recombinant protein of interest produced in a mammalian cell not comprising or encoding the artificial miRNA. In a preferred embodiment, the composition comprising the purified recombinant protein of interest has reduced hydrolase activity and/or reduced PS degrading activity.

[023] In certain embodiments, the artificial miRNA is the artificial miRNA according to the invention and/or the mammalian expression vector comprising a polynucleotide sequence encoding the artificial miRNA is the mammalian expression vector according to the invention. The two or more mammalian hydrolases are endogenous hydrolases of the mammalian cell, preferably the mammalian cell is a human or a rodent cell, more preferably a CHO cell.

[024] The invention further encompasses a composition comprising a recombinant protein of interest, wherein the recombinant protein of interest is obtainable by the method of the invention and the composition preferably further comprises PS (such as PS20 or PS80), more preferably at a concentration of 0.1 g/L or more. For instance, in such a composition comprising PS (PS20 or PS80) preferably less than about 10% of PS is degraded when the composition is stored at about 2°C to about 8°C for at least six months. The composition comprising the recombinant protein of interest has reduced hydrolase activity and/or reduced PS degrading activity, preferably the composition comprising the recombinant protein of interest has reduced hydrolase activity and/or reduced PS degrading activity compared to a composition comprising the recombinant protein of interest produced in a mammalian cell without introducing or comprising the artificial miRNA.

DESCRIPTION OF THE FIGURES

[025] **Figure 1: Protein sequence alignments of LPL with its eight homologs (Lipase 1a – 1h) and LPL with PLBL2.** Lipase amino acid sequences (LPL, Lipase 1a – 1h and PLBL2) of the CHO host cell line were extracted from a database. Alignment was conducted using the alignment type “Global alignment with free end gaps” of the Geneious Prime® Software. (A) Amino acids 140 – 320 of the LPL with its eight homologs (Lipase 1a - 1h) alignment. (B) Amino acids 240 – 450 of the LPL with PLBL2 alignment. Consensus: Black regions = sequences conserved between all lipases; grey = sequences partially conserved between lipases; light grey = less conserved sequences between lipases. Identity: The bars indicate the degree of homology between lipases. Lipase 1 = LPL; Lipase 2 = PLBL2; Lipase 1a = Pla1a; Lipase 1b = PNLIPRP1; Lipase 1c = PNLIPRP2; Lipase 1d = PNLIP; Lipase 1e = LipI; Lipase 1f = LipH; Lipase 1g = LipG; Lipase 1h = LipC.

[026] **Figure 2: Transcript sequence alignments of LPL with its eight homologs (Lipase 1a - 1h) and LPL with PLBL2 for identification of amiRNA binding sites.** Lipase transcript sequences (LPL, Lipase 1a – 1h and PLBL2) of the CHO host cell line were extracted from a database. Alignment was conducted using the alignment type “Global alignment with free end gaps” of the Geneious Prime® Software. (A) Transcript sequences 480 – 1020 of the LPL with its eight homologs (Lipase 1a - 1h) alignment. Designed amiRNAs (grey arrows): 9 = LH 9mer; NC = LH Non-canonical; 8 = LH 8mer. (B) Transcript sequences 620 – 1630 of the LPL with PLBL2 alignment. Designed amiRNAs (grey arrows): 6 = LP 6mer; 8 = LP 8mer. Consensus: Black regions = sequences conserved between all lipases; grey = sequences partially conserved between lipases; light grey = less conserved sequences between lipases. Identity: The bars indicate the degree of homology between lipases. Lipase 1 = LPL; Lipase 2 = PLBL2; Lipase 1a = Pla1a; Lipase 1b = PNLIPRP1; Lipase 1c = PNLIPRP2; Lipase 1d = PNLIP; Lipase 1e = LipI; Lipase 1f = LipH; Lipase 1g = LipG; Lipase 1h = LipC.

[027] **Figure 3: Binding of *de novo* designed amiRNAs against LPL and its eight homologs (Lipase 1a-1h).** Lipase transcript sequences (LPL, Lipase 1a – 1h) of the CHO host cell line were extracted from a database. Alignment was conducted using the alignment type “Global alignment with free end gaps” of the Geneious Prime® Software resulting in the consensus sequence numbering

indicated at the top of the figure. Designed amiRNAs are indicated as grey arrows. amiRNAs were designed with an aimed 100% sequence complementarity against LPL (highest preferences) and deviations are indicated. Black boxes are indicating nucleotides conserved between all lipase targets and thus 100% complementary between amiRNA and its targets, whereas mismatches between individual as well as multiple lipases and the amiRNA are depicted as dots. (A) Binding of LH 9mer, (B) LH NC, and (C) LH 8mer to LPL and its eight homologs. Lipase 1 = LPL; Lipase 2 = PLBL2; Lipase 1a = Pla1a; Lipase 1b = PNLIPRP1; Lipase 1c = PNLIPRP2; Lipase 1d = PNLIP; Lipase 1e = LipI; Lipase 1f = LipH; Lipase 1g = LipG; Lipase 1h = LipC.

[028] Figure 4: Binding of *de novo* designed amiRNAs against LPL and PLBL2. Lipase transcript sequences (LPL = Lipase 1, PLBL2 = Lipase 2) of the CHO host cell line were extracted from a database. Alignment was conducted using the alignment type "Global alignment with free end gaps" of the Geneious Prime® Software resulting in the consensus sequence numbering indicated at the top of the figure. Designed amiRNAs are indicated as grey arrows. amiRNAs were designed with 100% sequence complementarity against LPL (highest preferences). Black boxes are indicating nucleotides conserved between all lipase targets and thus 100% complementary between amiRNA and its targets, whereas mismatches between individual as well as multiple lipases and the amiRNA are depicted as dots. Gaps/Bulges are indicated as dashes. (A) Binding of LP 6mer, (B) Binding of LP 8mer to LPL and PLBL2.

[029] Figure 5: Analysis of cell growth and viability of CHO host cells transiently transfected with amiRNAs designed against multiple lipases. The CHO-K1 GS host cell line was transiently transfected with amiRNAs against LPL (Lipase 1) and its eight homologs (LH) or LPL and PLBL2 (Lipase 2) (LP). Cells were analyzed 3 days post amiRNA transfection via flow cytometry. (A) Viable cell concentration (VCC) and (B) viability of treated CHO host cells. NT siRNA = Transfection with non-targeting siRNA, LP = amiRNA against LPL and PLBL2 (6mer or 8mer), LH = amiRNA against LPL and its eight homologs (8mer, 9mer or Non-canonical), Mock = Transfection with nuclease-free water, Tox5 = Transfection with cell death triggering siRNA. Lipase 1 (LPL) siRNA = LPL knockdown positive control. Biological duplicates were measured (n = 2). For statistical analysis, an unpaired two-tailed t-test was applied (without * = not significant; * p ≤ 0.05; *** p ≤ 0.001).

[030] Figure 6: Normalized LPL concentrations in harvested cell culture fluid (HCCF) samples of CHO host cells transiently transfected with amiRNA designed against multiple lipases. The CHO-K1 GS host cell line was transiently transfected with amiRNAs against LPL (Lipase 1) and its eight homologs (LH) or LPL and PLBL2 (Lipase 2) (LP). HCCF samples were collected 3 days post transfection and LPL concentrations were measured with an enzyme-linked immunosorbent assay. Determined LPL concentrations were normalized (norm.) with viable cell concentrations. NT siRNA = Transfection with non-targeting siRNA, LP = amiRNA against LPL and PLBL2 (6mer or 8mer), LH = amiRNA against LPL and its eight homologs (8mer, 9mer or Non-canonical), Mock = Transfection with nuclease-free water, Tox5 = Transfection with cell death triggering siRNA, Lipase 1(LPL) siRNA = LPL

knockdown positive control. Biological duplicates were measured ($n = 2$). For statistical analysis, an unpaired two-tailed t-test was applied (without * = not significant; * $p \leq 0.05$).

[031] Figure 7: Normalized PLBL2 concentrations in HCCF samples of CHO host cells transiently transfected with amiRNA designed against multiple lipases. The CHO-K1 GS host cell line was transiently transfected with amiRNAs against LPL (Lipase 1) and its eight homologs (LH) or LPL and PLBL2 (Lipase 2) (LP). HCCF samples were collected 3 days post transfection and PLBL2 concentrations were measured with a bio-layer interferometry assay. Determined PLBL2 concentrations were normalized (norm.) with viable cell concentrations. NT siRNA = Transfection with non-targeting siRNA, LP = amiRNA against LPL and PLBL2 (6mer or 8mer), LH = amiRNA against LPL and its eight homologs (8mer, 9mer or Non-canonical), Mock = Transfection with nuclease-free water, Tox5 = Transfection with cell death triggering siRNA. Biological duplicates were measured ($n = 2$). For statistical analysis, an unpaired two-tailed t-test was applied (without * = not significant; * $p \leq 0.05$; ** $p \leq 0.01$).

[032] Figure 8: Normalized Pla1a concentrations in cell lysate samples of CHO host cells transiently transfected with amiRNA designed against multiple lipases. The CHO-K1 GS host cell line was transiently transfected with amiRNAs against LPL (Lipase 1) and its eight homologs (LH) or LPL and PLBL2 (Lipase 2) (LP). Cell lysate samples were prepared 3 days post transfection and Pla1a (Lipase 1a) concentrations were measured with an enzyme-linked immunosorbent assay. Determined Lipase 1a concentrations were normalized (norm.) with total protein concentrations determined via Bradford assay. NT siRNA = Transfection with non-targeting siRNA. LP = amiRNA against LPL and PLBL2 (6mer or 8mer), LH = amiRNA against LPL and its eight homologs (8mer, 9mer or Non-canonical), Mock = Transfection with nuclease-free water, Tox5 = Transfection with cell death triggering siRNA. Biological duplicates were measured ($n = 2$). For statistical analysis, an unpaired two-tailed t-test was applied (without * = not significant; * $p \leq 0.05$).

[033] Figure 9: Root cause analysis of amiRNA mediated knockdown of un-specific lipase targets. Lipase transcript sequences (Pla1a = Lipase 1a, PLBL2 = Lipase 2) of the CHO host cell line were extracted from a database. Potential amiRNA binding sites were identified using the "Add Primers to Sequence" function of the Geneious Prime® Software. (A) Off-target analysis of LP 8mer amiRNA in the Lipase 1a transcript sequence. (B) Off-target analysis of LH 9mer amiRNA in the PLBL2 transcript sequence. amiRNAs are indicated as grey arrows. Black boxes are indicating nucleotides with complementary binding to the mRNA, whereas mismatches between the lipase transcript sequence and the amiRNA are depicted as dots.

[034] Figure 10: Analysis of polysorbate degradation in HCCF samples of CHO host cells transiently transfected with amiRNAs designed against multiple lipases. CHO host cells were transiently transfected with *de novo* amiRNAs for post-translational regulation of LPL (Lipase 1) and PLBL2 (Lipase 2) (LP) or LPL and its eight homologs (LH). HCCF samples were collected 3 days post transfection. HCCF samples were spiked with PS20 or PS80 and incubated at room temperature in the dark for 0, 1, 3, 7, and 14 days. PS concentrations were measured by fluorescence micelle assay

(FMA). (A) PS20 concentrations of HCCF samples (initial PS20 concentration 0.4 mg/ml). (B) PS80 concentrations of HCCF samples (initial PS80 concentration 0.2 mg/ml). NT siRNA = Transfection with non-targeting siRNA, LP = amiRNA against LPL and PLBL2 (6mer or 8mer), LH = amiRNA against LPL and its eight homologs (8mer, 9mer or Non-canonical), Mock = Transfection with
 5 nuclease-free water, Lipase 1 (LPL) siRNA = LPL knockdown positive control. Biological duplicates were measured ($n = 2$). For statistical analysis, an unpaired two-tailed t-test was applied (without * = not significant; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

[035] Figure 11: Average polysorbate degradation rates in HCCF samples of CHO host cells transiently transfected with amiRNAs designed against multiple lipases. Average PS20 and
 10 PS80 degradation rates were calculated based on FMA data generated from HCCF samples of amiRNA transfected CHO host cells. amiRNAs targeting LPL (Lipase 1) and PLBL2 (Lipase 2) (LP) or LPL and its eight homologs (LH). Initial PS concentrations were subtracted by final measured PS concentrations and divided by the incubation time to calculate degradation rates per day. NT siRNA = Transfection with non-targeting siRNA, LP = amiRNA against LPL and PLBL2 (6mer or 8mer), LH =
 15 amiRNA against LPL and its eight homologs (8mer, 9mer or Non-canonical), Mock = Transfection with nuclease-free water, Lipase 1 (LPL) siRNA = LPL knockdown positive control. Biological duplicates were measured ($n = 2$). For statistical analysis, an unpaired two-tailed t-test was applied (without * = not significant; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

[036] Figure 12: Analysis of viability and growth performance of IgG1 or IgG4 CHO production cells transiently transfected with amiRNAs designed against multiple lipases. IgG1 (top) or IgG4
 20 (bottom) CHO production cell lines were transiently transfected with amiRNAs against LPL (Lipase 1) and PLA1A (Lipase 1a) (LH) or LPL and PLBL2 (Lipase 2) (LP). Cells were batch-cultivated and analyzed 3, 4 and 5 days after transfection via flow cytometry. (A) Viability 5 days post-transfection. (B) Integral of viable cell concentration (IVC) on day 5. NT siRNA = Transfection with non-targeting
 25 siRNA, LP = amiRNA against LPL and PLBL2 (6mer or 8mer), LH = amiRNA against LPL and PLA1A (9mer, 8mer or Non-canonical), Mock = Transfection with nuclease-free water, Tox5 = Transfection with cell death triggering siRNA. Biological duplicates were measured ($n = 2$). For statistical analysis, an unpaired two-tailed t-test was applied 627 (without * = not significant; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

[037] Figure 13: Analysis of cell specific productivity and product concentration of IgG1 or IgG4 CHO production cells transiently transfected with amiRNAs designed against multiple lipases. IgG1 (top) or IgG4 (bottom) CHO production cells were transiently transfected with amiRNAs
 30 against LPL (Lipase 1) and PLA1A (Lipase 1a) (LH) or LPL and PLBL2 (Lipase 2) (LP). HCCF samples were collected 5 days after transfection and product concentrations were determined using a Protein A based bio-layer interferometry assay. IVC and titer data were used to calculate specific productivities (QP) in pg/cell*day (pg/c*d). (A) Specific (spec.) productivity (pg/cell/day) 5 days post-transfection. (B) product concentration (mg/l) 5 days post-transfection. NT siRNA = Transfection with non-targeting
 35 siRNA, LP = amiRNA against LPL and PLBL2 (6mer or 8mer), LH = amiRNA against LPL and PLA1A

(9mer, 8mer or Non-canonical), Mock = Transfection with nuclease-free water, Tox5 = Transfection with cell death triggering siRNA. Biological duplicates were measured (n = 2).

DETAILED DESCRIPTION

5 [038] The term “comprises” or “comprising” means “including, but not limited to”. The term is intended to be open-ended, to specify the presence of any stated features, elements, integers, steps, or components, but not to preclude the presence or addition of one or more other features, elements, integers, steps, components, or groups thereof. The term “comprising” thus includes the more restrictive terms “consisting of” and “essentially consisting of”. With regard to sequences, the terms
10 “having a nucleotide/amino acid sequence of” and “comprising a nucleotide/amino acid sequence of” are used interchangeably and include the embodiment “consisting of the nucleic acid/amino acid sequence of”. Similarly, the term “encoding” or “encodes” is intended to be open-ended and allows the presence or addition of one or more other features, elements, or components. Furthermore, singular and plural forms are not used in a limiting way. As used herein, the singular forms “a”, “an”
15 and “the” designate both the singular and the plural, unless expressly stated to designate the singular only.

[039] The term “protein” is used interchangeably with “amino acid sequence” or “polypeptide” and refers to polymers of amino acids of any length. These terms also include proteins that are post-translationally modified through reactions that include, but are not limited to, glycosylation, acetylation,
20 phosphorylation, glycation, or protein processing. Modifications and changes, for example fusions to other proteins, amino acid sequence substitutions, deletions, or insertions, can be made in the structure of a polypeptide while the molecule maintains its biological functional activity. For example, certain amino acid sequence substitutions can be made in a polypeptide or its underlying nucleic acid coding sequence and a protein can be obtained with the same properties. A protein produced or
25 manufactured in a mammalian cell or encoded by a mammalian expression vector, particularly using the artificial miRNA according to the invention is also referred to as protein of interest. Preferably the protein of interest is a recombinant protein of interest.

[040] The term “recombinant protein” as used herein relates to a protein generated by recombinant techniques, such as molecular cloning and may also be referred to as recombinant protein of interest.
30 As used herein, the recombinant protein is the protein of interest, e.g., in a sample to be purified or in a mammalian host cell to be produced. Recombinant techniques bring together genetic material from multiple sources or create sequences that do not naturally exist. A recombinant protein is typically based on a sequence from a different cell or organism or a different species from the recipient host cell used for production of the protein in cell culture, e.g., a CHO cell or a human embryonic kidney
35 (HEK) 293 cell, or is based on an artificial sequence, such as a fusion protein. In the context of the present invention the recombinant protein of interest is preferably a therapeutic protein, such as an antibody, an antibody fragment, an antibody derived molecule (e.g., scFv, bi- or multispecific antibodies) or a fusion protein (e.g., an Fc fusion protein). Thus, in one embodiment the recombinant

protein of interest is selected from the group consisting of an antibody, an antibody fragment, an antibody derived molecule, glycoproteins and a fusion protein.

[041] The term “nucleic acid sequence” is used interchangeably with “polynucleotide” or “polynucleotide sequence” and refers to DNA or RNA of any length. In the context of an expression
5 vector, particularly a plasmid and integration into the host cell’s genome, the person skilled in the art would understand that it refers to a DNA sequence or molecule. In the context of the miRNA the person skilled in the art would understand that it refers to an RNA sequence or molecule or to a DNA sequence encoding the miRNA.

[042] The term “mammalian cell” as used herein refers to cells, particularly cell lines, derived from a
10 mammal. In the present invention a “mammalian cell” particularly encompasses human or rodent cells, such as HEK 293 cells or derivatives thereof or CHO cells and derivatives thereof. In most cases it refers to a CHO cell or derivatives thereof. Cells as referred to herein are cells maintained in culture and do not relate to primary cells, but cell lines or cell line-derived cells, i.e., to immortalized cells.

[043] The term “drug substance”, abbreviated as DS, as used herein refers to the formulated active
15 pharmaceutical ingredient (API) with excipients. The API has the therapeutic effect in the body as opposed to the excipients, which assist with the delivery of the API. In the case of biologic therapeutics, the formulated API with excipients typically means the API in the final formulation buffer at a concentration of at least the highest concentration used in the final dosage form, also referred to as drug product.

[044] The term “drug product”, abbreviated as DP, as used herein refers to the final marketed dosage
20 form of the drug substance for example a tablet or capsule or in the case of biologics typically the solution for injection in the appropriate containment, such as a vial or syringe. The drug product may also be in a lyophilized form. An antibody is preferably provided in an aqueous formulation in a glass vial or in a glass syringe.

[045] The term “polysorbate 20” as used herein refers to a non-ionic polysorbate-type surfactant,
25 which is a laurate ester of sorbitol and its anhydrides, copolymerized with approximately 20 moles of ethylene oxide for each mole of sorbitol and sorbitol anhydrides (polyoxyethylene (20) sorbitan monolaurate; CAS number: 9005-64-5) as defined in ©2020 The United States Pharmacopeial Convention (Official May 1, 2020). It is also known as Tween 20. Its stability and relative non-toxicity
30 allow it to be used as a surfactant and emulsifier in a number of domestic, scientific analyses. Polysorbate 20 can be used as washing agent in immunoassays, Western blots and enzyme-linked immunosorbent assay (ELISA). It can further be used in pharmacological applications, such as pharmaceutical formulations, particularly for biologics, such as antibodies and Fc-fusion proteins. Particularly it helps to prevent non-specific antibody binding interactions.

[046] The term “polysorbate 80” as used herein refers to a non-ionic polysorbate-type surfactant,
35 which is a mixture of partial esters of fatty acids mainly oleic acid, with sorbitol and its anhydrides ethoxylated with approximately 20 moles of ethylene oxide for each mole of sorbitol and sorbitol

anhydrides (polyoxyethylene (20) sorbitan monooleate, CAS number: 9005-65-6) as defined in ©2015 The United States Pharmacopeial Convention (Stage 6 Harmonized, Official May 1, 2016). It is also known as Tween 80 and has a similar use as polysorbate 20.

[047] The term “contaminating” or “contamination” as used herein refers to the presence of an undesired and/or unintentional substance, such as substances that are immunogenic and/or with PS degrading enzyme activity, e.g., enzymes with lipolytic activities accompanying HCPs and/or at least one protein or substance having a hydrolytic activity, such as a lipase activity, which can be regarded only as a trace component in comparison to other predominantly produced proteins for medical treatments such as antibodies or antibody-like compounds. In the context of the present invention, a hydrolytic and particularly a lipase activity is undesired due to its PS degrading potential that may be co-purified with the recombinant protein. This applies especially to finally formulated protein preparations which advantageously comprise such unwanted factors only to less than 1% (w/w), preferably less than 0.1% (w/w), more preferably less than 0.01% (w/w) in comparison to total protein content, i.e., in the drug product.

[048] The term “polysorbate degrading enzyme activity” or “polysorbate degrading activity” as used herein, refers to the activity of a substance, typically a protein (enzyme) derived from the host cell, that catalyzes the hydrolysis of an ester bond in PS. This includes HPCs, specifically enzymes with hydrolase activity, such as lipase activity and/or carboxylesterase activity. The term “lipase activity” as used herein refers to the activity of a protein (enzyme) that catalyzes the hydrolysis of an ester bond in a lipid, such as fatty acid esters. The term “carboxylesterase activity” as used herein refers to the activity of a substance, typically a protein (enzyme) that catalyzes the hydrolysis of an ester bond in a carboxylic ester. A lipase or a carboxylesterase is a hydrolase enzyme that splits esters into an acid and an alcohol in a chemical reaction with water, also referred to as hydrolysis. Many lipases and carboxylesterases belong to the class of carboxylic ester hydrolases (EC 3.1.1). While carboxylesterases form a separate class of carboxylesterase (EC 3.1.1.1), lipases may be, without being limited thereto, a triacylglycerol lipase (EC 3.1.1.3), a phospholipase A2 (EC 3.1.1.4), a lysophospholipase (EC 3.1.1.5), an (EC 3.1.1.23), galactolipase (EC 3.1.1.26), phospholipase A1 (EC 3.1.1.32), lipoprotein lipase (EC 3.1.1.34) or hormone-sensitive lipase (EC 3.1.1.79); a phosphoric diester hydrolase (EC 3.1.4) such as phospholipase D (EC 3.1.4.4), a phosphoinositide phospholipase C (EC 3.1.4.11), glycosylphosphatidylinositol phospholipase D (EC 3.1.4.50) or N-acetylphosphatidylethanolamine-hydrolyzing phospholipase D (EC 3.1.4.54); or glycosphingolipid deacylase (EC 3.5.1.69). The term “hydrolase activity” as used herein is the more general term, including lipases, but also refers to hydrolysis of compounds other than lipids, such as the sialic acid acetyltransferase (SIAE) that catalyze removal of O-acetyl ester groups from sialic acids by hydrolysis and thioester hydrolase (EC 3.1.2), such as palmitoyl protein thioesterase 1 (PPT1). The term “hydrolase acting on ester bonds” as used herein relates to enzymes (particularly lipases or carboxylesterases) having hydrolase activity that catalyze the hydrolysis of an ester bond, wherein “acting on ester bonds” means cleaving ester bonds.

[049] The term “therapeutic protein” as used herein refers to proteins that can be used in medical treatment of humans and/or animals. These include, but are not limited to antibodies, growth factors, blood coagulation factors, vaccines, interferons, hormones, and fusion proteins. In the context of the present invention the therapeutic protein is an antibody, including an antibody-derived molecule.

5 [050] The term “produced” as used herein relates to the production of a recombinant protein of interest, specifically an antibody, in a mammalian cell, specifically a CHO cell, in cell culture. The person skilled in the art knows how to produce recombinant proteins and particularly antibodies in cells using fermentation. The production of recombinant proteins comprises cultivating a mammalian cell, such as a CHO cell, expressing the recombinant protein of interest in cell culture. Cultivating the
10 cell expressing the recombinant protein in cell culture comprises maintaining the cell in a suitable medium and under conditions that allow growth and/or protein production/expression. The recombinant protein of interest may be produced by fed-batch or continuous cell culture. Thus, the mammalian cell, such as the CHO cell, may be cultivated in a fed-batch or continuous cell culture or a combination thereof, preferably in a fed-batch cell culture.

15 [051] The term “expressing a recombinant protein of interest” or “expressing an antibody” as used herein refers to a cell comprising a DNA sequence coding for the recombinant protein of interest, such as an antibody, which is transcribed and translated into the protein sequence including post-translational modifications, i.e., resulting in the production of the recombinant protein of interest, such as an antibody in cell culture.

20 [052] The term “about” as used herein refers to a variation of 10 % of the value specified, for example, about 50 % comprises a variation from 45 to 55 %.

[053] The term “two or more” and “at least two” or equivalents are used synonymously herein and refer to two or more than two. The same applies to equivalents for other numbers, such as three or more or four or more.

25 [054] The term “yield” as used herein refers to the amount of the antibody following purification relative to the amount of the antibody before purification, such as in the starting material. The term “step yield” refers to the amount of the antibody following a certain purification step relative to the amount of the antibody before said purification step.

The term “siRNAs” as used herein refers to short double-stranded RNAs typically composed of 21–23
30 base pairs that is fully complementary to its target mRNA. An siRNA often comprises two nucleotide overhangs at the 3' ends, but multiple variations in length and overhangs are tolerated. siRNA consists of an active (guide) strand and a complementary inactive (passenger) strand. siRNAs are regarded as exogenous RNAs that enter the endogenous RNAi pathway. In contrast to endogenous miRNAs, exogenous siRNAs have no nuclear phase during processing but enter the same processing pathway
35 as dsRNAs at the step of Dicer association. As an alternative, siRNAs can be introduced into a cell by expression of a small harpin RNA (shRNA) (also referred to as short hairpin RNA) using vectors.

shRNA is a precursor of siRNA. The shRNAs enter the endogenous RNAi machinery for processing into single-stranded siRNAs.

[055] The term "miRNAs" or "microRNA" as used herein refers to endogenous RNAs that are produced from within the cells and have partial or imperfect complementarity to their target mRNA and bind to multiple mRNAs to inhibit their expression. miRNAs are naturally occurring RNAs that are transcribed from their genes by RNA polymerase II. miRNAs are naturally expressed as long primary miRNA transcripts (pri-miRs) from miRNA genes. After partial cleavage by the microprocessor complex in the nucleus, the stem loop precursor miRNA (pre-miRNA) is exported to the cytoplasm, where it is further processed by Dicer into a double-stranded RNA consisting of the active, or mature, strand and the inactive passenger strand. The loop region of the precursor miRNA is removed and a 21-24 bp double stranded RNA (dsRNA) is produced consisting of passenger and guide strand. The passenger strand has no further function and thus is degraded by the cell. The mature miRNA (miRNA guide strand) is incorporated into the RNA-induced silencing complex (RISC) to initiate gene silencing. In contrast to the perfect complementarity between an siRNA and its target mRNA, a miRNA has imperfect complementarity, binding mainly through the seed region sequence at its 5' end. A key difference between siRNAs and miRNAs is that an siRNA is specific for a single target site in a single mRNA, and, therefore, inhibits the expression of one target gene, whereas a miRNA has multiple targets and can regulate multiple genes because mRNA recognition requires binding to the much shorter seed region sequence rather than the entire 21-23 nucleotide sequence of an siRNA. To initiate RNAi, an siRNA must be fully complementary to its target mRNA, whereas miRNA can be partially complementary and hence binds to multiple mRNAs to inhibit their expression, and their mechanisms of action are different: siRNAs cleave mRNA, whereas miRNAs inhibit translation and/or initiate degradation of mRNAs.

[056] The term "artificial microRNA", "artificial miRNA" or "amiRNA" as used herein refers to artificial RNAs comprising a *de novo* designed (i.e., *in silico* designed, non-naturally occurring) guide stand sequence that binds to multiple mRNAs to inhibit their expression. As used herein the artificial miRNA binds to at least one natural mammalian cellular mRNA with imperfect complementarity to inhibit its expression. Artificial miRNAs can be designed by using a natural or artificial scaffold, as double stranded RNA (dsRNA) or as mature miRNA (miRNA guide strand). Artificial miRNAs can also be transcribed by RNA polymerase III. While the term artificial miRNA is sometime also used for recombinantly or synthetically produced miRNAs comprising a natural miRNA guide strand sequence, the artificial miRNA according to the present invention comprises an artificial miRNA guide strand sequence, i.e., a *de novo* designed, not naturally occurring (not previously identified) miRNA guide strand sequence.

[057] The term "expression" as used herein refers to transcription and/or translation of a heterologous polynucleotide sequence within a host cell. The level of expression of a polynucleotide sequence, such as a polynucleotide sequence encoding a hydrolase acting on ester bonds or a protein of interest in a host cell may be determined based on either the amount of corresponding mRNA that is present

in the cell, or the amount of the desired polypeptide/ protein of interest encoded by the selected sequence as in the present examples. For example, mRNA transcribed from a selected sequence can be quantified by Northern blot hybridization, ribonuclease RNA protection, *in situ* hybridization to cellular RNA or by polymerase chain reaction (PCR). Proteins encoded by a selected sequence can be quantitated by various methods, e.g. by ELISA, by Western blotting, by radioimmunoassays, by immunoprecipitation, by assaying for the biological activity of the protein, by immunostaining of the protein followed by flow cytometry analysis or by homogeneous time-resolved fluorescence (HTRF) assays. The level of expression of a non-coding RNA, such as a miRNA can be quantified by PCR, such as qPCR.

[058] The term "harvested cell culture fluid" as used herein refers to the cell culture fluid (CCF) from which the cells have been removed during harvest, typically by centrifugation or filtration. Thus, the harvested cell culture fluid refers to the cell culture supernatant following harvest, i.e., cell removal, particularly for secreted proteins.

[059] In this proof-of-concept study, three different model lipases based on their PS degradation potential reported in literature as well as their presence in internal drug product mass spectrometry analyses were selected, including lipoprotein lipase (LPL), putative phospholipase B-like-2 (PLBL2) and phospholipase A1 member A (PLA1A) (Chiu et al., 2017, Biotechnology and Bioengineering, 114(5), 1006-1015; Dixit et al., 2016, Journal of Pharmaceutical Sciences, 105(5), 1657-1666; Graf et al., 2021, Journal of Pharmaceutical Sciences, 110(11), 3558-3567; Li et al., 2021, Analytical Chemistry, 93(23), 8161-8169). Described is the application of *in silico* designed, non-naturally occurring amiRNAs to knock down multiple lipases simultaneously in CHO cells and thus reducing PS degradation. Therefore, micro-conserved regions across the mRNA sequences of LPL, PLA1A and PLBL2 were harnessed for the identification of target sites for simultaneous amiRNA binding. The novel amiRNA technology a novel and promising way in cell line engineering for targeted pathway engineering based on binding to conserved sequences of target transcripts.

Artificial microRNA (miRNA) targeting two or more mammalian hydrolases

[060] In one aspect, the present invention relates to an artificial microRNA (miRNA) comprising an artificial miRNA guide strand sequence targeting two or more mammalian hydrolases acting on ester bonds. Targeting two or more mammalian hydrolases acting on ester bonds means binding to two or more mRNAs encoding two or more mammalian hydrolases acting on ester bonds, respectively, and inhibiting their expression in a mammalian cell (host cell). In other words, the artificial miRNA guide strand binds to two or more mRNAs each encoding a mammalian hydrolase acting on ester bonds and inhibits their expression in a mammalian cell (host cell). The two or more hydrolases are endogenous hydrolases of a mammalian cell. Preferably the mammalian cell is a rodent cell or a human cell, more preferably the mammalian cell is a rodent cell, such as a CHO cell. Thus, in certain embodiments, the two or more mammalian hydrolases acting on ester bonds are rodent or human hydrolases, preferably the two or more mammalian hydrolases are rodent hydrolases such as

hydrolases from CHO cells. In certain embodiments, the two or more mammalian hydrolases are three or more mammalian hydrolases, four or more mammalian hydrolases, five or more mammalian hydrolases, six or more mammalian hydrolases, seven or more mammalian hydrolases or eight or more mammalian hydrolases.

5 [061] The artificial miRNA guide strand sequence has perfect or imperfect complementarity to target sequences of at least two mRNAs encoding the two or more mammalian hydrolases. The two or more mammalian hydrolases may also be referred to as the two or more targeted mammalian hydrolases. The artificial miRNA guide strand sequence has imperfect complementarity to the target sequence of at least one of the at least two mRNAs encoding the two or more mammalian hydrolases, preferably
10 more than one of the at least two mRNAs encoding the two or more mammalian hydrolases. In one embodiment the artificial miRNA guide strand sequence has perfect complementarity to the target sequence of one mRNA of the at least two mRNAs encoding the two or more mammalian hydrolases. Thus, the artificial miRNA guide strand sequence has perfect complementarity to the target sequence of one mRNA of the at least two mRNAs encoding the two or more mammalian hydrolases and
15 imperfect complementarity to the target sequence(s) of the remaining mRNAs of the at least two mRNAs encoding the two or more (targeted) mammalian hydrolases. In certain embodiments the artificial miRNA guide strand has imperfect complementarity to the target sequence(s) of all of the at least two mRNAs encoding the two or more (targeted) mammalian hydrolases, or the artificial miRNA guide strand has imperfect complementarity to the target sequence(s) of all or all but one of the at least two mRNAs encoding the two or more (targeted) mammalian hydrolases. Preferably, the artificial
20 microRNA (miRNA) according to the invention comprises an artificial miRNA guide strand sequence targeting three, four, five, six, seven, eight, nine or more mammalian hydrolases acting on ester bonds. In certain embodiments the artificial miRNA guide strand has imperfect complementarity to the target sequence(s) of all or all but one of the mRNAs encoding the three, four, five, six, seven, eight, nine or
25 more (targeted) mammalian hydrolases. The person skilled in the art would understand that the artificial miRNA guide strand sequence may have perfect complementarity to the target sequence of one mRNA of the mRNAs encoding the three, four, five, six, seven, eight, nine or more mammalian hydrolases.

[062] The term "perfect complementarity" as used herein means that in two sequences when they are
30 aligned and/or hybridized antiparallel to each other, the nucleotide bases at each position in the sequence will be complementary (no mismatch). It may also be referred to as "fully complementary" or "full complementarity". In the context of the present invention the term complementarity is used for the antiparallel alignment of RNA strand sequences, particularly between miRNA guide strand sequences and mRNA target sequences. Given that there are four choices for each base in the strand,
35 a 20 bp – 22 bp length for a guide strand sequence leads to more than 4×10^{12} possible combinations. Considering that the human genome is about ~3.1 billion bases (3.1×10^{12} bases) in length, this means that each miRNA guide strand sequence of 20 bp to 22 bp should only find a match with perfect complementarity once in the entire human genome.

[063] The term “imperfect complementarity” or “partially complementary” is used herein synonymously and means that in the two sequences when they are aligned and/or hybridized antiparallel to each other, the nucleotide base(s) in at least one position in the sequence will not be complementary (one or more mismatches). The term aligned as used herein refers to a theoretical way of arranging sequences, while the term hybridized as used herein refers to a phenomenon in which single stranded DNA or RNA molecules anneal to complementary DNA or RNA. In other words, imperfect complementarity describes two sequences that hybridize under physiological conditions and contain one or more mismatches. Imperfect complementarity may also be defined by less than 100% sequence complementarity, such as 96% or less sequence complementarity, such as 50% to 96%, preferably 55% to 96% sequence complementarity, more preferably 60 to 96% sequence complementarity.

[064] The target sequences may be located anywhere in the mRNA sequence of the at least two mRNAs encoding the two or more mammalian hydrolases, such as in the 3' untranslated regions (3'UTR), the coding regions or the 5' untranslated regions (5'UTR) of the at least two mRNAs encoding the two or more mammalian hydrolases, preferably the 3' untranslated regions or the coding region of the at least two mRNAs encoding the two or more mammalian hydrolases, more preferably in the coding region of the at least two mRNA encoding the two or more mammalian hydrolases, even more preferably within micro-conserved regions in the coding regions of the at least two mRNAs encoding the two or more mammalian hydrolases. Typically, the target sequences of all of the at least two mRNAs encoding the two or more mammalian hydrolases are either in the 3' untranslated regions or in the coding regions thereof, preferably in the coding region of the at least two mRNA encoding the two or more mammalian hydrolases, even more preferably within micro-conserved regions in the coding regions of the at least two mRNAs encoding the two or more mammalian hydrolases.

[065] In certain embodiments, the target sequences are located in a conserved region of the amino acid sequence of the two or more mammalian hydrolases. In certain preferred embodiments, the target sequences are located in a conserved region of the at least two mRNAs encoding the two or more mammalian hydrolases, preferably in a conserved region of the coding region of the at least two mRNAs encoding the two or more mammalian hydrolases. The term “conserved region” as used herein refers to identical or similar nucleotide sequences in the at least two mRNAs encoding the two or more mammalian hydrolases. Wherein similar sequences means more than 55% percent of pairwise identity. Pairwise identity refers to the percentage of pairwise residues that are identical in the alignment, including gap versus non-gap residues, but excluding gap versus gap residues. Micro-conserved regions are very small stretches of identical or similar sequences of at least 21 nucleotides, preferably with at least 55% sequence identity.

[066] The artificial miRNA according to the invention targeting two or more mammalian hydrolases reduces activity of said two or more hydrolases in a mammalian cell. The reduced activity is typically due to an inhibited (or prevented) expression, either by mRNA cleavage and/or inhibited translation of mRNA. In certain embodiments, the artificial miRNA reduces hydrolase activity in a mammalian cell,

such as in a human or rodent cell, preferably a rodent cell, more preferably a CHO cell. In preferred embodiments, the artificial miRNA reduces PS degrading hydrolase activity and/or prevents expression of at least two endogenous hydrolases in a mammalian cell and/or the HCCF obtained from said mammalian cell, such as a human or rodent cell. Reduced activity in this context means
5 reduced by at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or 100% compared to a mammalian cell not containing the artificial miRNA according to the invention. Hydrolase activity or PS degrading hydrolase activity in a mammalian cell is preferably determined in the harvested cell culture fluid (HCCF). Moreover, the artificial miRNA of the invention prevents hydrolase activity and/or polysorbate degrading activity associated with a
10 recombinant protein of interest produced in a mammalian cell. Prevents expression or prevents hydrolase activity and/or polysorbate degrading activity means any reduction in expression and/or associated with the recombinant protein of interest produced in a mammalian cell comprising said artificial miRNA compared to the mammalian cell not comprising said artificial miRNA, such as by at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least
15 90% or 100% compared to the mammalian cell not comprising said artificial miRNA.

[067] miRNA typically has multiple targets and can regulate multiple genes, because its artificial miRNA guide strand sequence can bind to the target sequences (and inhibit expression) via imperfect complementarity. The artificial miRNA guide strand sequence of the artificial miRNA of the invention has typically 19 to 25 nucleotides, preferably 20 to 24 nucleotides, more preferably 21 to 23
20 nucleotides, even more preferably 22 to 23. Binding to the target sequence(s) of the mRNA requires binding of the 5' seed region and optionally a 3' complementary region rather than the entire guide strand sequence. Thus, preferably the artificial miRNA guide strand sequence comprises at least a 5' seed region and a 3' complementary region. In certain embodiments, the artificial miRNA guide strand sequence has not perfect complementarity to at least one of the at least two mRNAs encoding the two
25 or more mammalian hydrolases and the miRNA guide strand has a 5' seed region and a 3' complementary region that undergoes base pairing with said target sequence(s), preferably a 5' seed region starting at nucleotide 2 (nt 2) from the 5' end of the artificial miRNA guide strand sequence. Overall, the artificial miRNA guide strand sequence may have at least 55%, preferably at least 60%, more preferably at least 65% sequence complementarity with the target sequence(s) of the at least
30 two mRNAs encoding the two or more mammalian hydrolases. In certain embodiments, the miRNA guide strand sequence has at least 13, preferably 14, more preferably 15 Watson-Crick base pairings (consecutive and non-consecutive) with the target sequence(s) of the at least two mRNAs encoding the two or more mammalian hydrolases. The term "at least 55% sequence complementarity" as used herein means the sequence of the complementary strand of the miRNA guide strand has at least 55%,
35 preferably at least 60%, more preferably 65% sequence identity with the target sequence(s) or the antisense transcript of the target sequence(s) has at least 55%, preferably at least 60%, more preferably 65% sequence identity with the miRNA guide strand sequence. In certain embodiments, the artificial miRNA guide strand sequence has a content of G and C complementary to the target

sequence(s) of 35%-45% (GC complementarity of 35-45%), preferably of 35-42%. Thus, an artificial miRNA guide strand sequence of 22 to 23 nucleotides comprises 8-10, preferably 8-9 G or C that are complementary to the target sequence(s), i.e., undergo base pairing with the target sequence(s). In certain preferred embodiments, the first nucleotide at the 5' end of the artificial miRNA guide strand sequence (i.e., the nucleotide 5' of the 5' seed region) comprises a nucleotide (comprises a nucleobase) selected from the group consisting of adenine (A) and uracil (U), preferably the nucleotide is U.

[068] In certain embodiments the artificial miRNA guide strand sequence hybridizes to the target sequences of the at least two mRNAs encoding the two or more mammalian hydrolases independently without the formation of a bulge loop and/or with formation of a maximum of two bulge loops, preferably without the formation of a bulge loop and/or with formation of a maximum of one bulge loop, more preferably without bulge loop formation. In certain embodiments, the 5' seed region of the artificial miRNA guide strand sequence hybridizes to the target sequences of the at least two mRNAs encoding the two or more mammalian hydrolases independently without the formation of a bulge loop and/or with formation of a maximum of one bulge loop, more preferably without bulge loop formation. The term "bulge loop" as used herein refers to a structure in a polynucleotide duplex in which one strand contains a nonterminal extra sequence that is not able to base-pair with the second strand, thereby forming a bulge on one side of the duplex.

[069] The two or more mammalian hydrolases acting on ester bonds may be any mammalian hydrolases, particularly two or more mammalian lipases or carboxylesterases and/or two or more PS degrading mammalian hydrolases. In certain embodiments at least one of the two or more mammalian hydrolases are selected from the group consisting of lipoprotein lipase (LPL, such as having the RNA sequence of SEQ ID NOs: 6 or 7), phospholipase B-like 2 (PLBL2, such as having the RNA sequence of SEQ ID NO: 8 (rna-XM_027414155.1)), phospholipase A1 (Pla1a, such as having the RNA sequence of SEQ ID NOs: 9 or 10 (rna-XM_027412692.1 or rna-XM_035444560.1, respectively)), inactive pancreatic lipase-related protein 1 (PNLIPRP1, such as having the RNA sequence of SEQ ID NO: 11 (CgPICR_007696)), inactive pancreatic lipase-related protein 2 (PNLIPRP2; such as having the RNA sequence of SEQ ID NO: 12 (rna-XM_027406893.1)), pancreatic triacylglycerol lipase precursor (PNLIP, such as having the RNA sequence of SEQ ID NOs: 13 or 14 (rna-XM_035441745.1 or rna-XM_035441744.1, respectively)), lipase member I precursor (LipI, such as having the RNA sequence of SEQ ID NO: 15 (rna-XM_035444258.1)), lipase member H precursor (LipH, such as having the RNA sequence of SEQ ID NOs: 16, 17, 18 or 19 (rna-XM_035444694.1, rna-XM_027413008.1, rna-XM_027413009.1 or rna-XM_027413010.1, respectively)), lipase member G precursor (LipG, such as having the RNA sequence of SEQ ID NO: 20 (rna-XM_035438714.1)), lipase member C precursor (LipC, such as having the RNA sequence of SEQ ID NO: 21 (rna-XM_027411221.1)) and transcript variants or isoforms thereof. Preferably, one of the two or more mammalian hydrolases is lipoprotein lipase (LPL). In certain embodiments the two or more mammalian hydrolases are selected from the group consisting of lipoprotein lipase (LPL),

phospholipase B-like 2 (PLBL2), phospholipase A1 (Pla1a), inactive pancreatic lipase-related protein 1 (PNLIPRP1), inactive pancreatic lipase-related protein 2 (PNLIPRP2), pancreatic triacylglycerol lipase precursor (PNLIP), lipase member I precursor (LipI), lipase member H precursor (LipH), lipase member G precursor (LipG), lipase member C precursor (LipC) and transcript variants or isoforms thereof. Preferably, one of the two or more mammalian hydrolases is lipoprotein lipase (LPL).
[070] All exemplified artificial miRNA guide strand sequences were designed to bind to LPL. The artificial miRNA binding locations are indicated in Table 1 and in the two LPL sequences below.

Table 1

		amiRNA Binding Location				
SEQ ID NO:	Length	<i>LP 6mer</i>	<u>LP 8mer</u>	<u>LH 8mer</u>	<u>LH 9mer</u>	<u>LH Non-canonical</u>
6	1871	719-741	1337-1359	934-955	563-584	708-730
7	3820	718-740	1336-1358	933-954	562-583	707-729

LPL, lipoprotein lipase (isoform X1), >rna-XM_027388880.1 CriGri_PICRH_1.0; (SEQ ID NO: 6)
ACCGTCAGATTCGATTCCTCCTCCTCCGAGGAATTCTGTCCCTTCCAGCTGCCCTGCCCTCCCC
TCTTTAAAGGTTGACTTGCCCTGCGGCGCTCTACCGCGCTCCAGCCCTTTGGCGCCTCCTGCT
CAACCCCTTGCTACACGTATCTGCTCCAGCCCGCCCGCCGTCGCGCCCGCGCTCGCCCGC
TCGTCCGCACCGCGCCGCGCACTTCCACAGCAAAGCAGAAGGACGCCCAGGGAGATGGAGA
GCAAAGCCCTGCTCCTGGTGGCTCTGGGAGTGTGGCTCCAGAGTTTGACCGCCTCCCAAGGA
GGGGTGGCCGCAGCAGACGGGGAAGAGATTTTACAGACATTGAAAGTAAATTTGCCCTAAGG
ACCCCTGATGACACGGCTGAGGACAATTGCCACCTCATTCCCGGAATAGCAGAATCTGTGTCTA
ACTGCCACTTCAACCACAGCAGCAAGACCTTCGTTGTGATCCATGGCTGGACGGTGACAGGAA
TGTATGAGAGTTGGGTGCCAACTTGTGGCTGCCCTGTACAAGAGAGAACCTGACTCCAACG
TCATTGTGGTGGACTGGCTGTATCGGGCCAGCAACACTATCCAGTGTGGCTGGCTACACCA
AGCTGGTGGGAAATGATGTGGCCAGGTTCACTGATGGAGGAAGAGTTTAACTACCCCC
TGGACAATGTCCACCTCTTGGGGTATAGCCTTGGAGCTCATGCTGCTGGTGTGGCAGGAAGTC
TGACCAACAAGAAGGTTAACAGAATCACTGGCTTGGATCCAGCTGGGCCTAACTTTGAGTATGC
AGAGGCCCCCAGTCGCTTTCTCCTGATGATGCAGATTTCTGACGCTTACATACATTACACC
AGAGGGTCACCTGGCCGAAGTATTGGGATCCAGAAACCAGTAGGACATGTTGACATTTATCCC
AATGGAGGCACATTTCCAGCCAGGATGCAACATTGGGGAAGCCATTCTGTGATTGCAGAGCGG
GGCCTTGGAGATGTGGACCAGCTGGTAAAGTGCTCCCATGAGCGATCCATTATCTCTTCATTG
ACTCCTTGCTGAATGAGGAAAACCCAGCAAGGCTTACAGGTGCAACTCCAAGGAAGCCTTTG
AGAAAGGGCTCTGCCTGAGCTGCAGAAAGAATCGATGCAACAATGTGGGCTATGAGATCAACA
AGGTCAAGAGCCAAAAGGAGCAGCAAGATGTACCTGAAGACTCGCTCTCAGATGCCCTACAAAG
TCTTCCACTACCAAGTCAAAATTCATTTTCTGGGACTGAGAGTGACAAGCAGCTCAACCAGGC
CTTTGAGATTTCTCTGTATGGCACAGTGGCCGAGAGTGAGAATATCCCCTTCACTCTGCCTGAG
GTCTCCACAAATAAAACCTACTCCTTCTGATTATACGGAGGTGGACATCGGAGAACTGCTGA
TGATGAAGCTCAAGTGAAGAGTGATTCCTACTTCAGCTGGTCCGACTGGTGGAGCAGCCCCG
GCTTTGTCTGATCGAGAAGATTCTGGGTGAAAGCAGGAGAGACTCAGAAAAAGGTCATCTTCTGTG
CTAGGGAGAAAAGTGTCTCATCTGCAGAAAGGGAAGGACTCCGCACTGTTTGTGAAGTGCCATG
ACAAGTCTCTGAAGAAGTCTGGCTGCTGCTGGCTTGTCTCCGTGACCTCCAGGGCTAATCCA
CATGGCAGCTGGCAGCAGACTCTTCCAGCACGTCAATGCTAAGTGGAGACCAAGCATGTGAC
CTTCCATCTCCTCTCCCAAGTTCTCGGGATATGCTGTTTTATCCCCCCCCCACTCCACTGCC
AGACTCCAAGTGCTAATGGGCCACACATATTAAAGGAA

LPL, lipoprotein lipase (isoform X2), >rna-XM_027388881.1 CriGri_PICRH_1.0; (SEQ ID NO: 7)

CCGTCAGATTTCGATTCTCCTCCTCCGAGGAATTCTGTCCCTTCCAGCTGCCCTGCCCTCCCCT
CTTTAAAGGTTGACTTGCCCTGCGGCGCTCTACCGCGCTCCAGCCCTTTGGCGCCTCCTGCTC
AACCCCTTGCTACACGTATCTGCTCCAGCCCCGCCCGCCGTCCGCCCGCCCGCTCGCCCGCT
CGTCCGCAACCGCGCCGCGCACTTCCCACAGCAAAGCAGAAGGACGCCAGGGAGATGGAGA
5 GCAAAGCCCTGCTCCTGGTGGCTCTGGGAGTGTGGCTCCAGAGTTTGACCGCCTCCGAAGGA
GGGGTGGCCGCGAGCAGACGGGGGAAGAGATTTTACAGACATTGAAAGTAAATTTGCCCTAAGG
ACCCCTGATGACACGGCTGAGGACAATTGCCACCTCATTCCCGGAATAGCAGAATCTGTGTCTA
ACTGCCACTTCAACCACAGCAGCAAGACCTTCGTTGTGATCCATGGCTGGACGGTGACAGGAA
TGTATGAGAGTTGGGTGCCCCAACTTGTGGCTGCCCTGTACAAGAGAGAACCTGACTCCAACG
10 TCAATTGTGGTGGACTGGCTGTATCGGGCCCAGCAACACTATCCAGTGTGGCTGGCTACACCA
AGCTGGTGGGAAATGATGTGGCCAGGTTTCATCAACTGGATGGAGGAAGAGTTTAACTACCCCC
TGGACAATGTCCACCTCTTGGGGTATAGCCTTGGAGCTCATGCTGCTGGTGTGGCAGGAAGTC
TGACCAACAAGAAGGTTAACAGAATCACTGGCTTGGATCCAGCTGGGCCTAACTTTGAGTATGC
AGAGGCCCCCAGTCGCCCTTCTCCTGATGATGCAGATTTCTAGACGTCTTACATACATTACC
15 AGAGGGTCACCTGGCCGAAGTATTGGGATCCAGAAACCAGTAGGACATGTGACATTTATCCC
AATGGAGGCACTTTCCAGCCAGGATGCAACATTGGGGAAGCCATTTCGTGTGATTGCAGAGCGG
GGCCTTGGAGATGTGGACCAGCTGGTAAAGTGTCCCATTGAGCGATCCATTATCTCTTCATTG
ACTCCTTGCTGAATGAGGAAAACCCAGCAAGGCTTACAGGTGCAACTCCAAGGAAGCCTTTG
AGAAAGGGCTCTGCCTGAGCTGCAGAAAGAATCGATGCAACAATGTGGGCTATGAGATCAACA
20 AGGTCAGAGCCAAAAGGAGCAGCAAGATGTACCTGAAGACTCGCTCTCAGATGCCCTACAAAG
TCTTCCACTACCAAGTCAAAATTCATTTTTCTGGGACTGAGAGTGACAAGCAGCTCAACCAGGC
CTTTGAGATTCTCTGTATGGCACAGTGGCCGAGAGTGAGAATATCCCCCTTCACTCTGCCTGAG
GTCTCCACAAATAAAACCTACTCCTTCTGATTTATACGGAGGTGGACATCGGAGAACTGCTGA
TGATGAAGCTCAAGTGAAGAGTGATTCTCCTACTTCAGCTGGTTCGGACTGGTGGAGCCCGG
25 GCTTTGTCTATCGAGAAGATTCGGGTGAAAGCAGGAGAGACTCAGAAAAAGGTCATCTTCTGTG
CTAGGGAGAAAGTGTCTCATCTGCAGAAGGGAAAGGACTCCGCAGTGTTTGTGAAGTGCCATG
ACAAGTCTCTGAAGAAGTCTGGCTGATACTGAACAAACCAACAAGAGAAGAAAGCACAAGAGTT
CTTTGAAGACTGAAGTAAACGAAGTAAATTTTATAAAAAAGAAAAAAACAATACCCTTGTTTGGG
TGTTGAAAGTGGGTTTTCTGAGTATTAATCCCAGCTCTATCTTGTAGTTAAGTTAGAAGACA
30 GGCTCAAATACTAAACGTGGCTAATACAAGGTGAGGAGTCTCATGGCCAATGGCATGTCTTCC
CGCATCAAAGGACAGCAGATAGGAGAAGCATGGTGCCTTTTGTCCCATAGAAGGAATCATTTG
TTCCCAATAAATAGGACTCCTTCTGTGACCCGATTGGTCATGGTCGAAAAAATGAGTGAGAGT
GAGGGCCTCTTATTTTGTAGAAATCTGAGGCTTTAACTGAGACCTTTTCAAGTTTTCTTGTGG
CATGAGTCAGATCCATTTCTTCAGCAGTTGAAATACCTGGCCTTTGTAAGTCTTCTCACCA
35 TTGTGAGGAAGCAAAAAAAAAAAAAAAAAAGAAAGAAAGAAAGAAACGTAATCAGAGATGTATGACT
TAGCAAAAGCAATGGAGGCCAGAGCTCATGAAGTCTGAGCCTTGTTCACCATGACAAAGTA
CAAGTCAACAGAGATATAGAACTAGATTGAGTAATTCTAAAGAGACTTGAATTTTACAGCTTAA
TCCCTCCATGTTTTAAAGTTTGTCTTATATTTTAACTTGTCTCTGAGTAGACATTGAAAAATGA
GCTTATAATTCAGGTGACACATAAATTGAAATGAAGGAAAATAACACCTAGTCTGATTTTACTTTT
40 TTTTTTTTTTTTTTTTGAAGAAAAGTCTCGTTTTTTTCCACTCTGTAGCCAAGCCTGACCCAGAAC
TCAGCTACGTTGTTGAGGCTTGCCATGAACTCTCAGTGATCCTCCTGCCTTAGCTTTCTGAGTG
CTGGGATTATTAGCATGAACCACACCTGGCTTGATACCATAACTTTATCCTCAGATTTTCTCTAT
TTGTTTTTCATCAACTCGAACACATTCAACAGCCAATAGTTAGTGTTTAGTTTGAGACTCTTCTCG
GCCATGCCTCTGGCACACTTCTAACACATCACATTTCGTTTCTAGTTTAGATGTGATCAAGGTCAA
45 TTTCTGCAGCATGCAATGTACAAAGTTTAGATCGTGATCATTTTACCACATAAAAGTTGAAGTTAT
TAGAAAAAGGGTTATCAATGCTTGTAATTTGTTGTGTACACATAAGGGGGTGCCTCAGCTGTGA
TAGCCACAGGAAGTACCTGGCGATTGCCACTGTTTCATTTTGCCTAAAAACAAAAACAAAA
CAAAGCCAATCAAATGTGAGTGTATGATTATTTATGAACTAGATCTTATATTTTCAAGATATTTT
ACTATATATTAATAAAGTGATAAAGAGTGCTATGCCAGAGGCTATTGCTGCAAAACAAAAC
50 GTGAAACTGTAGGTGTCTGAACACCTACCCACAGACAAAGCCCCACAAGTATAGCTGTCAATTCT
GTGTCACTTGGAAAGGGAAAAAGAGTCAAGGGACGTAAGGAGAGTGTGAGAGTAGTAGTTCCG
GATATGCTGGAATGTTAGCCCTTGTAGGAGAAGGGTGGTTGTGCCTATGTGATTAGGACAAAA
GTGACTGATTTTCATCAGGCTTCCAGTCAATTCTAACAATAAAATGATGTGCAATTTGTCACTGGC
ATCCCCCTTATTGCTAATTCATTGACTTTGTACATTTAGATTTGGATCAAGGGCTCACAAATTTCA
55 AAGATTACGCCAACCTGTACATAGCACTGGACATTCTGGCTTCTAAATCGTGCGCGTGCACACA
CACATGTATACGTGTACATATACACATACATATGTATCTCAATGATGCTTTGGCTTTACATTTTAT
TTATTAGCTGTAAATATATGTGTGGGTGTGTAAGAGAACTTGTAACATGGGAAAAGCTGTTGTG

TAGATTTGTGGTGCTAAGTTTGTGTGTGTCTCCATCAGTGATGGTCTGCCTCACTGAGCTAACTT
 ACTCTGGTAACTAATACAGTGAAATAGGCTTTTAAAAGAGGAAAAGAAATTTACCTATGTGAAG
 AAACGGAATCTGCTTTTAATAAAACCGACATTTTATCATGA

5 [071] In certain embodiments, one of the two or more mammalian hydrolases is LPL and the target sequence(s) in the mRNA encoding LPL is within nucleotides 560-590, 700-745, 930-960 or 1330-1360 of SEQ ID NOs: 6 or 7, preferably within nucleotides 560-590, 705-745, or 930-960 of SEQ ID NOs: 6 or 7. Suitable artificial miRNA guide strand sequences in the context of the present invention are, e.g., an artificial miRNA guide strand sequence having the nucleotide sequence of SEQ ID NO:
 10 1 (LP 8mer), SEQ ID NO: 2 (LP 6mer), SEQ ID NO: 3 (LH 8mer), SEQ ID NO: 4 (LH 9mer) and/or SEQ ID NO: 5 (LH Non-canonical).

[072] The inventors identified two types of artificial miRNA guide strand sequences that are effective in reducing expression and/or activity of hydrolases. A first sequence type comprising a strong 5' seed region, wherein strong means high GC-content, a high degree of complementarity ($\geq 62.5\%$ sequence
 15 complementarity, preferably $\geq 75\%$ sequence complementarity or no more than 3 mismatches in 8 nucleotides, preferably no more than 2 mismatches in 8 nucleotides) and a weak or normal 3' complementary region that can compensate for mismatches in the seed region, if present. A second sequence comprising a weaker 5' seed region either due to a lower GC-content or a shorter complementary region, particularly such as having consecutive mismatches at the end of the region
 20 and a strong 3' complementary region that compensates for the weaker binding and/or mismatches in the 5' seed region.

[073] The term "5' seed region" as used herein refers to nucleotide 2 to 9 of the artificial miRNA guide strand sequence. A miRNA has imperfect complementarity to its target sequence and typically has a higher degree of complementarity in the 5' seed region compared to the remaining sequence, up to
 25 perfect complementarity in the 5' seed region. The nucleotide 5' of the 5' seed region (nucleotide 1) is typically an A or an U and is preferably not complementary to its target sequence.

[074] The term "3' complementary region" as used herein refers to the last 6 nucleotides in the artificial miRNA guide strand sequence, also referred to as nucleotide -1 to -6 (nt -1, nt -2, nt -3, nt -4, nt -5 and nt -6), wherein nucleotide -1 is the last nucleotide (or the first nucleotide counted from the 3' end)
 30 and nucleotide -6 is the 6th nucleotide counted from the 3' end of the artificial miRNA guide strand sequence. Typically, this region has a relatively low GC-content, such as a GC-content of $\leq 50\%$ and often has two or more mismatches. A higher degree of complementarity, particularly GC complementarity, as well as the last nucleotide being complementary to its target sequence increase the strength of a 3' complementary region.

35 [075] In certain embodiments, the miRNA guide strand sequence has imperfect complementarity to the target sequence(s) of at least one of the at least two mRNAs encoding the two or more mammalian hydrolases and wherein said miRNA guide strand sequence comprises a 5' seed region starting at nucleotide 2 (nt 2) and a 3' complementary region that undergoes base pairing with said target sequence(s), wherein the 5' seed region comprises (i) a sequence of 8 nucleotides (nt 2-9)

complementary to the target sequence(s) and having 0, 1, 2 or 3 mismatches with the target sequence(s), and (ii) a GC-content of 55-70% wherein all or all but one or all but two G or C are complementary to the target sequence(s); and wherein the 3' complementary region comprises (i) a sequence of the last 6 nucleotides (i.e., nt -6 to nt-1) complementary to the target sequence(s) and
5 having 1, 2 or 3 mismatches with the target sequence(s), and (ii) a GC-content of $\leq 50\%$ in the last 6 nucleotides, wherein 1 or 2 of the complementary nucleotides are G or C. Examples of suitable artificial miRNA guide strand sequences are a nucleotide sequence of SEQ ID NO: 2 (LP 6mer), SEQ ID NO: 4 (LH 9mer) or SEQ ID NO: 5 (LH Non-canonical).

[076] The term "GC content" as used herein relates to the percentage of nitrogenous bases in a DNA
10 or RNA molecule (in the context of the present invention mainly RNA molecule) that are either G or C. The person skilled in the art will understand that 1 or 2 of the nucleotides are G or C means that 1 or 2 of the complementary nucleotides contain the nucleobase G or C. A nucleotide consists of a nucleobase, a five-carbon sugar (also referred to as nucleoside) and a phosphate and hence a nucleotide is defined and referred to by its nucleobase for simplicity. Moreover, the nucleobases are
15 referred to by their commonly used abbreviations, adenine (A), cytosine (C), guanine (G), thymidine (T) and uracil (U).

[077] Preferably, the 5' seed region comprising a sequence of 8 nucleotides (nt 2-9) complementary to the target sequence(s) has 0, 1 or 2 mismatches with the target sequence(s), more preferably 0 or 1 mismatches. Also, the 5' seed region has a GC-content of 55-70%, such as 62.5% (or 5 G or C out
20 of 8 nucleotides) and preferably wherein all or all but one G or C are complementary to the target sequence(s). In some embodiments, the 5' seed region is longer, such as comprising at least 10 nucleotides (at least nt 2-11) or at least 11 nucleotides (at least nt 2-12), otherwise as described herein.

[078] In certain embodiments the 5' seed region as disclosed herein comprises no mismatch with the
25 target sequence(s) within nucleotides 2-3, preferably within nucleotides 2-4, more preferably within nucleotides 2-5, even more preferably within nucleotides 2-7, and/or the 5' seed sequence comprises at least 2 times 3-4 consecutive base pairings with the target sequence(s). In a preferred embodiment the mismatch(es) in the 5' seed region with the target sequence(s) is/are not consecutive mismatches. In certain embodiments the first nucleotide at the 5' end of the artificial miRNA guide strand sequence
30 (i.e., the nucleotide 5' of the 5' seed region) comprises a nucleotide (comprises a nucleobase) selected from the group consisting of A and U, preferably the nucleotide is U.

[079] The 3' complementary region as disclosed herein comprises a sequence of the last 6 nucleotides (i.e., nt -6 to nt-1) complementary to the target sequence(s) having no more than three mismatches in the last 6 nucleotides of the miRNA guide strand and the target sequence, such as
35 having 1, 2 or 3 mismatches with the target sequence(s), preferably 1 or 2 mismatches with the target sequence(s). In preferred embodiments, the mismatches in the 3' complementary region are not consecutive mismatches. Also, the 3' complementary region has a GC-content of $\leq 50\%$ in the last 6 nucleotides, such as 1, 2 or 3 G or C, preferably 2 or 3 G or C (33% to 50% GC content), wherein 1

or 2 of the complementary nucleotides are G or C. The person skilled in the art will understand that increasing the number of mismatches and/or consecutive mismatches and/or a lower number of complementary nucleotides that are G or C renders the 3' complementary region weaker and that decreasing the number of mismatches and/or no consecutive mismatches and/or a higher number of complementary nucleotides that are G or C renders the 3' complementary region stronger. Moreover, a stronger 3' complementary region can potentially compensate for a weaker 5' seed region.

[080] In certain alternative embodiments, the miRNA guide strand sequence has imperfect complementarity to the target sequence(s) of at least one of the at least two mRNAs encoding the two or more mammalian hydrolases and wherein said miRNA guide strand sequence comprises a 5' seed region starting at nucleotide 2 (nt 2) and a 3' complementary region that undergoes base pairing with said target sequence(s), wherein the 5' seed region comprises (i) a sequence of 8 nucleotides (nt 2-9), wherein at least the first 4 consecutive nucleotides (at least nt 2-5) are complementary to the target sequence(s), wherein the sequence of 8 nucleotides comprises at least one mismatch with the target sequence(s), and (ii) a GC-content of 55-75%, wherein at least 4 of the consecutive nucleotides are complementary to the target sequence(s) are G or C; and wherein the 3' complementary region comprises (i) a sequence of the last 6 nucleotides complementary to the target sequence(s) and having 0 or 1 mismatches with the target sequence(s), and (ii) a GC-content of 30-50% in the last 6 nucleotides, preferably wherein at least 2 of the complementary nucleotides are G or C. Examples of suitable artificial miRNA guide strand sequences are a nucleotide sequence of SEQ ID NO: 1 (LP 8mer) or SEQ ID NO: 2 (LP 6mer).

[081] According to the alternative embodiment, the 5' seed region comprises a sequence of 8 nucleotides (nt 2-9), wherein at least the first 4 consecutive nucleotides (at least nt 2-5) are complementary to the target sequence(s), wherein the sequence of 8 nucleotides comprises at least one mismatch with the target sequence(s). Thus, the at least one mismatch is in nucleotides 6-9 and the at least one mismatch may be 1, 2, 3 or 4 mismatch(es). In certain embodiments, the 5' seed region comprises a sequence of 8 nucleotides (nt 2-9), wherein at least the first 6 consecutive nucleotides (at least nt 2-7) are complementary to the target sequence(s), wherein the sequence of 8 nucleotides comprises at least one mismatch with the target sequence(s). Thus, the at least one mismatch is in nucleotides 8-9 and the at least one mismatch may be 1 or 2 mismatch(es). As disclosed herein, the GC content in the 5' seed region is 55-75% (such as 62.5 to 75%, or such as 5 or 6 G or C out of 8 nucleotides), wherein at least 4 of the consecutive nucleotides complementary to the target sequence(s) are G or C. For example, 5 or 6 of the consecutive nucleotides complementary to the target sequence(s) are G or C. Preferably, at least the first 4 consecutive nucleotides are complementary to the target sequence(s) and are G or C. In a specific embodiment at least the first 4 consecutive nucleotides (nt 2-5) are complementary to the target sequence(s) and are G or C and the sequence of 8 nucleotides comprises at least two mismatches with the target sequence(s) in nucleotides 6-9, preferably 3 or 4 mismatches in nucleotides 6-9. In certain embodiments the first nucleotide at the 5' end of the artificial miRNA guide strand sequence (i.e., the nucleotide 5' of the 5'

seed region) comprises a nucleotide (comprises a nucleobase) selected from the group consisting of A and U, preferably the nucleotide is U.

[082] As disclosed herein, the corresponding 3' complementary region comprises (i) a sequence of the last 6 nucleotides complementary to the target sequence(s) and having 0 or 1 mismatches with the target sequence(s). Preferably, the last nucleotide (nt -1) is complementary to the target sequence(s) and/or the penultimate nucleotide is a mismatch, more preferably the last nucleotide is complementary to the target sequence(s) and the penultimate nucleotide is a mismatch. As disclosed herein, the GC-content in the 3' complementary region is 30-50% (such as 33% to 50%, or such as 2 or 3 G or C out of 8 nucleotides) in the last 6 nucleotides, wherein at least 2 of the complementary nucleotides are G or C, preferably wherein 2 of the complementary nucleotides are G or C. The term complementary nucleotides as used herein means complementary to the target sequence(s).

[083] The artificial miRNA guide strand sequences according to the invention are *de novo* designed, which means not naturally occurring in mammalian cells or organisms. Thus, the sequence is generated by rational design based on sequence alignment of target sequences on mRNA and/or protein level, preferably on mRNA level. The aim of the present invention is to provide the proof-of-concept that an artificial miRNA comprising an artificial miRNA guide strand sequence can be used for a cell line engineering approach for simultaneous targeting of multiple hydrolases acting on ester bonds, such as multiple lipases, in a mammalian host cell, such as CHO cells. The aim is to reduce hydrolase expression and hence hydrolase activity, PS degrading enzyme activity, PS degradation and/or particle formation in the mammalian host cell, HCCF and in a purified recombinant protein of interest produced in said mammalian host cell. Particularly, the aim is reducing the expression of two or more hydrolases acting on ester bonds (such as lipases and/or carboxylesterases) in order to reduce the presence of the two or more hydrolases acting on ester bonds and/or PS degrading activity in the mammalian host cell and/or HCCF and/or in a purified recombinant protein of interest produced in said mammalian host cell, or in a composition thereof. In addition, some lipases, such as PLBL2, were reported to be immunogenic in patients. Thus, reducing the expression of two or more hydrolases acting on ester bonds (such as lipases and/or carboxylesterases) in a mammalian host cell may also result in reduced immunogenic activity in a purified recombinant protein of interest produced in the mammalian host cell, or a composition thereof. It has been observed that the hydrolytic activity profile co-purified with a protein of interest varies with the biopharmaceutical and hence the contaminating co-purified hydrolases (lipases and carboxylesterases) are believed to vary between protein of interest, cell lines, culture conditions and cell clones. Thus, an approach targeting multiple targets would be beneficial.

[084] Highly conserved transcripts of lipases were therefore targeted by *de novo* designed artificial microRNAs (amiRNA). As proof-of-concept, it has been shown that artificial (*de novo* designed) miRNAs were able to simultaneously knock-down lipases via imperfect mRNA target binding and hence that artificial miRNAs comprising an artificial (*de novo* designed) miRNA guide strand sequence can be used for stable expression along with the transgene of interest to target and reduce expression

of multiple (two or more) hydrolases acting on ester bonds (such as lipases or carboxylesterases) in a mammalian host cell.

[085] The artificial miRNA according to the invention may be a pri-miRNA, a pre-miRNA, a double stranded mature miRNA (comprising the passenger strand and the guide strand) or a single stranded
5 miRNA guide strand. In certain embodiments, the artificial miRNA is a single or double stranded miRNA mimic (miR-Mimic). miRNA mimics are chemically synthesized double stranded RNAs which mimic mature miRNAs after transfection into cells and may be provided as double stranded miRNA mimic or as single stranded miRNA, which requires an additional annealing step with the passenger strand prior to transfection. Customized miRNA mimics are available with proprietary chemical
10 modifications in order to improve their performance as well as stability and to prevent binding activity of the passenger strand from various suppliers on demand, such as from Horizon, Qiagen or Thermo Fisher.

Expression vectors encoding artificial miRNA targeting two or more hydrolases

15 [086] In another aspect, the present invention relates to a mammalian expression vector comprising a polynucleotide sequence encoding the artificial miRNA according to the invention. The mammalian expression vector may further comprise at least one gene of interest encoding a recombinant protein of interest. Alternatively, the recombinant protein of interest may also be encoded by a separate expression vector.

20 [087] In certain embodiments, the mammalian expression vector comprises a polynucleotide sequence encoding two or more of the artificial miRNAs according to the invention, wherein the artificial miRNAs are identical or different. The two miRNAs may be encoded by the same or separate expression cassettes.

[088] The mammalian expression vector according to the invention is for expression of the
25 heterologous sequence in mammalian cells, i.e., it is adapted for expression in mammalian cells, i.e., the artificial miRNA and optionally further a protein of interest. Thus, the mammalian expression vector according to the invention is characterized in that it comprises the polynucleotide encoding the polynucleotide sequence encoding the artificial miRNA operably linked to a mammalian promoter. Typically, the mammalian expression vector comprises a mammalian cassette comprising the
30 polynucleotide sequence encoding the artificial miRNA operably linked to a mammalian promoter. Mammalian promoters regulate transcription in mammalian cells. Exemplary mammalian promoters, without being limited thereto are simian virus 40 (SV40) early promoter, cytomegalovirus (CMV) immediate-early promoter, rous sarcoma virus (RSV) promoter, adenovirus promoter (e.g., the adenovirus major late promoter (AdMLP), human ubiquitin C promoter (UBC), human elongation factor
35 1 α promoter (EF1A), CHO-derived elongation factor-1 (CHEF-1) mouse phosphoglycerate kinase 1 promoter (PGK) and chicken β -actin promoter coupled to CMV early enhancer (CAGG), and strong mammalian promoters such as native immunoglobulin and actin promoters or the natural promoter of

the heterologous polynucleotide, such as the polynucleotide encoding the protein of interest. The mammalian expression vector may further comprise bacterial sequences, such as an origin of replication and resistance genes for vector amplification in bacterial cells.

[089] The term “expression cassette” as used herein is a distinct component of a DNA, particularly vector DNA, consisting of one or more coding polynucleotide sequences and the regulatory sequences controlling their expression in a transfected or transduced cell. An expression cassette comprises at least three components: a promoter sequence, an open reading frame (such as a polynucleotide coding for a protein of interest), and termination sequence. It may further comprise means controlling the expression of the gene product(s) (mRNA encoding the protein of interest), such as an enhancer.

10 In mammalian expression vectors comprising a polynucleotide encoding a protein of interest, the termination sequence is referred to as 3'UTR and usually contains a polyadenylation site. The expression cassette directs the cell's machinery to make RNA and may therefore also be referred to as transcriptional cassette. The miRNA may be expressed within an expression cassette of a recombinant protein of interest or preferably of a selectable marker, i.e., within an intron, out of which

15 the pre-miRNA is spliced and processed until the mature miRNA is generated by the cellular machinery. Alternatively, it may be expressed separately on the same expression vector or on a separate expression vector and driven by an own promoter.

[090] Exemplary mammalian promoters, without being limited thereto are simian virus 40 (SV40) early promoter, cytomegalovirus (CMV) immediate-early promoter, rous sarcoma virus (RSV) promoter, adenovirus promoter (e.g., the adenovirus major late promoter (AdMLP), human ubiquitin C promoter (UBC), human elongation factor 1 α promoter (EF1A), CHO-derived elongation factor-1 (CHEF-1) mouse phosphoglycerate kinase 1 promoter (PGK) and chicken β -actin promoter coupled to CMV early enhancer (CAGG), and strong mammalian promoters such as native immunoglobulin and actin promoters or the natural promoter of the heterologous polynucleotide, such as the polynucleotide

25 encoding the protein of interest. Examples for polyadenylation signals are BGH polyA, SV40 late or early polyA; alternatively, 3'UTRs of immunoglobulin genes etc. can be used. The skilled person will further understand that the 3'UTR may be engineered to support high expression levels, e.g., by removing instability elements, such as AREs (adenylate-uridylate rich elements).

[091] The recombinant protein of interest may be any protein, but is typically a therapeutic protein.

30 The term “therapeutic protein” as used herein refers to proteins that can be used in medical treatment of humans or animals. These include, but are not limited to cytokines, growth factors, hormones, blood coagulation factors, vaccines, interferons, fusion proteins, antibodies, antibody-derived molecules and an antibody mimetic. In certain embodiments, the therapeutic protein is selected from the group consisting of a cytokine, a hormone, a fusion protein, an antibody, an antibody-derived molecule and

35 an antibody mimetic. The term “gene of interest” as used herein refers to the polynucleotide sequence (DNA sequence) encoding the recombinant protein of interest.

[092] In some embodiments, the gene product (miRNA or protein of interest) may be placed under the control of an amplifiable genetic selection marker, such as dihydrofolate reductase (DHFR) or glutamine synthetase (GS). The amplifiable selection marker gene can be on the same expression vector as the gene of interest and/or the polynucleotide sequence encoding the artificial miRNA of the invention. Alternatively, the amplifiable selection marker gene and the secreted therapeutic protein expression cassette can be on different expression vectors but integrate in close proximity into the host cell's genome. Two or more vectors that are co-transfected simultaneously, for example, often integrate in close proximity into the host cell's genome. Further, amplification of the genetic region containing the secreted therapeutic protein expression cassette may be mediated by adding the amplification agent (e.g., methotrexate (MTX) for DHFR or methionine sulfoximine (MSX) for GS) into the cultivation medium.

[093] Sufficiently high stable levels of the gene product in the host cell or the producer cell may be achieved, e.g., by cloning multiple copies of a polynucleotide encoding the artificial miRNA of the invention and/or the protein of interest into an expression vector. Cloning multiple copies of a polynucleotide into an expression vector and amplifying the expression cassette (encoding for the artificial miRNA or the recombinant protein of interest) may further be combined.

[094] Mammalian expression vectors may include but are not limited to plasmid vectors, cosmids, artificial/mini-chromosomes (e.g. ACE), Bacterial Artificial Chromosomes (BAC) or viral vectors, such as lentivirus, retrovirus, adenovirus, adeno-associated virus, herpes simplex virus, and retrovirus derived vectors. Preferably, the mammalian expression vector is a plasmid vector, a Bacterial Artificial Chromosome (BAC) or a viral vector (e.g., a lentiviral vector). Said mammalian expression vector may be introduced into the mammalian host cell via transfection or transduction, respectively, and is preferably stably integrated into the host cell genome. The person skilled in the art knows suitable plasmids, BACs or viral vectors and that a plasmid may further comprise elements for targeted integration (homology regions), including transposon recognition sequences and inverted terminal repeats, upstream and downstream of the polynucleotide sequence encoding the artificial miRNA and/or the protein of interest (and optionally a selection marker such as GS).

[095] Means for cloning microRNA into an expression vector are known to the person skilled in the art. For example, one or more microRNAs may be cloned as polynucleotides encoding engineered pre-miRNA sequences (i.e. short hairpins) into a mammalian expression vector, such as pcDNA6.2-GW/miR or pcDNA6.2-GW/EmGFP-miR from Invitrogen (see manual BLOCK-iT™ Pol II miR RNAi Expression Vector Kits). Said vector may encode one or more copies of the same or different miRNAs. In brief, the mature miRNA sequence is cloned into a given sequence encoding an optimized hairpin loop sequence and 3' and 5' flanking regions derived from the murine miRNA mir-155 (Lagos-Quintana et al., 2002). The flanking regions are present on the vector and a DNA oligonucleotide is designed, which encodes the miRNA guide strand sequence, the mentioned loop and the antisense sequence of the respective mature miRNA with a two nucleotide depletion to generate an internal loop in the hairpin stem. Furthermore, overhangs are added for cloning at both ends. Hairpin structure may be

analyzed using the online tool mfold (M. Zuker, Mfold web server for nucleic acid folding and hybridization prediction, Nucleic Acids Res. 31(13), 3406-3415, 2003). DNA strands are annealed and ligated into the 3'UTR of emerald GFP reporter protein gene as described by the manufacturer. A vector containing more than one miRNA may be generated applying the chaining method. The negative control miRNA (supplied by the manufacturer) and the siLacZ may be used as appropriate negative controls. Alternative vectors that may be used in the present invention for miRNA expression, without being limited thereto, are pCMV-MIR (Origene), pmR-ZsGreen1 (clontech) and shMIMIC lentiviral miRNA vector (ThermoScientific).

[096] The recombinant protein of interest may be any protein and is typically a therapeutic protein. The term "therapeutic protein" as used herein refers to proteins that can be used in medical treatment of humans or animals. These include, but are not limited to cytokines, growth factors, hormones, blood coagulation factors, vaccines, interferons, fusion proteins, antibodies, antibody-derived molecules, and an antibody mimetic. In certain embodiments, the therapeutic protein is selected from the group consisting of a cytokine, a hormone, a fusion protein, an antibody, an antibody-derived molecule, and an antibody mimetic.

[097] In certain embodiments the protein of interest is an antibody. In cases where the protein of interest is an antibody, the mammalian expression vector comprises a polynucleotide comprising a coding sequence for a variable region of the heavy chain and/or a coding sequence for a variable region of the light chain of the antibody. In certain embodiments, the eukaryotic expression vector comprises a polynucleotide comprising a coding sequence for a heavy chain and/or a coding sequence for a light chain of the antibody. Thus, the polynucleotide comprising a coding sequence for a variable region of the heavy chain and the polynucleotide comprising a coding sequence of a variable region of the light chain may be expressed on the same mammalian expression vector or on separate mammalian expression vectors. The expression vector may comprise a multi-cistronic expression cassette, such as a bi-cistronic expression cassette, and/or multiple expression cassettes. A multi-cistronic expression cassette comprises more than one open reading frames separated by sequences coding for an RNA element that allows for translation initiation, such as an internal ribosomal entry site (IRES). In a multi-cistronic expression cassette, the two or more open reading frames are under the control of the same promoter. The polynucleotide encoding at least a variable region of the heavy chain and the polynucleotide encoding at least a variable region of the light chain may therefore be expressed within the same expression cassette (separated e.g., by an IRES sequence) or by two separate expression cassettes.

Mammalian cells comprising artificial miRNA targeting two or more hydrolases

[098] In yet another aspect, the present invention relates to a mammalian cell, preferably a CHO cell comprising the artificial miRNAs of the invention or a polynucleotide encoding the artificial miRNA of the invention or a mammalian expression vector of the invention. The mammalian cell may be any

mammalian cell suitable for protein expression in cell culture, such as a human or rodent cell. Preferably, the mammalian cell is a CHO cell.

[099] The mammalian cell comprising the artificial miRNAs of the invention has reduced expression of two or more mammalian hydrolases compared to a mammalian cell not comprising the artificial miRNA of the invention. In certain embodiments, the mammalian cell comprising the artificial miRNA of the invention and/or a recombinant protein of interest produced and isolated from said mammalian cell has reduced hydrolase activity and/or PS degrading activity compared to a mammalian cell and/or a recombinant protein of interest produced and isolated from said mammalian cell not comprising the artificial miRNA of the invention. The mammalian cell comprising the artificial miRNA of the invention and/or the recombinant protein of interest produced and isolated from said mammalian cell may also have reduced PS degrading activity of two or more mammalian hydrolases compared to the mammalian cell and/or a recombinant protein of interest produced and isolated from said mammalian cell not comprising the artificial miRNA of the invention. The person skilled in the art would understand that a recombinant protein produced and isolated from said mammalian cell includes a fluid or composition comprising said recombinant protein, such as HCCF a purified or partially purified recombinant protein or a purified and formulated composition (drug substance or drug product). Isolated includes purification as well as separation, such as by centrifugation or filtration of the cell culture supernatant. In certain embodiments, the artificial miRNA prevents expression of at least two endogenous hydrolases in the mammalian cell and/or prevents hydrolase activity and/or polysorbate degrading activity associated with a recombinant protein of interest produced in the mammalian cell. Associated with a recombinant protein of interest produced in a mammalian cell means co-purified with the recombinant protein of interest produced in the mammalian cell and/or in a composition comprising the recombinant protein of interest produced in the mammalian cell. Reduced hydrolase activity may be determined, without being limited thereto, by means of a fluorescent assay as for example described in WO 2022/049294 for detecting lipase activity using 4-methylumbelliferyl decanoate (4-MUD) as substrate and/or in EP 22161135 for detecting hydrolases such as carboxylesterases using 1-octanoyloxy-pyrene-3,6,8-trisulfonic acid (OPTS) as substrate. PS concentrations may be determined, e.g., using the fluorescence micelle assay (FMA) as described herein. Thus, for determining reduced PS degrading activity samples, such as HCCF samples (diluted), may be spiked with PS20 (e.g., cPS20 = 0.4 mg/ml) or PS80 (e.g., cPS80 = 0.2 mg/ml) following storage at room temperature (about 22°C) at day 0, 1, 3, 7 and 14, and frozen at -70°C prior to analysis using the FMA. For the FMA 10 µl sample are mixed with 240 µl FMA reagent (150 mM NaCl, 50 mM Tris, 0.2% Acetonitrile, 5 µM NPN, 0.0015% Brij-35, pH 8) and subsequently incubated for 1 min at 35°C. Fluorescent signal, positively correlating with PS concentration, may be measured at 420 nm following excitation of the dye with a 350 nm laser using a microplate reader (such as from Infinite® 200 PRO Tecan, Männedorf, Switzerland). PS concentrations are quantified using standard curves for PS20 (such as ranging from 0.1 to 0.6 mg/ml) and/or for PS80 (such as ranging from 0.05 to 0.3 mg/ml). Reduction compared to control (samples from cells without the artificial miRNA) may

be determined by comparing PS20 or PS80 in mg/ml at various days or by comparing the degradation rate (mg/ml/day).

[100] The mammalian cell according to the invention may further express a recombinant protein of interest, preferably a therapeutic protein, more preferably a therapeutic antibody. The recombinant protein of interest may be stably or transiently expressed, preferably the recombinant protein of interest is stably expressed.

[101] In certain embodiments the mammalian cell according to the invention is transfected or transduced with the mammalian expression vector of the invention. Preferably the mammalian cell is stably transfected or transduced with the mammalian expression vector of the invention. Methods for transfecting or transducing mammalian cells are well known in the art and include semi-targeted, targeted and random integration. Alternatively, the mammalian cell according to the invention is transfected with the artificial miRNA of the invention or with a mammalian expression vector comprising a polynucleotide sequence encoding the artificial miRNA of the invention. Methods for transfecting mammalian cells with miRNA or with a mammalian expression vector are well known in the art.

[102] The recombinant protein of interest may be any protein and is typically a therapeutic protein. The term "therapeutic protein" as used herein refers to proteins that can be used in medical treatment of humans or animals. These include, but are not limited to cytokines, growth factors, hormones, blood coagulation factors, vaccines, interferons, fusion proteins, antibodies, antibody-derived molecules, and an antibody mimetic. In certain embodiments, the therapeutic protein is selected from the group consisting of a cytokine, a hormone, a fusion protein, an antibody, an antibody-derived molecule, and an antibody mimetic.

[103] A preferred recombinant protein of interest is an antibody, including fragments and derivatives thereof. Typically, an antibody is monospecific, but an antibody may also be multispecific (symmetric or asymmetric). Thus, the present invention may be used for the production of monospecific antibodies, multispecific antibodies, or fragments thereof, preferably of antibodies (monospecific), bispecific antibodies, trispecific antibodies or fragments thereof, preferably antigen-binding fragments thereof. Exemplary antibodies within the scope of the present invention include but are not limited to anti-CD2, anti-CD3, anti-CD20, anti-CD22, anti-CD30, anti-CD33, anti-CD37, anti-CD40, anti-CD44, anti-CD44v6, anti-CD49d, anti-CD52, anti-EGFR1 (HER1), anti-EGFR2 (HER2), anti-GD3, anti-IGF, anti-VEGF, anti-TNFalpha, anti-IL2, anti-IL-5R or anti-IgE antibodies, and are preferably selected from the group consisting of anti-CD20, anti-CD33, anti-CD37, anti-CD40, anti-CD44, anti-CD52, anti-HER2/neu (erbB2), anti-EGFR, anti-IGF, anti-VEGF, anti-TNFalpha, anti-IL2 and anti-IgE antibodies.

[104] The term "antibody", "antibodies", or "immunoglobulin(s)" is used herein in the broadest sense and encompasses various antibody structures, including but not limited to monoclonal antibodies, polyclonal antibodies, monospecific antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they exhibit the desired antigen-binding activity. There are various

classes of immunoglobulins: IgA, IgD, IgE, IgG, IgM, IgY, IgW. Preferably the antibody is an IgG antibody, more preferably an IgG1 or an IgG4 antibody.

[105] Antibodies can be of any species and include chimeric and humanized antibodies. "Chimeric" antibodies are molecules in which antibody domains or regions are derived from different species. For example, the variable region of heavy and light chain can be derived from rat or mouse antibody and the constant regions from a human antibody. In "humanized" antibodies only minimal sequences are derived from a non-human species. Often only the complementarity-determining region (CDR) amino acid residues of a human antibody are replaced with the CDR amino acid residues of a non-human species such as mouse, rat, rabbit or llama. Sometimes a few key framework amino acid residues with impact on antigen binding specificity and affinity are also replaced by non-human amino acid residues.

[106] Typically, antibodies are tetrameric polypeptides composed of two pairs of a heterodimer each formed by a heavy and a light chain. Stabilization of both the heterodimers as well as the tetrameric polypeptide structure occurs via interchain disulfide bridges. Each chain is composed of structural domains called "immunoglobulin domains" or "immunoglobulin regions" whereby the terms "domain" or "region" are used interchangeably. Each domain contains about 70 – 110 amino acids and forms a compact three-dimensional structure. Both heavy and light chain contain at their N-terminal end a "variable domain" or "variable region" with less conserved sequences which is responsible for antigen recognition and binding. The variable region of the light chain is also referred to as "VL" and the variable region of the heavy chain as "VH".

[107] An "antibody fragment" or "antigen-binding fragments" refers to a molecule other than an intact antibody that comprises a portion of an intact antibody that binds the antigen to which the intact antibody binds. Examples of antibody fragments include but are not limited to Fv, Fab, Fab', Fab'-SH, F(ab')₂; diabodies; linear antibodies; single-chain variable fragment antibody molecules (e.g. scFv); and multispecific antibodies formed from antibody fragments. Fab fragments consist of the variable regions of both chains, which are held together by the adjacent constant region. These may be formed by protease digestion, e.g., with papain, from conventional antibodies, but similarly Fab fragments may also be produced by genetic engineering. Further antibody fragments include F(ab')₂ fragments, which may be prepared by proteolytic cleavage with pepsin.

[108] Using genetic engineering methods, it is possible to produce shortened antibody fragments which consist only of the variable regions of the heavy (VH) and of the light chain (VL). These are referred to as Fv fragments (Fragment variable = fragment of the variable part). Since these Fv-fragments lack the covalent bonding of the two chains by the cysteines of the constant chains, the Fv fragments are often stabilized. It is advantageous to link the variable regions of the heavy and of the light chain by a short peptide fragment, e.g. of 10 to 30 amino acids, preferably 15 amino acids. In this way a single peptide strand is obtained consisting of VH and VL, linked by a peptide linker. An antibody protein of this kind is known as a scFv. Examples of scFv-antibody proteins are known to the person

skilled in the art. Thus, antibody fragments and antigen-binding fragments further include Fv-fragments and particularly scFv.

[109] In recent years, various strategies have been developed for preparing scFv as a multimeric derivative. This is intended to lead, in particular, to recombinant antibodies with improved pharmacokinetic and biodistribution properties as well as with increased binding avidity. In order to achieve multimerisation of the scFv, scFv were prepared as fusion proteins with multimerisation domains. The multimerisation domains may be, e.g. the CH3 (CH3 = 3rd constant heavy chain domain) region of an IgG or coiled coil structure (helix structures) such as Leucine-zipper domains. However, there are also strategies in which the interaction between the VH/VL regions of the scFv is used for the multimerisation (e.g. dia-, tri- and pentabodies). By diabody the skilled person means a bivalent homodimeric scFv derivative. The shortening of the linker in a scFv molecule to 5 – 10 amino acids leads to the formation of homodimers in which an inter-chain VH/VL-superimposition takes place. Diabodies may additionally be stabilized by the incorporation of disulfide bridges. Examples of diabody-antibody proteins are known from the prior art.

[110] By minibody the skilled person means a bivalent, homodimeric scFv derivative. It consists of a fusion protein which contains the CH3 region of an immunoglobulin, preferably IgG, most preferably IgG1 as the dimerisation region which is connected to the scFv via a Hinge region (e.g. also from IgG1) and a linker region. Examples of minibody-antibody proteins are known from the prior art.

[111] By triabody the skilled person means a: trivalent homotrimeric scFv derivative. ScFv derivatives wherein VH-VL is fused directly without a linker sequence lead to the formation of trimers.

[112] The skilled person will also be familiar with so-called miniantibodies which have a bi-, tri- or tetravalent structure and are derived from scFv. The multimerisation is carried out by di-, tri- or tetrameric coiled coil structures. In a preferred embodiment of the present invention, the gene of interest is encoded for any of those desired polypeptides mentioned above, preferably for a monoclonal antibody, a derivative or fragment thereof.

[113] Further encompassed is a single-domain antibody (sdAb), also be referred to as nanobody, which is an antibody fragment of a single monomeric variable antibody domain. Single-domain antibodies are typically engineered from heavy chain antibodies found in camelids (V_HH fragments) or cartilaginous fishes (V_{NAR} fragments).

[114] The immunoglobulin fragments composed of the CH2 and CH3 domains of the antibody heavy chain are called "Fc fragments", "Fc region" or "Fc" because of their crystallization propensity (Fc = fragment crystallizable). These may be formed by protease digestion, e.g. with papain or pepsin from conventional antibodies but may also be produced by genetic engineering. The N-terminal part of the Fc fragment might vary depending on how many amino acids of the hinge region are still present.

[115] Antibodies comprising an antigen-binding fragment and an Fc region may also be referred to as full-length antibody. Full-length antibody may be monospecific and multispecific antibodies. Multispecific antibodies are antibodies which have at least two different antigen-binding sites each of

which bind to different epitopes. A multispecific antibody includes, without being limited thereto, bispecific and trispecific antibodies. A bispecific antibody has two different binding sites. Multispecific antibodies also include antibody formats other than full-length antibodies such as antibody-derived molecules.

5 [116] Bispecific antibodies typically combine antigen-binding specificities for target cells (e.g., malignant B cells) and effector cells (e.g., T cells, NK cells or macrophages) in one molecule. Exemplary bispecific antibodies, without being limited thereto are symmetric antibodies with an additional scFv fused at the Fc part for second target, diabodies, BiTE (Bi-specific T-cell Engager) formats and DART (Dual-Affinity Re-Targeting) formats. The diabody format separates cognate
10 variable domains of heavy and light chains of the two antigen binding specificities on two separate polypeptide chains, with the two polypeptide chains being associated non-covalently. The DART format is based on the diabody format, but it provides additional stabilization through a C-terminal disulfide bridge. Trispecific antibodies are monoclonal antibodies which combine three antigen-binding specificities. They may be built on bispecific-antibody technology that reconfigures the antigen-
15 recognition domain of two different antibodies into one bispecific molecule or may be based on a DART architecture with an scFv at the Fc part of one chain. For example, trispecific antibodies have been generated that target CD38 on cancer cells and CD3 and CD28 on T cells. Multispecific (bi- and tri-specific) antibodies are particularly difficult to produce with high product quality.

[117] The term "antibody-derived molecule" as used herein refers to any molecule comprising at least
20 an antigen-binding moiety that is structurally related to antibodies. It includes modified full-length mono- or bispecific antibodies further modified with an additional antigen binding moiety or smaller antibody formats including the ones described herein.

[118] The term "antibody mimetic" as used herein refers to proteins that bind to specific antigens in a manner similar to antibodies, but that are not structurally related to antibodies. Antibody mimetic
25 includes, without being limited thereto an anticalin, an affibody, an adnectin, a monobody, a DARPin, an affimer, and an affitin.

[119] A single-domain antibody (sdAb) may also be referred to as nanobody. The person skilled in the art will understand that the protein may comprise more than one antigen-binding domain and hence may be multivalent, preferably bivalent (e.g., a bivalent sdAb or a bivalent anticalin or any other
30 bivalent antibody mimetic).

[120] Another preferred therapeutic protein is a fusion protein, such as an Fc-fusion protein. Thus, the invention can be advantageously used for production of fusion proteins, such as Fc-fusion proteins. The effector part of the fusion protein can be the complete sequence or any part of the sequence of a natural or modified heterologous protein. The immunoglobulin constant domain sequences may be
35 obtained from any immunoglobulin subtypes, such as IgG1, IgG2, IgG3, IgG4, IgA1 or IgA2 subtypes or classes such as IgA, IgE, IgD or IgM. Preferentially they are derived from human immunoglobulin, more preferred from human IgG and even more preferred from human IgG1 and IgG2. Non-limiting examples of Fc-fusion proteins are MCP1-Fc, ICAM-Fc, EPO-Fc and scFv fragments or the like

coupled to the CH2 domain of the heavy chain immunoglobulin constant region comprising the N-linked glycosylation site. Fc-fusion proteins can be constructed by genetic engineering approaches by introducing the CH2 domain of the heavy chain immunoglobulin constant region comprising the N-linked glycosylation site into another expression construct comprising for example other immunoglobulin domains, enzymatically active protein portions, or effector domains. Thus, an Fc-fusion protein according to the present invention comprises also a single chain Fv fragment linked to the CH2 domain of the heavy chain immunoglobulin constant region comprising, e.g., the N-linked glycosylation site.

[121] The term "cytokine" refers to small proteins, which are released by cells and act as intercellular mediators, for example influencing the behavior of the cells surrounding the secreting cell. Cytokines may be secreted by immune cells or other cells, such as T-cells, B-cells, NK cells and macrophages. Cytokines may be involved in intercellular signaling events, such as autocrine signaling, paracrine signaling and endocrine signaling. They may mediate a range of biological processes including, but not limited to immunity, inflammation, and hematopoiesis. Cytokines may be chemokines, interferons, interleukins, lymphokines or tumor necrosis factors.

[122] As used herein, "growth factor" refers to proteins or polypeptides that are capable of stimulating cell growth.

[123] The artificial miRNA, the polynucleotide or the expression vector encoding the artificial miRNA of the invention is introduced into the mammalian cell used for production of the protein of interest.

[124] Preferred mammalian cells for heterologous or recombinant protein production are rodent cells or human cells. The mammalian cell (also referred to as mammalian host cell) is therefore preferably a human or rodent cell, more preferably a rodent cell (e.g., a mouse or a hamster cell), even more preferably a hamster cell, such as a CHO cell. Suitable rodent cells may be e.g., hamster cells, particularly BHK21, BHK TK-, CHO, CHO-K1, CHO-DXB11 (also referred to as CHO-DUKX or DuxB11), a CHO-S cell and CHO-DG44 cells or the derivatives/progenies of any of such cell line. Particularly preferred are CHO cells, such as CHO-DG44, CHO-K1 and BHK21, and even more preferred are CHO-DG44 and CHO-K1 cells. GS-deficient derivatives of the mammalian cell, particularly of the CHO-DG44 and CHO-K1 cell are also encompassed. In one embodiment of the invention the mammalian cell is a CHO cell, preferably a CHO-DG44 cell, a CHO-K1 cell, a CHO DXB11 cell, a CHO-S cell, a CHO GS deficient cell or a derivative thereof. Suitable human cells are HEK293 or HEK293T cells. The host cells may also be murine cells such as murine myeloma cells, such as NS0 and Sp2/0 cells or the derivatives/progenies of any of such cell line.

[125] Preferred examples of mammalian cells or mammalian cell lines are CHO cells (such as DG44 and K1), NS0 cells, HEK293 cells (such as HEK293 cells and HEK293T cells) and BHK21 cells. Preferably the mammalian cells or mammalian cell lines are adapted to growth in suspension. In a preferred embodiment the mammalian cells or mammalian cell line is a CHO cell. In certain

embodiments the mammalian cell is a HEK293 cell or a CHO cell or a HEK293 cell or a CHO cell derived cell, preferably the mammalian cell is a CHO cell or a CHO derived cell.

[126] Preferably, CHO cells that allow for efficient cell line development processes are metabolically engineered, such as by GS knockout and/or DHFR knockout to facilitate selection with MSX or MTX, respectively. Commonly used CHO cells for large-scale industrial production are often engineered to improve their characteristics in the production process, or to facilitate selection of recombinant cells. Such engineering includes, but is not limited to increasing apoptosis resistance, reducing autophagy, increasing cell proliferation, altered expression of cell-cycle regulating proteins, chaperone engineering, engineering of the unfolded protein response (UPR), engineering of secretion pathways and metabolic engineering.

[127] Non-limiting examples of mammalian cells which can be used in the meaning of this invention are also summarized in Table 2. However, derivatives/progenies of those cells, other mammalian cells, including but not limited to human, mice, rat, monkey, and rodent cell lines, can also be used in the present invention, particularly for the production of biopharmaceutical proteins.

Table 2: Exemplary mammalian production cell lines

Cell line	Order Number
NS0	ECACC No. 85110503
Sp2/0-Ag14	ATCC CRL-1581
BHK21	ATCC CCL-10
BHK TK-	ECACC No. 85011423
HaK	ATCC CCL-15
2254-62.2 (BHK-21 derivative)	ATCC CRL-8544
CHO	ECACC No. 8505302
CHO wild type	ECACC 00102307
CHO-K1	ATCC CCL-61
CHOZN®	Merck SAFC GS -/- and DHFR -/-
CHO-DUKX (= CHO duk-, CHO/dhfr-, CHO-DXB11)	ATCC CRL-9096
CHO-DUKX 5A-HS-MYC	ATCC CRL-9010
CHO-DG44	Urlaub G, <i>et al.</i> , 1983. <i>Cell</i> . 33:405-412.
CHO Pro-5	ATCC CRL-1781
CHO-S	Life Technologies A1136401; CHO-S is derived from CHO as disclosed in Deaven LL and Petersen DF, 1973, <i>Chromosoma (Berl.)</i> 41: 129-144; and Lewis NE <i>et al.</i> , 2013, <i>Nature Biotechnology</i> 31: 759-765
V79	ATCC CCC-93
B14AF28-G3	ATCC CCL-14
HEK 293	ATCC CRL-1573
COS-7	ATCC CRL-1651
U266	ATCC TIB-196
HuNS1	ATCC CRL-8644
CHL	ECACC No. 87111906
CAP ¹	Wölfel J, <i>et al.</i> , 2011. <i>BMC Proc.</i> 5(Suppl 8):P133.
PER.C6®	Pau <i>et al.</i> , 2001. <i>Vaccines</i> . 19: 2716-2721.

H4-II-E	ATCC CRL-1548 ECACC No.87031301 Reuber, 1961. <i>J. Natl. Cancer Inst.</i> 26:891-899. Pitot HC, <i>et al.</i> , 1964. <i>Natl. Cancer Inst. Monogr.</i> 13:229-245.
H4-II-E-C3	ATCC CRL-1600
H4TG	ATCC CRL-1578
H4-II-E	DSM ACC3129
H4-II-Es	DSM ACC3130

¹CAP (CEVEC's Amniocyte Production) cells are an immortalized cell line based on primary human amniocytes. They were generated by transfection of these primary cells with a vector containing the functions E1 and pIX of adenovirus 5. CAP cells allow for competitive stable production of recombinant proteins with excellent biologic activity and therapeutic efficacy as a result of authentic human post-translational modification.

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[128] Cells are most preferred, when being established, adapted, and completely cultivated under serum free conditions, and optionally in media, which are free of any protein/peptide of animal origin and/or chemically defined. Commercially available media such as Ham's F12 (Sigma, Deisenhofen, Germany), RPMI-1640 (Sigma), Dulbecco's Modified Eagle's Medium (DMEM; Sigma), Minimal Essential Medium (MEM; Sigma), Iscove's Modified Dulbecco's Medium (IMDM; Sigma), CD-CHO (Invitrogen, Carlsbad, CA), serum-free CHO Medium (Sigma), and protein-free CHO Medium (Sigma) are exemplary appropriate nutrient solutions. Any of the media may be supplemented as necessary with a variety of compounds, non-limiting examples of which are recombinant hormones and/or other recombinant growth factors (such as insulin, transferrin, epidermal growth factor, insulin like growth factor), salts (such as sodium chloride, calcium, magnesium, phosphate), buffers (such as HEPES), nucleosides (such as adenosine, thymidine), glutamine, glucose or other equivalent energy sources, antibiotics and trace elements. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. For the growth and selection of genetically modified cells expressing a selectable gene a suitable selection agent is added to the culture medium.

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Use of artificial miRNAs targeting two or more hydrolases

[129] In yet another aspect, the invention relates to the use of the mammalian cell according to the invention for producing a recombinant protein of interest, preferably a therapeutic protein, more preferably a therapeutic antibody.

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[130] In yet another aspect, the invention relates to the use of **the artificial miRNA** according to the invention in a mammalian cell for preventing expression of at least two endogenous hydrolases in the mammalian cell and/or preventing hydrolase activity and/or polysorbate degrading activity associated with a recombinant protein of interest produced in the mammalian cell. Wherein associated means co-purified with the recombinant protein of interest produced in the mammalian cell and/or in a composition comprising the recombinant protein of interest produced in the mammalian cell. In a related aspect, the invention relates to the use of the artificial miRNA according to the invention for

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reducing hydrolase activity and/or PS degrading activity in a mammalian cell and/or in a composition comprising a recombinant protein of interest produced in a mammalian cell comprising said miRNA. Wherein reduced means compared to control, such as compared to a (the same) mammalian cell not comprising the miRNA of the invention, preferably a (the same) mammalian cell mock transfected (or
5 transduced) with nuclease-free water or non-targeting siRNA (NT siRNA) or a non-targeting or empty vector variant. Thus, in certain embodiments the use of the artificial miRNA according to the invention is for reducing PS degrading activity in a composition comprising a recombinant protein of interest, wherein the recombinant protein of interest is produced in a mammalian cell comprising said artificial miRNA.

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Methods for manufacturing or producing a protein of interest using artificial miRNAs targeting two or more hydrolases and for manufacturing or producing compositions comprising a protein of interest produced by these methods

[131] In yet another aspect, the invention relates to a method for manufacturing a protein of interest comprising the steps of: (a) introducing an expression vector comprising a gene of interest encoding a recombinant protein of interest into a mammalian cell; (b) introducing an artificial miRNA comprising an artificial miRNA guide strand sequence targeting two or more mammalian hydrolases acting on ester bonds into the mammalian cell, wherein the artificial miRNA is introduced before, simultaneously or after step (a) into the mammalian cell, wherein the artificial miRNA is introduced as RNA or as a
20 mammalian expression vector comprising a polynucleotide sequence encoding said artificial miRNA; (c) cultivating said mammalian cell under conditions that allow expression of the recombinant protein of interest; and (d) harvesting the recombinant protein of interest in the HCCF. The artificial miRNA prevents expression of at least two endogenous hydrolases in the mammalian cell and/or preventing hydrolase activity and/or polysorbate degrading activity associated with the recombinant protein of
25 interest produced in the mammalian cell. Wherein associated means in the HCCF or co-purified with the recombinant protein of interest produced in the mammalian cell and/or in a composition comprising the recombinant protein of interest produced in the mammalian cell.

[132] The artificial miRNA comprising an artificial miRNA guide strand sequence binds to the mRNAs (at least two mRNAs) encoding the two or more mammalian hydrolases acting on ester bonds and inhibit their expression in the mammalian cell (host cell). The two or more hydrolases are endogenous
30 hydrolases of the mammalian cell and hence the mRNAs are endogenous mRNAs expressing the two or more hydrolases acting on ester bonds. Preferably the mammalian cell is a rodent cell or a human cell, more preferably the mammalian cell is a rodent cell, such as a CHO cell. Thus, in certain embodiments, the two or more mammalian hydrolases acting on ester bonds are rodent or human
35 hydrolases, preferably the two or more mammalian hydrolases are rodent hydrolases such as hydrolases from CHO cells. In certain embodiments, the two or more mammalian hydrolases are three or more mammalian hydrolases, four or more mammalian hydrolases or five or more mammalian hydrolases.

[133] The term "introducing an artificial miRNA" refers to any means of inserting the artificial miRNA into the mammalian cell and includes transfection of RNA and transfection or transduction of a polynucleotide sequence encoding said artificial miRNA or of a mammalian expression vector comprising a polynucleotide sequence encoding said artificial miRNA. Likewise, the term "a
5 mammalian cell comprising an artificial miRNA" as used herein means a mammalian cell comprising the artificial miRNA transiently transfected as RNA as well as a mammalian cell comprising a polynucleotide either stably inserted into the genome or transiently transfected that encodes and expresses the artificial miRNA in the mammalian cell.

[134] The HCCF of step (d) has reduced hydrolase activity and/or reduced PS degrading activity, preferably the HCCF has reduced hydrolase activity and/or reduced PS degrading activity compared to HCCF comprising the recombinant protein of interest produced in a mammalian cell not comprising (or encoding) the artificial miRNA (miRNA). Methods for determining reduced hydrolase activity and/or reduced PS degrading activity are known in the art and include the methods described herein. The HCCF of step (d) may also have reduced levels of two or more mRNAs encoding two or more
15 hydrolase acting on ester bonds compared to HCCF comprising the recombinant protein of interest produced in a mammalian cell not comprising (or encoding) the artificial miRNA (miRNA). mRNA levels can be easily determined using reverse transcription polymerase chain reaction (RT-PCR), preferably using quantitative PCR (qPCR). Combined RT-PCR and qPCR (quantitative RT-PCR or real-time RT-PCR) are routinely used for analysis of gene expression.

[135] The PS degrading activity in the HCCF may be reduced by 20% or more, 30% or more, 50% or more, or 70% or more compared to a HCCF derived from a mammalian cell not comprising (or encoding) the artificial miRNA. Wherein the mammalian cell (i.e., the same mammalian cell) not comprising the artificial miRNA, such as the same mammalian cell mock transfected (or transduced), such as transfected (or transduced) with nuclease-free water or non-targeting siRNA (NT siRNA) or a
25 non-targeting or empty vector variant. The mammalian cell not comprising the artificial miRNA may also be referred to as control or control cell. The person skilled in the art will understand that the mammalian cells are cultured under the same conditions that allow expression of the recombinant protein of interest and the recombinant protein of interest is harvested using the same method. The reduced PS degrading activity may refer to reduction of PS concentration (PS20 and/or PS80) at a certain day, such as day 7 or day 14 at room temperature and may be provided as absolute or relative values, including % of control or to reduction in PS degradation rate provided in mg/ml/day and may be provided as absolute or relative values, including % of control.

[136] The method according to the invention may further comprises the following steps: (a) purifying the recombinant protein of interest; and (b) formulating the purified recombinant protein of interest into
35 a composition, preferably into a pharmaceutical composition. A pharmaceutical composition refers to a composition comprising the recombinant protein of interest and at least one pharmaceutically acceptable excipient.

[137] In certain embodiments the pharmaceutical composition comprises PS, such as PS20 or PS80. Thus, PS20 or PS80 and optionally further excipients may be added. In certain embodiments the composition comprises PS and less than about 10%, less than about 5% or less than about 2% or less than about 1% of PS is degraded when the composition is stored at about 2°C to about 8°C, preferably about 5°C, for at least six months, and/or less than about 15%, less than about 10%, less than about 5% or less than about 2 % of PS is degraded when stored at about 2°C to about 8°C, preferably about 5°C for at least 12 months. The temperature for determining storage stability as outlined above is 2°C to 8°C, preferably 4°C to 6°C, more preferably about 5°C. In one embodiment about 0.2 % (w/v) or more PS20 or PS80, more preferably about 0.4 % (w/v) or more PS20 or PS80, more preferably about 0.4 % (w/v) PS20 is added.

[138] A composition comprising the purified recombinant protein of interest of step (e) or the composition of step (f) has reduced hydrolase activity and/or reduced PS degrading activity, preferably the composition comprising the purified recombinant protein of interest of step (e) or the composition of step (f) has reduced hydrolase activity and/or reduced PS degrading activity compared to a composition comprising the purified recombinant protein of interest produced in a mammalian cell not comprising or encoding the artificial miRNA. The hydrolase activity or reduced PS degrading activity may be due to contaminating HCPs co-purified with the protein of interest, such as hydrolases acting on esterase bonds. The composition comprising the purified recombinant protein of interest may also have reduced immunogenic activity. The artificial miRNA according to the invention prevents expression of at least two endogenous hydrolases in the mammalian cell and/or prevents hydrolase activity and/or polysorbate degrading activity associated with a recombinant protein of interest produced in the mammalian cell. Wherein associated means co-purified with the recombinant protein of interest produced in the mammalian cell and/or in a composition comprising the recombinant protein of interest produced in the mammalian cell. The composition in this context may be any composition comprising the recombinant protein of interest (such as an antibody), particularly HCCF, a composition comprising a purified recombinant protein of interest of step (d) or a composition of step (e), particularly a pharmaceutical composition comprising pharmaceutically acceptable excipients.

[139] Some lipases, such as PLBL2, were reported to be immunogenic in patients. Thus, reducing the expression of two or more hydrolases acting on ester bonds (such as lipases and/or carboxylesterases) in a mammalian host cell may also result in reduced immunogenic activity in a purified recombinant protein of interest produced in the mammalian host cell, or a composition thereof. Thus, the artificial miRNAs and the methods of the invention may further improve patient safety.

[140] In certain embodiments, the expression vector comprising a gene of interest encoding a recombinant protein of interest is introduced by transfection or transduction, preferably by transfection. Preferably the gene of interest is stably introduced by transfection or transduction. More preferably, the expression vector comprising a gene of interest encoding a recombinant protein of interest is stably transfected into the mammalian cell.

[141] The artificial miRNA is introduced as RNA or as mammalian expression vector comprising a polynucleotide sequence encoding the artificial miRNA. Wherein the artificial miRNA is introduced as RNA by transient transfection or as expression vector comprising a polynucleotide sequence encoding said artificial miRNA by transfection or transduction, preferably by stable transfection or transduction, more preferably by stable transfection. In certain embodiments, the artificial miRNA is introduced into the mammalian cell by stably transfecting or transducing an expression vector comprising a polynucleotide sequence encoding the artificial miRNA, optionally wherein the expression vector comprising a polynucleotide sequence encoding the artificial miRNA further comprises the gene of interest encoding the recombinant protein of interest. Stable integration, i.e. transfection or transduction includes semi-targeted, targeted and random integration. The expression vector and/or the artificial miRNA introduced according to the method of the invention is preferably the mammalian expression vector and/or the artificial miRNA according to the invention or the expression vector comprises a polynucleotide sequence encoding the artificial miRNA according to the invention. The disclosure with regard to the artificial miRNA, the mammalian expression vector and/or the mammalian cell according to the invention similarly applies to the method of the invention, where applicable.

[142] More specifically, in certain embodiments, the two or more mammalian hydrolases are three or more mammalian hydrolases, four or more mammalian hydrolases, five or more mammalian hydrolases, six or more mammalian hydrolases, seven or more mammalian hydrolases or eight or more mammalian hydrolases.

[143] The artificial miRNA guide strand sequence has imperfect complementarity to the target sequence of at least one of the at least two mRNAs encoding the two or more mammalian hydrolases, preferably more than one of the at least two mRNAs encoding the two or more mammalian hydrolases. Thus, the artificial miRNA guide strand sequence has perfect complementarity to the target sequence of one mRNA of the at least two mRNAs encoding the two or more mammalian hydrolases and imperfect complementarity to the target sequence(s) of the remaining mRNAs of the at least two mRNAs encoding the two or more (targeted) mammalian hydrolases. In certain embodiments the artificial miRNA guide strand has imperfect complementarity to the target sequence(s) of all of the at least two mRNAs encoding the two or more (targeted) mammalian hydrolases, or the artificial miRNA guide strand has imperfect complementarity to the target sequence(s) of all or all but one of the at least two mRNAs encoding the two or more (targeted) mammalian hydrolases. Preferably, the artificial microRNA (miRNA) according to the invention comprises an artificial miRNA guide strand sequence targeting three, four, five, six, seven, eight, nine or more mammalian hydrolases acting on ester bonds. In certain embodiments the artificial miRNA guide strand has imperfect complementarity to the target sequence(s) of all or all but one of the mRNAs encoding the three, four, five, six, seven, eight, nine or more (targeted) mammalian hydrolases, preferably CHO hydrolases. The person skilled in the art would understand that the artificial miRNA guide strand sequence may have perfect complementarity to the target sequence of one mRNA of the mRNAs encoding the three, four, five, six, seven, eight, nine or more mammalian hydrolases, preferably CHO hydrolases.

[144] The target sequences may be located anywhere in the mRNA sequence of the at least two mRNAs encoding the two or more mammalian hydrolases, preferably the 3' untranslated regions or the coding region of the at least two mRNAs encoding the two or more mammalian hydrolases, more preferably in the coding region of the at least two mRNA encoding the two or more mammalian hydrolases, even more preferably within micro-conserved regions in the coding regions of the at least two mRNAs encoding the two or more mammalian hydrolases. Typically, the target sequences of all of the at least two mRNAs encoding the two or more mammalian hydrolases are either in the 3' untranslated regions or in the coding regions thereof, preferably in the coding region of the at least two mRNA encoding the two or more mammalian hydrolases, even more preferably within micro-conserved regions in the coding regions of the at least two mRNAs encoding the two or more mammalian hydrolases.

[145] In certain embodiments, the target sequences are located in a conserved region of the amino acid sequence of the two or more mammalian hydrolases. In certain preferred embodiments, the target sequences are located in a conserved region of the at least two mRNAs encoding the two or more mammalian hydrolases, preferably in a conserved region of the coding region of the at least two mRNAs encoding the two or more mammalian hydrolases. The term "conserved region" as used herein refers to identical or similar nucleotide sequences in the at least two mRNAs encoding the two or more mammalian hydrolases. Wherein similar sequences means more than 55% percent of pairwise identity. Pairwise identity refers to the percentage of pairwise residues that are identical in the alignment, including gap versus non-gap residues, but excluding gap versus gap residues. Micro-conserved regions are very small stretches of identical or similar sequences of at least 21 nucleotides, preferably with at least 55% sequence identity.

[146] The artificial miRNA guide strand sequence of the artificial miRNA used in the method of the invention has typically 19 to 25 nucleotides, preferably 20 to 24 nucleotides, more preferably 21 to 23 nucleotides, even more preferably 22 to 23. Binding to the target sequence(s) of the mRNA requires binding of the 5' seed region and optionally a 3' complementary region rather than the entire guide strand sequence. Thus, preferably the artificial miRNA guide strand sequence comprises at least a 5' seed region and a 3' complementary region. In certain embodiments, the artificial miRNA guide strand sequence has not perfect complementarity to at least one of the at least two mRNAs encoding the two or more mammalian hydrolases and the miRNA guide strand has a 5' seed region and a 3' complementary region that undergoes base pairing with said target sequence(s), preferably a 5' seed region starting at nucleotide 2 (nt 2) from the 5' end of the artificial miRNA guide strand sequence. Overall, the artificial miRNA guide strand sequence may have at least 55%, preferably at least 60%, more preferably at least 65% sequence complementarity with the target sequence(s) of the at least two mRNAs encoding the two or more mammalian hydrolases. In certain embodiments, the miRNA guide strand sequence has at least 13, preferably 14, more preferably 15 Watson-Crick base pairings (consecutive and non-consecutive) with the target sequence(s) of the at least two mRNAs encoding the two or more mammalian hydrolases. Thus, an artificial miRNA guide strand sequence of 22 to 23

nucleotides comprises 8-10, preferably 8-9 G or C that are complementary to the target sequence(s), i.e., undergo base pairing with the target sequence(s). In certain preferred embodiments, the first nucleotide at the 5' end of the artificial miRNA guide strand sequence (i.e., the nucleotide 5' of the 5' seed region) comprises a nucleotide (comprises a nucleobase) selected from the group consisting of adenine (A) and uracil (U), preferably the nucleotide is U.

[147] The two or more mammalian hydrolases acting on ester bonds may be any mammalian hydrolases, particularly two or more mammalian lipases or carboxylesterases and/or two or more PS degrading mammalian hydrolases.

[148] The inventors identified two types of artificial miRNA guide strand sequences that are effective in reducing expression and/or activity of hydrolases. A first sequence type comprising a strong 5' seed region, wherein strong means high GC-content, a high degree of complementarity ($\geq 62.5\%$ sequence complementarity, preferably $\geq 75\%$ sequence complementarity or no more than 3 mismatches in 8 nucleotides, preferably no more than 2 mismatches in 8 nucleotides) and a weak or normal 3' complementary region that can compensate for mismatches in the seed region, if present. A second sequence comprising a weaker 5' seed region either due to a lower GC-content or a shorter complementary region, particularly such as having consecutive mismatches at the end of the region and a strong 3' complementary region that compensates for the weaker binding and/or mismatches in the 5' seed region.

[149] In certain embodiments of the method according to the invention, the miRNA guide strand sequence has imperfect complementarity to the target sequence(s) of at least one of the at least two mRNAs encoding the two or more mammalian hydrolases and wherein said miRNA guide strand sequence comprises a 5' seed region starting at nucleotide 2 (nt 2) and a 3' complementary region that undergoes base pairing with said target sequence(s), wherein the 5' seed region comprises (i) a sequence of 8 nucleotides (nt 2-9) complementary to the target sequence(s) and having 0, 1, 2 or 3 mismatches with the target sequence(s), and (ii) a GC-content of 55-70% wherein all or all but one or all but two G or C are complementary to the target sequence(s); and wherein the 3' complementary region comprises (i) a sequence of the last 6 nucleotides (i.e., nt -6 to nt-1) complementary to the target sequence(s) and having 1, 2 or 3 mismatches with the target sequence(s), and (ii) a GC-content of $\leq 50\%$ in the last 6 nucleotides, wherein 1 or 2 of the complementary nucleotides are G or C. Examples of suitable artificial miRNA guide strand sequences are a nucleotide sequence of SEQ ID NO: 2 (LP 6mer), SEQ ID NO: 4 (LH 9mer) or SEQ ID NO: 5 (LH Non-canonical), preferably artificial miRNA guide strand sequences used in the method according to the invention are a nucleotide sequence of SEQ ID NO: 2 (LP 6mer) or SEQ ID NO: 5 (LH Non-canonical).

[150] In certain alternative embodiments of the method according to the invention, the miRNA guide strand sequence has imperfect complementarity to the target sequence(s) of at least one of the at least two mRNAs encoding the two or more mammalian hydrolases and wherein said miRNA guide strand sequence comprises a 5' seed region starting at nucleotide 2 (nt 2) and a 3' complementary region that undergoes base pairing with said target sequence(s), wherein the 5' seed region comprises

- (i) a sequence of 8 nucleotides (nt 2-9), wherein at least the first 4 consecutive nucleotides (at least nt 2-5) are complementary to the target sequence(s), wherein the sequence of 8 nucleotides comprises at least one mismatch with the target sequence(s), and (ii) a GC-content of 55-75%, wherein at least 4 of the consecutive nucleotides are complementary to the target sequence(s) are G or C; and wherein the 3' complementary region comprises (i) a sequence of the last 6 nucleotides complementary to the target sequence(s) and having 0 or 1 mismatches with the target sequence(s), and (ii) a GC-content of 30-50% in the last 6 nucleotides, preferably wherein at least 2 of the complementary nucleotides are G or C. Examples of suitable artificial miRNA guide strand sequences are a nucleotide sequence of SEQ ID NO: 1 (LP 8mer) or SEQ ID NO: 2 (LP 6mer).
- 5 [151] The artificial miRNA guide strand sequences according to the method of the invention are *de novo* designed, which means not naturally occurring (or identified) in mammalian cells or organisms. Thus, the sequence is generated by rational design based on sequence alignment of target sequences on mRNA and/or protein level, preferably on mRNA level. The aim of the present invention is to provide the proof-of-concept that an artificial miRNA comprising an artificial miRNA guide strand sequence
- 10 can be used for a cell line engineering approach for simultaneous targeting of multiple hydrolases acting on ester bonds, such as multiple lipases, in a mammalian host cell, such as CHO cells.
- [152] The artificial miRNA according to the method of the invention may be a pri-miRNA, a pre-miRNA, a double stranded mature miRNA (comprising the passenger strand and the guide strand) or a single stranded miRNA guide strand. In certain embodiments, the artificial miRNA is a single or
- 15 double stranded miRNA mimic (miR-Mimic). miRNA mimics are chemically synthesized double stranded RNAs which mimic mature miRNAs after transfection into cells and may be provided as double stranded miRNA mimic or as single stranded miRNA, which requires an additional annealing step with the passenger strand prior to transfection. Customized miRNA mimics are available with proprietary chemical modifications in order to improve their performance as well as stability and to
- 20 prevent binding activity of the passenger strand from various suppliers on demand, such as from Horizon, Qiagen or Thermo Fisher.
- [153] The two or more mammalian hydrolases acting on ester bonds targeted by the artificial miRNA according to the invention are endogenous hydrolases of the mammalian cell, preferably the mammalian cell is a human or a rodent cell, more preferably a rodent cell, such as a CHO cell.
- 25 [154] The protein of interest, such as an antibody is produced in mammalian cells (e.g., CHO cells) in cell culture according to the method of the invention. Following expression, the recombinant protein is harvested and further purified. The recombinant protein of interest, such as an antibody, may be recovered from the culture medium as a secreted protein in the HCCF or from a cell lysate (i.e., the fluid containing the content of a cell lysed by any means, including without being limited thereto
- 30 enzymatic, chemical, osmotic, mechanical and/or physical disruption of the cell membrane and optionally cell wall) and purified using techniques described herein. According to the invention the method comprises harvesting the recombinant protein of interest in the HCCF. Preferably the recombinant protein of interest (e.g., an antibody) is recovered (i.e. purified) from the HCCF following
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cell separation, such as by filtration and/or centrifugation. Thus, in certain embodiments the harvest includes centrifugation and/or filtration to produce a HCCF. Methods for purification are well known in the art and include chromatographic methods, such as affinity chromatography, ion exchange chromatography and/or hydrophobic interaction chromatography. Moreover, filtration such as
5 membrane and/or depth filtration may be applied.

[155] Also encompassed by the present invention is a method for manufacturing a cell line stably expressing the artificial miRNA of the invention comprising the steps of (a) stably introducing a mammalian expression vector comprising a polynucleotide sequence encoding an artificial miRNA comprising an artificial miRNA guide strand sequence targeting two or more mammalian hydrolases
10 on ester bonds into the mammalian cell, and (b) selecting a mammalian cell clone stably expressing said artificial miRNA, and optionally using said cell for cell line generation comprising (c) introducing an expression vector comprising a gene of interest encoding a recombinant protein of interest into the mammalian cell stably expressing said artificial miRNA.

[156] In yet another aspect the invention relates to a composition comprising a recombinant protein of interest, wherein the recombinant protein of interest is obtainable by the method according to the
15 invention. Preferably the composition further comprises PS (PS20 or PS80), preferably at a concentration of 0.1 g/L or more. In certain embodiments, the composition comprises PS and less than about 10% of PS is degraded when the composition is stored at about 2°C to about 8°C, preferably about 5°C, for at least six months.

[157] The composition comprising the recombinant protein of interest according to the invention has reduced hydrolase activity and/or reduced PS degrading activity. Preferably the composition comprising the recombinant protein of interest has reduced hydrolase activity and/or reduced PS degrading activity compared to a composition comprising the recombinant protein of interest produced in a mammalian cell not comprising (or encoding) the artificial miRNA.

[158] In view of the above, it will be appreciated that the invention also encompasses the following items:

[159] Item 1 provides an artificial microRNA (miRNA) comprising an artificial miRNA guide strand sequence targeting two or more mammalian hydrolases acting on ester bonds.

[160] Item 2 further specifies the artificial miRNA of item 1, wherein the two or more hydrolases are
30 endogenous hydrolases of a mammalian cell, preferably of a human or a rodent cell, more preferably a rodent cell, even more preferably of a CHO cell.

[161] Item 3 further specifies the artificial miRNA of item 1 or 2, wherein the artificial miRNA guide strand sequence has perfect or imperfect complementarity to target sequences of at least two mRNAs encoding the two or more mammalian hydrolases.

[162] Item 4 further specifies the artificial miRNA of item 3, wherein the artificial miRNA guide strand
35 sequence has imperfect complementarity to the target sequence(s) of at least one of the at least two mRNAs encoding the two or more mammalian hydrolases, preferably more than one of the at least

two mRNAs encoding the two or more mammalian hydrolases. Preferably, the artificial miRNA guide strand sequence has imperfect complementarity to all or all but one target sequences of the at least two mRNAs encoding the two or more mammalian hydrolases.

[163] Item 5 further specifies the artificial miRNA of item 3 or 4, wherein the target sequences are located in the 3'UTR or in the coding regions of the at least two mRNAs encoding the two or more mammalian hydrolases, preferably in the coding regions of the at least two mRNAs encoding the two or more mammalian hydrolases, more preferably within micro-conserved regions in the coding regions of the at least two mRNAs encoding the two or more mammalian hydrolases.

[164] Item 6 further specifies the artificial miRNA of item 5, wherein the target sequences are located in a conserved region of the at least two mRNAs encoding the two or more mammalian hydrolases, preferably in a conserved region of the coding region of the at least two mRNAs encoding the two or more mammalian hydrolases.

[165] Item 7 further specifies the artificial miRNA of any one of items 1 to 6, wherein the artificial miRNA targeting two or more mammalian hydrolases reduces activity of said two or more hydrolases in a mammalian cell, preferably a human or a rodent cell, more preferably a rodent cell, even more preferably a CHO cell.

[166] Item 8 further specifies the artificial miRNA of any one of the preceding items, wherein the artificial miRNA guide strand sequence has 19 to 25 nucleotides, preferably 20 to 24 nucleotides, more preferably 21 to 23 nucleotides.

[167] Item 9 further specifies the artificial miRNA of any one of the preceding items, wherein the artificial miRNA guide strand sequence hybridizes to the target sequences of the at least two mRNAs encoding the two or more mammalian hydrolases independently without the formation of a bulge loop and/or with formation of a maximum of two bulge loops, preferably without bulge loop formation.

[168] Item 10 further specifies the artificial miRNA of any one of items 1 to 9, wherein the artificial miRNA guide strand sequence has at least 55% sequence complementarity with the target sequence(s) of the at least two mRNAs encoding the two or more mammalian hydrolases; and/or wherein the miRNA guide strand sequence has at least 13 Watson-Crick base pairings with the target sequence(s) of the at least two mRNAs encoding the two or more mammalian hydrolases.

[169] Item 11 further specifies the artificial miRNA of any one of items 1 to 10, wherein one of the two or more mammalian hydrolases is lipoprotein lipase (LPL), and preferably wherein one of the two or more mammalian hydrolases is LPL and the target sequence(s) in the mRNA encoding LPL is within nucleotides 560-590, 700-745, 930-960 or 1330-1360 of SEQ ID NOs: 6 or 7, preferably within nucleotides 560-590, 705-745, or 930-960 of SEQ ID NOs: 6 or 7.

[170] Item 12 further specifies the artificial miRNA of any one of items 1 to 11 wherein the two or more mammalian hydrolases are selected from the group consisting of lipoprotein lipase (LPL), phospholipase B-like 2 (PLBL2), phospholipase A1 (Pla1a), inactive pancreatic lipase-related protein 1 (PNLIPRP1), inactive pancreatic lipase-related protein 2 (PNLIPRP2), pancreatic triacylglycerol

lipase precursor (PNLIP), lipase member I precursor (LipI), lipase member H precursor (LipH), lipase member G precursor (LipG), lipase member C precursor (LipC) and isoforms thereof.

[171] Item 13 further specifies the artificial miRNA of any one of items 1 to 12, wherein the artificial miRNA guide strand sequence has the nucleotide sequence of SEQ ID NO: 1 (LP 8mer), SEQ ID NO: 2 (LP 6mer), SEQ ID NO: 3 (LH 8mer), SEQ ID NO: 4 (LH 9mer) or SEQ ID NO: 5 (LH Non-canonical).

[172] Item 14 further specifies the artificial miRNA of any one of the preceding items, wherein the artificial miRNA guide strand sequence has imperfect complementarity to the target sequence(s) of at least one of the at least two mRNAs encoding the two or more mammalian hydrolases and wherein said miRNA guide strand sequence comprises a 5' seed region starting at nucleotide 2 (nt 2) and a 3' complementary region that undergo base pairing with said target sequence(s), wherein the 5' seed region comprises (i) a sequence of 8 nucleotides (nt 2-9) complementary to the target sequence(s) and having 0, 1, 2 or 3 mismatches within the target sequence(s), preferably 0, 1 or 2 mismatches, more preferably 0 or 1 mismatches, and (ii) a GC-content of 55-70% wherein all or all but one or all but two G or C are complementary to the target sequence(s); and wherein the 3' complementary region comprises (i) a sequence of the last 6 nucleotides complementary to the target sequence(s) and having 1, 2 or 3 mismatches with the target sequence(s), preferably wherein the mismatches are not consecutive mismatches, and (ii) a GC-content of $\leq 50\%$ in the last 6 nucleotides, wherein 1 or 2 of the complementary nucleotides are G or C.

[173] Item 15 further specifies the artificial miRNA of item 14, wherein (a) the 5' seed region comprises no mismatch within nucleotides 2-3, preferably within nucleotides 2-4, more preferably within nucleotides 2-5, even more preferably within nucleotides 2-7, and/or (b) the 5' seed sequence comprises at least 2 times 3-4 consecutive base pairings with the target sequence(s), and/or (c) wherein the mismatches in the 5' seed region are not consecutive mismatches.

[174] Item 16 further specifies the artificial miRNA of item 14 or 15, wherein the artificial miRNA guide strand sequence has the nucleotide sequence of SEQ ID NO: 2 (LP 6mer), SEQ ID NO: 4 (LH 9mer) or SEQ ID NO: 5 (LH Non-canonical).

[175] Item 17 further specifies the artificial miRNA of any one of items 1 to 13, wherein the artificial miRNA guide strand sequence has imperfect complementarity to the target sequence(s) of at least one of the at least two mRNAs encoding the two or more mammalian hydrolases and wherein said miRNA guide strand sequence comprises a 5' seed region starting at nucleotide 2 (nt 2) and a 3' complementary region that undergoes base pairing with said target sequence(s), wherein the 5' seed region comprises (i) a sequence of 8 nucleotides (nt 2-9), wherein at least the first 4 consecutive nucleotides (at least nt 2-5), preferably at least the first 6 consecutive nucleotides (at least nt 2-7) are complementary to the target sequence(s), wherein the sequence of 8 nucleotides comprises at least one mismatch with the target sequence(s), and (ii) a GC-content of 55-75%, wherein at least 4 of the consecutive nucleotides are complementary to the target sequence(s) are G or C; and wherein the 3' complementary region comprises (i) a sequence of the last 6 nucleotides complementary to the target sequence(s) and having 0 or 1 mismatches with the target sequence(s), preferably wherein the last

nucleotide is complementary to the target sequence(s) and/or the penultimate nucleotide is a mismatch, and (ii) a GC-content of 30-50% in the last 6 nucleotides, preferably wherein at least 2 of the complementary nucleotides are G or C.

5 [176] Item 18 further specifies the artificial miRNA of items 17, wherein the artificial miRNA guide strand sequence has the nucleotide sequence of SEQ ID NO: 1 (LP 8mer) or SEQ ID NO: 2 (LP 6mer).

[177] Item 19 further specifies the artificial miRNA of any one of items 1 to 18 wherein the first nucleotide at the 5' end of the artificial miRNA guide strand sequence comprises a nucleotide selected from the group consisting of A and U, preferably the nucleotide is U.

10 [178] Item 20 further specifies the artificial miRNA of any one of items 1 to 19, wherein the artificial miRNA is a pri-miRNA, a pre-miRNA, a double stranded mature miRNA (comprising the passenger strand and the guide strand) or a single stranded miRNA guide strand.

[179] Item 21 further specifies the artificial miRNA of any one of items 1 to 20, wherein the artificial miRNA guide strand sequence is *de novo* designed.

15 [180] Item 22 further specifies the artificial miRNA of any one of items 1 to 21, wherein the artificial miRNA is a single or double stranded miRNA mimic.

[181] Item 23 provides a mammalian expression vector comprising a polynucleotide sequence encoding the artificial miRNA of any of items 1 to 21.

[182] Item 24 further specifies the mammalian expression vector of item 23 to further comprise at least one gene of interest encoding a recombinant protein of interest.

20 [183] Item 25 further specifies the mammalian expression vector of item 23 or 24, wherein the mammalian expression vector comprises a polynucleotide sequence encoding two or more of the artificial miRNAs of any of items 1 to 21 wherein the artificial miRNAs are identical or different.

25 [184] Item 26 provides a mammalian cell, preferably a CHO cell, comprising the artificial miRNAs of any one of items 1 to 22, or a polynucleotide encoding the artificial miRNA of any one of items 1 to 22, or a mammalian expression vector of any one of items 23 to 25.

[185] Item 27 further specifies the mammalian cell of item 26, wherein the mammalian cell is a human cell or a rodent cell, preferably a CHO cell.

30 [186] Item 28 further specifies the mammalian cell of item 26 or 27, wherein the mammalian cell further expresses a recombinant protein of interest, preferably a therapeutic protein, more preferably a therapeutic antibody.

[187] Item 29 further specifies the mammalian cell of item 28, wherein the recombinant protein of interest is stably expressed.

35 [188] Item 30 further specifies the mammalian cell of any one of items 26 to 29, wherein (a) the mammalian cell has reduced expression of two or more mammalian hydrolases compared to a mammalian cell not comprising the artificial miRNA of any one of items 1 to 22; (b) the mammalian cell and/or a recombinant protein of interest produced and isolated from said mammalian cell has a

reduced hydrolase activity compared to a mammalian cell and/or a recombinant protein of interest produced and isolated from said mammalian cell not comprising the artificial miRNA of any one of items 1 to 22; (c) the mammalian cell and/or a recombinant protein of interest produced and isolated from said mammalian cell has reduced PS degrading activity of two or more mammalian hydrolases compared to a mammalian cell and/or a recombinant protein of interest produced and isolated from said mammalian cell not comprising the artificial miRNA of any one of items 1 to 22; and/or (d) the mammalian cell and/or a recombinant protein of interest produced and isolated from said mammalian cell has reduced PS degrading activity compared to a mammalian cell and/or a recombinant protein of interest produced and isolated from said mammalian cell not comprising the artificial miRNA of any one of items 1 to 22; and/or (e) the artificial miRNA prevents expression of at least two endogenous hydrolases in the mammalian cell and/or prevents hydrolase activity and/or polysorbate degrading activity associated with a recombinant protein of interest produced in the mammalian cell.

[189] Item 31 further specifies the mammalian cell of any one of items 26 to 30, wherein the mammalian cell is transfected or transduced with the mammalian expression vector of any one of items 23 or 25, preferably stably transfected or transduced, including semi-targeted, targeted and random integration.

[190] Item 32 further specifies the mammalian cell of any one of items 26 to 30, wherein the mammalian cell is transfected with the artificial miRNA of any one of items 1 to 22 or a mammalian expression vector comprising a polynucleotide sequence encoding the artificial miRNA of any of items 1 to 21.

[191] Item 33 provides a use of the mammalian cell of any one of items 26 to 32 for producing a recombinant protein of interest.

[192] Item 34 provides a use of the artificial miRNA of any one of items 1 to 22 in a mammalian cell for preventing expression of at least two endogenous hydrolases in the mammalian cell and/or preventing hydrolase activity and/or polysorbate degrading activity associated with a recombinant protein of interest produced in the mammalian cell.

[193] Item 35 provides a use of the artificial miRNA of any one of items 1 to 22, for reducing PS degrading activity in a composition comprising a recombinant protein of interest, wherein the recombinant protein of interest is produced in a mammalian cell comprising said artificial miRNA.

[194] Item 36 provides a method for manufacturing a protein of interest comprising the steps of: (a) introducing an expression vector comprising a gene of interest encoding a recombinant protein of interest into a mammalian cell; (b) introducing an artificial miRNA comprising an artificial miRNA guide strand sequence targeting two or more mammalian hydrolases acting on ester bonds into the mammalian cell, wherein the artificial miRNA is introduced before, simultaneously or after step (a) into the mammalian cell, wherein the artificial miRNA is introduced as RNA or as a mammalian expression vector comprising a polynucleotide sequence encoding said artificial miRNA; (c) cultivating said

mammalian cell under conditions that allow expression of the recombinant protein of interest; and (d) harvesting the recombinant protein of interest in the HCCF.

[195] Item 37 further specifies the method of item 36, wherein the HCCF has reduced hydrolase activity and/or reduced PS degrading activity, preferably the HCCF has reduced hydrolase activity and/or reduced PS degrading activity compared to HCCF comprising the recombinant protein of interest produced in a mammalian cell not comprising or encoding the artificial miRNA (miRNA).

[196] Item 38 further specifies the method of item 36 or 37, wherein the PS degrading activity in the HCCF is reduced by 30% or more, 50% or more, or 70% or more compared to a HCCF derived from a mammalian cell not comprising or encoding the artificial miRNA.

[197] Item 39 further specifies the method of any one of items 36 to 38, wherein the method further comprises the following steps: (e) purifying the recombinant protein of interest; and optionally (f) formulating the purified recombinant protein of interest into a composition, preferably into a pharmaceutical composition.

[198] Item 40 further specifies the method of item 39, wherein the composition comprises PS (PS20 or PS80) and wherein less than about 10% of PS is degraded when the composition is stored at about 2°C to about 8°C for at least six months.

[199] Item 41 further specifies the method of item 39 or 40, wherein a composition comprising the purified recombinant protein of interest according to step (e) or the composition of step (f) has reduced hydrolase activity and/or reduced PS degrading activity, preferably the composition comprising the purified recombinant protein of interest has reduced hydrolase activity and/or reduced PS degrading activity compared to a composition comprising the purified recombinant protein of interest produced in a mammalian cell not comprising or encoding the artificial miRNA.

[200] Item 42 further specifies the method of any one of items 36 to 41, wherein the expression vector comprising a gene of interest encoding a recombinant protein of interest is introduced by transfection or transduction.

[201] Item 43 further specifies the method of any one of items 36 to 42, wherein the artificial miRNA is introduced as RNA by transient transfection or as expression vector comprising a polynucleotide sequence encoding said artificial miRNA by transfection or transduction.

[202] Item 44 further specifies the method of item 43, wherein the artificial miRNA is introduced into the mammalian cell by stably transfecting or transducing an expression vector comprising a polynucleotide sequence encoding the artificial miRNA, optionally wherein the expression vector comprising a polynucleotide sequence encoding the artificial miRNA further comprises the gene of interest encoding the recombinant protein of interest.

[203] Item 45 further specifies the method of any one of items 36 to 44, wherein the expression vector comprising a polynucleotide sequence encoding the artificial miRNA is the mammalian expression vector of any one of items 23 to 25.

[204] Item 46 further specifies the method of any one of items 36 to 45, wherein the artificial miRNA is the artificial miRNA of any one of items 1 to 22.

[205] Item 47 further specifies the method of any one of items 36 to 46, wherein the two or more mammalian hydrolases are endogenous hydrolases of the mammalian cell, preferably the mammalian cell is a human or a rodent cell, more preferably a CHO cell.

[206] Item 48 provides a composition comprising a recombinant protein of interest, wherein the recombinant protein of interest is obtainable by the method of any one of items 35 to 46 and PS, preferably at a concentration of 0.1 g/L or more.

[207] Item 49 further specifies the composition of item 48, wherein the composition comprises PS (PS20 or PS80) and wherein less than about 10% of PS is degraded when the composition is stored at about 2°C to about 8°C for at least six months.

[208] Item 50 further specifies the composition of item 48 or 49, wherein the composition comprising the recombinant protein of interest has reduced hydrolase activity and/or reduced PS degrading activity, preferably the composition comprising the recombinant protein of interest has reduced hydrolase activity and/or reduced PS degrading activity compared to a composition comprising the recombinant protein of interest produced in a mammalian cell without introducing or comprising the artificial miRNA.

EXAMPLES

Material and Methods

Cell lines and cultivation

[209] A CHO-K1 cell line with a biallelic GS knockout (CHO-K1 GS) was used for the performed experiments, as well as CHO-K1 GS cell expressing different recombinant products.

[210] CHO cells were adapted for growth in chemically defined and animal component-free media in suspension culture. For standard cultivation, cells were cultured in 60 ml culture media using 250 ml shaking flasks (Corning, Corning, USA) and incubated at 37°C, 5% CO₂ and 125 rpm (50 mm orbit) in a Multitron incubator (Infors, Bottmingen, Switzerland). CHO cells were passaged in a 2/2/3-day rhythm using respective seeding densities (0.4 x 10⁶ / 0.2 x 10⁶ cells/ml (2d/3d)).

Measurement of viable cell concentration and viability

[211] For the measurement of viable cell concentration (VCC) and viability of cells cultured in shaking flasks, the Innovatis CedexTM (Roche Diagnostics, Mannheim, Germany) was used. Per measurement, 1 ml cell suspension was pipetted in the designated sample vessel and fully automated measurement was initiated. The image-based technology makes use of the Trypan Blue (Life Technologies, Carlsbad, CA USA) exclusion method for cell counting. Dead cells with a damaged cell

membrane will be stained by Trypan Blue, whereas live cells with an intact cell membrane exclude the dye (Strober 2001).

[212] For the measurement of VCC and viability of cells cultured in 96-half-deep well (DW) plates (EnzyScreen BV, Heemstede, the Netherlands) or in spin tubes (TPP, Trasadingen, Switzerland), a NovoCyte flow cytometer (Acea, San Diego, USA) was used. Per measurement, 25 μ l cell suspension was mixed with 75 μ l propidium iodide solution (PI; c = 6,67 μ g/ml) (Sigma Aldrich, Steinheim, Germany) for viability staining. Subsequently, 1×10^4 events were analyzed via plotting of forward scatter (488 nm laser, 488/10 nm filter) and side scatter (488 nm laser, 488/10 nm filter). Furthermore, the PI content of cells was measured by an additional laser-filter combination (488 nm laser, 660/20 nm filter). PI intercalates into double stranded DNA of dead cells and thus function as indicator for cell apoptosis (Lecoeur 2002). The VCC was used to calculate the integral of viable cell concentration (IVC) after batch cultivation of amiRNA transfected CHO production cell lines.

Post-transcriptional gene regulation via double stranded RNA

[213] Double stranded RNA (dsRNA) molecules such as amiRNA mimics and siRNAs, applied as positive and negative controls, were used for post-transcriptional gene regulation in CHO cell lines. Hence, dsRNAs were transfected using the NeonTM Transfection System with the associated 10 μ l NeonTM Kit (ThermoFisher, Waltham, USA). One day prior to transfection, CHO cells were passaged with a seeding density of 0.6×10^6 cells/ml. Per transfection, 1.5×10^5 cells were centrifuged at 200 x g for 7 min and washed with 1 x Phosphate buffered saline (PBS) (Life Technologies, Waltham, USA). Cells were resuspended in resuspension buffer R (10 μ l NeonTM Kit) and mixed with 9 pmol dsRNA to obtain a 10 μ l transfection mix in total. Furthermore, 3 ml electroporation buffer E (10 μ l NeonTM Kit) was pipetted into the electroporation tube of the transfection station. The electroporation buffer was replaced prior to the electroporation of a new dsRNA and the electroporation tube was exchanged after 10 transfections. Cells were electroporated with following transfection settings: 1700 V, 20 ms, 1 pulse. Cells were transferred in 290 μ l pre-warmed media in a square 96-half-DW microplate and incubated for 2 h in a static incubator (Thermo Fisher, Waltham, USA) at 37°C and 5% CO₂. Following static incubation, the microplate was incubated in a shaking incubator at 37°C, 5% CO₂, 90% humidity and 225 rpm (50 mm orbit) until harvest on day 3 (72 h). For transfections in 1 ml scale, 3-times the number of cells and dsRNA were applied per transfection. Cultivation was performed in 50 ml spin tubes placed vertically in a shaking incubator at 37°C, 5% CO₂, 90% humidity and 180 rpm (50 mm orbit). dsRNAs used in the present work are listed in Table 3.

Table 3: Transfected miRNAs/siRNAs with respective sequence, target(s) and supplier.

miRNA/siRNA	Target(s)	Sequence (5' – 3')	Supplier
LP 8mer	LPL PLBL2	UCGGCCACUGUGCCAUACAGAGA (SEQ ID NO: 1)	ThermoFisher
LP 6mer	LPL PLBL2	CCAGCAGCAUGAGCUCCAAGGCU (SEQ ID NO: 2)	ThermoFisher
LH 8mer	LPL homologs	GCCUCCAUUGGGAUAAAUGUCA (SEQ ID NO: 3)	ThermoFisher
LH 9mer	LPL homologs	UCCAGUCCACCACAAUGACGUU (SEQ ID NO: 4)	ThermoFisher
LH non-canonical	LPL homologs	AGCUCCAAGGCUAUACCCCAAGA (SEQ ID NO: 5)	ThermoFisher
LPL siRNA	LPL	Mix of 2 sequences Mm_Lpl_5: UUAACUAACAAGAUAGAGCUG (SEQ ID NO: 22) Mm_Lpl_6: UUUUUUGUGGAAACCUCGGG (SEQ ID NO: 23)	Qiagen
AllStars Negative control siRNA	-	Unspecified	Qiagen
Tox5 miRNA (positive control)	-	Not specified	ThermoFisher

Cell lysis via freeze and thaw

- [214] For cell lysis, 1.0×10^6 cells were pelleted at $300 \times g$ for 7 min and resuspended in 11.2 μ l PBS supplemented with cCompleteTM (Sigma Aldrich, Steinheim, Germany). Cell suspension was frozen for 2 min in liquid nitrogen, thawed in a water bath at 36°C and thoroughly vortexed. This freeze and thaw cycle was repeated 3 times. For harvest, the lysate was centrifuged in a benchtop centrifuge for 10 min at 4°C and full speed. The lysate was transferred in a new reaction tube and stored at -70°C. Total protein content of cell lysate samples was quantified via Bradford protein assay.

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LPL sandwich enzyme-linked immunosorbent assay

- [215] Lipase enzyme-linked immunosorbent assay (ELISA) for the quantification of LPL was performed using a commercially available ELISA kit (Cloud-Clone Corp). A standard dilution series in

the range of 1.56 ng/ml - 100 ng/ml was prepared by diluting the 100 ng/ml LPL stock solution with standard diluent. Anti-LPL antibody pre-coated wells were filled with 100 µl standard or undiluted HCCF, sealed with a plate sealer and incubated for 1 h at 37°C. After incubation, the liquid was removed from each well and 100 µl detection reagent A (biotin-conjugated secondary antibody) was added per well. The sealed plate was incubated for 1 h at 37°C. Wells were washed 3 x with 350 µL wash solution and 100 µl detection reagent B (Avidin conjugated Horseradish Peroxidase) was added per sample. Incubation of the sealed plate was performed at 37°C for 30 min following 5 washing steps as described above. Subsequently, 90 µl tetramethylbenzidine (TMB) substrate solution was added to each well, the plate was sealed and incubated for 20 min at 37°C. The enzyme-substrate reaction caused a color shift in LPL positive wells and was stopped by the addition of 50 µl sulphuric acid stop solution. The color change was measured spectrophotometrically at a wavelength of 450 nm using an Infinite® M 200 microplate reader and the LPL concentration was calculated using the standard curve.

Pla1a (lipase 1a) sandwich enzyme-linked immunosorbent assay

[216] As an example for multi-lipase downregulation by amiRNAs, quantification of Pla1a (Lipase 1a) was performed by ELISA using a commercially available kit (Elabscience). A standard dilution series in the range of 3.13 ng/ml - 200 ng/ml was prepared by diluting the 200 ng/ml LPL stock solution with standard diluent. Anti-Pla1a antibody pre-coated wells were filled with 100 µl standard or diluted cell lysate, sealed with a plate sealer and incubated for 90 min at 37°C. After incubation, the liquid was removed from each well and 100 µl detection reagent A (biotin-conjugated secondary antibody) was added per well. The sealed plate was incubated for 1 h at 37°C. Wells were washed 3 x with 350 µL wash solution and 100 µl detection reagent B (Avidin conjugated Horseradish Peroxidase) was added per sample. Incubation of the sealed plate was performed at 37°C for 30 min following 5 washing steps as described above. Subsequently, 90 µl tetramethylbenzidine (TMB) substrate solution was added to each well, the plate was sealed and incubated for 20 min at 37°C. The enzyme-substrate reaction caused a color shift in Pla1a positive wells and was stopped by the addition of 50 µl sulphuric acid stop solution. The color change was measured spectrophotometrically at a wavelength of 450 nm using an Infinite® M 200 microplate reader and the Pla1a concentration was calculated using the standard curve.

PLBL2 quantification via bio-layer interferometry

[217] PLBL2 measurement was conducted using the Octet HTX system. A standard dilution series in the range of 0.2 ng/ml – 200 ng/ml was prepared, diluting purified CHO PLBL2 standard (confidential) with assay buffer (10 mM HEPES, 250 mM NaCl, 0.3% BSA, 0.05% CHAPS, pH 7.4). Fluorescein-anti-PLBL2 antibody (confidential) and biotin-anti-PLBL2 antibody (confidential) were diluted separately in assay buffer in order to obtain a working concentration of 5 µg/ml. Per sample, 80 µl of the PLBL2-antibody solutions were pipetted in a black 384 well plate (Greiner Bio-One, Stonehouse,

United Kingdom), the sample plate. Samples were diluted in assay buffer and 80 µl of diluted sample along with 80 µl assay buffer were each added in a well of the sample plate. Another black 384 well plate was used as detection plate, containing assay buffer, HRP labeled anti-FITC antibody in assay buffer (c = 2 µg/ml) (Sigma Aldrich, Steinheim, Germany), peroxide buffer (ThermoFisher, Waltham, USA) and Metal Enhanced diaminobenzidine peroxidase (DAB) (ThermoFisher, Waltham, USA) diluted 1:10 in peroxide buffer. Analogous to the sample plate, 80 µl reagent was pipetted in a well for each measured sample. Finally, the sample plate and detection plate were placed in the Octet System and fully automatic measurement was initiated. The layer thickness positively correlates with the PLBL2 concentration in the sample.

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Fluorescence micelle assay

[218] The fluorescence micelle assay (FMA) was employed to determine PS. PS20 and PS80 stock solutions (c = 100 mg/ml) (Croda International plc, Snaith, United Kingdom) were sterile filtered using a 0.2 µm PVDF filter (Merck Millipore, Burlington, USA). Next, PS was diluted in respective medium, where PS20 was diluted 1:245 and PS80 1:490 to obtain the PS-medium working solution. HCCF was mixed 1:50 with the working solution to obtain PS20 spiked samples (cPS20 = 0.4 mg/ml) and PS80 spiked samples (cPS80 = 0.2 mg/ml). 200 µl was aliquoted in 5 tubes in a cooling rack. One tube, representing sample t0, was immediately frozen at -70°C. Remaining tubes were incubated at room temperature in the dark and frozen at -70°C after 1, 3, 7, 14 days. Prior to measurement, standard curves ranging from 0.1 to 0.6 mg/ml for PS20 and from 0.05 to 0.3 mg/ml for PS80 were generated. For PS measurement, 10 µl sample was mixed with 240 µl FMA reagent (150 mM NaCl, 50 mM Tris, 0.2% Acetonitrile, 5 µM NPN, 0.0015% Brij-35, pH 8) and subsequently incubated for 1 min at 35°C. During incubation, fluorescent dye N-phenyl-1-naphtylamine (NPN) intercalates in PS formed micelles. Fluorescent signal, positively correlating with PS concentration, was measured at 420 nm following excitation of the dye with a 350 nm laser using an Infinite® 200 PRO microplate reader (Tecan, Männedorf, Switzerland).

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Determination of monoclonal antibody concentration

[219] Product concentration of mAb in HCCF was measured using an Octet HTX system (Forte Bio Inc, Menlo Park, USA). HCCF of IgG1 and IgG4 producing CHO cell lines was diluted in respective cell culture media and mAb concentration was determined using Protein A sensors.

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Example 1: Artificial miRNA design: General design parameters

[220] Artificial miRNAs (amiRNAs) were designed based on state-of-the-art knowledge on endogenous miRNA binding mechanisms. In recent years, several critical key features were identified, which are commonly used for the prediction of miRNA target binding. Following analysis and assessment of these features, a list of general design parameters applicable for the design of amiRNAs was created. Furthermore, ranges for amiRNA characteristics were defined and ranked accordingly. A summary of amiRNA design criteria and ranking of the parameters is given in Table 4.

Table 4: Summary of amiRNA design criteria and parameter ranking

Parameter	Description	Ranking		
		high	middle	low
Seed length [bp]	Target binding in 5'-end of amiRNA	> 6	5	< 5
3'-end compensation	Target binding in 3'-end of amiRNA	> 55%	55%-40%	< 40%
MFE [kcal/mol]	Minimal free hybridization energy between amiRNA and target site	< -28	-20 to -28	> -20
Total number of matching nucleotides [bp]	Total number of perfectly matching nucleotides	> 15	8-12	< 8
Bulges	Number of bulges caused by missing opposite nucleotides	0	1-3	> 3
A at mRNA Pos1	mRNA sequences exhibiting an adenosine opposite to position 1 in amiRNA	> 60%	20-60%	< 20%
Perfect seed matches	Number of mRNA sequences exhibiting a perfect seed match with amiRNA	> 4	2-4	< 2
BLAST hits	BLAST hits of amiRNA with CHO host cell transcriptome	< 10	10-50	> 50
Length [bp]	amiRNA sequence length	21-23	20 or 24	< 20 > 24
GC content	Percentage of guanine and cytosines in amiRNA	40-60%	35-39% 61-65%	< 35% > 65%
Restriction sites	Restriction sites interfering in plasmid cloning for stable amiRNA expression (Msc I, BamH I, Bgl II, Sal I, Xho I, Dra I)	0	-	> 1

Design of artificial miRNAs against lipases

[221] Based on previous observations, LPL (Lipase 1) and PLBL2 (Lipase 2) were hypothesized to be involved in PS degradation in DS/DP and thus particle formation in formulated biotherapeutic drugs. Therefore, genomic and transcriptomic sequence information were extracted from database, which contains CHO-specific omics data. Moreover, homologs were identified based on amino acid sequence homology with LPL (BlastP performed in Genedata Selector with default settings) and sequence information of these proteins were extracted. All further bioinformatic analysis including amino acid as well as transcript sequence alignments, mRNA translation and amiRNA design were carried out using the Geneious Prime® Software. Additionally, minimal free hybridization energy (MFE) between designed amiRNAs and their respective target mRNA sequences was evaluated based on the publicly available RNAhybrid tool (Rehmsmeier et al., RNA (New York, N.Y.), 2004, 10(10): 1507-1517).

[222] Two groups of multilipase targets were defined: (a) LPL and PLBL2 (LP) as well as (b) LPL and its eight homologs (LH) (Lipase 1a – Lipase 1h). Based on transcript alignment, seed regions shared between the lipases were identified and used to design amiRNAs applying features commonly used for prediction of miRNA target binding. Thereby, all amiRNAs were designed to be 100% complementary against the LPL mRNA sequence. No further preference for amiRNA design against a certain lipase was applied. In order to identify suitable amiRNAs capable of multilipase knockdown (KD).

Table 5: Binding parameters for amiRNA directed against LPL and PLBL2 and LPL and its homologs.

Parameter	amiRNAs against Lipase 1 and Lipase 2 (LP)		amiRNAs against Lipase 1 and its homologs (LH)		
	LP 6mer	LP 8mer	LH 8mer	LH 9mer	LH non-canonical
Seed length [bp]	6	8	8	9	5
3'-end compensation [%]	69	47	-	-	-
MFE [kcal/mol]	-32.0	-28.8	-27.0	-28.0	-26.0
Total number of matching nucleotides [bp]	17	16	9	9	10
Bulges	0	2	0	0	0
A at mRNA Pos1 [%]	0	100	55	66	22
Perfect seed matches	-	-	3	3	5
Length [bp]	23	23	22	22	23
GC content [%]	61	57	45	50	52

Artificial miRNA design to target multiple hydrolases/lipases using single amiRNAs

[223] Although PS degrading properties of PLBL2 were not confirmed, this enzyme was still included in the multilipase KD approach, because it is a frequently reported contaminant in antibody products and has the potential to cause HCP-mediated immunogenic reactions in patients. Therefore, an alignment with amino acid sequences of LPL and PLBL2 was performed (Figure 1B).

[224] The alignment of LPL and its eight homologs unraveled stretches of highly conserved amino acid sequences between the proteins (Figure 1A). These conserved segments are separated by amino acid sequences without or with low homology between proteins. Especially in the regions of amino acids 230 to 270 of the consensus numbering shown in Figure 1A, highly conserved segments were found. With respect to the LPL with PLBL2 alignment on amino acid sequence level, less sequence homology was identified in comparison to the alignment of LPL and its relatives (Figure 1 B). In Figure 1, only amino acid regions were highlighted showing high homology between enzymes.

[225] For the *de novo* design of amiRNAs, alignments on transcript level were further performed (Figure 2). Manual *de novo* amiRNA design was applied in highly conserved transcript regions based on the design criteria presented in Table 4.

[226] Transcript alignments of LPL against its homologs and LPL against PLBL2 are depicted in Figure 2 A and B, respectively. Thereby, conserved nucleotides are depicted as blocks of various height depending on homology in Figure 2 (identity line). The transcript alignment of LPL and its homologs showed multiple sites with increased homology separated by stretches which were conserved between target proteins. In contrast to transcript alignment of LPL and its relatives, homology in the alignment of LPL with PLBL2 was found to be more randomly distributed across the entire transcript sequences. In Figure 2, only transcript regions were highlighted showing high homology between enzymes. Highly conserved regions on transcript level, which were identified by the alignment approach, served as target seed regions for the design of the amiRNAs. Three amiRNAs were designed against LPL and its eight homologs (LH): LH 9mer, LH Non-canonical and LH 8mer. Furthermore, two amiRNAs were designed binding LPL and PLBL2 (LP): LP 6mer and LP 8mer.

[227] A more detailed scheme of amiRNA design against multiple lipases simultaneously is given in Figure 3 (LPL and its homologs) and Figure 4 (LPL and PLBL2). Therefore, theoretical amiRNA bindings sites in the different lipases were highlighted. For multi-lipase amiRNA design, preference was given to LPL as the main target due to its high potential to degrade PS. Hence, amiRNAs were designed to be near 100% complementary against the LPL target enzyme (LH 9mer has only 95% complementarity). However, gaps in amiRNA binding towards LPL were allowed. Moreover, amiRNA design was applied in a way that it was tried to achieve as much complementarity as possible with regards to the remaining lipase targets. Thus, nucleotides in Figure 3 and 4 that are conserved between targets were depicted as black boxes, whereas non-identical nucleotides were depicted as dots and gaps between the mRNA target and the *de novo* designed amiRNA were marked as dashes

(Figure 4B). Detailed design and binding parameters of all amiRNAs are summarize in Table 5. In brief, 22-23 bp long amiRNAs with different seed length from 5-9 nucleotides and a GC content of 45-61% were designed. Furthermore, 3' end compensation for LP amiRNAs were kept above 47%. MFE values, which describe the binding energy between amiRNA and mRNA, were generally below -26 kcal/mol. The total number of matching nucleotides were 16-17 bp regarding LP amiRNAs and 9-10 bp for LH amiRNAs. Except for LP 8mer, none of the theoretical amiRNA binding sites exhibited bulges (marked as dashes in Figure 4). Both targeted mRNA sequences in LP 8mer binding exhibited an A at position 1, whereas in LP 6mer binding sites no A were found at position 1 of the mRNA. For LH amiRNAs, 22-66% of the targeted mRNAs exhibited an A at mRNA position 1. Of totally eight homologs the LH amiRNAs exhibited a perfect seed match for three to five homologs.

[228] With respect to amiRNA design against LPL and its homologs, all amiRNA binding sites were generated with preference towards an optimal seed binding across targets (Figure 3). Thus, more amiRNA complementary nucleotides (black boxes) were obtained at the 5' end of the amiRNA sequences. The main differences between designed LH amiRNAs were the target regions and the respective seed length. LH 9mer was designed to bind with a nine-nucleotide seed length (Figure 3 A). Therefore, 100% seed complementarity was achieved against the transcripts of Lipase 1a, Lipase 1b and Lipase 1d. Mismatch amiRNA:mRNA binding in the 9mer seed region was obtained for all remaining lipase targets. For LH Non-canonical a 11 nucleotide long seed region was defined (Figure 3B). However, the LH Non-canonical amiRNA did not show seed binding greater six consecutive complementary nucleotides for all lipases except for Lipase 1g. Similar to LH 9mer, the LH 8mer amiRNA was created with at least eight complementary nucleotides in the seed region (Figure 3C). Thereby, 100% seed complementarity was established against the transcripts of Lipase 1a, Lipase 1d and Lipase 1f. With regard to the remaining targets, target binding in the seed region was achieved with 1 – 2 mismatches between amiRNA and the respective mRNA. In addition, amiRNA binding capacity towards its targets was increased by 3' end compensation in the amiRNA sequence incorporating complementary nucleotides shared between mRNA and the *de novo* miRNA molecule. Compensation at amiRNA 3' end was performed for all LH RNA molecules.

[229] Bindings sites of amiRNAs targeting LPL and PLBL2 are shown in Figure 4. Similar as for LH amiRNAs, preference was given to LPL for LP amiRNA design. Two LP amiRNAs were designed binding in different regions of the lipases. Furthermore, variations in seed length and 3' end target binding compensation were applied during the *de novo* design of LP amiRNAs. Besides six complementary nucleotides in the seed region, LP 6mer was designed to bind PLBL2 via six consecutive nucleotides in the 3' end of the amiRNA sequence (Figure 4 A). In comparison, LP 8mer was created with eight complementary nucleotides in the seed region. However, compensation at the amiRNA 3' end was not as strong as for the LP 6mer (Figure 4 B). Additionally, the LP 8mer amiRNA was designed with two gaps (middle and 3' end of amiRNA sequence) for PLBL2 target binding (marked with dashes (-) in the alignment), resulting in a one nucleotide bulge at those sites.

[230] In summary, three miRNAs were designed against LPL and its homologs and two *de novo* RNA molecules were generated targeting LPL and PLBL2 simultaneously. Designed amiRNAs were further processed for evaluation of their functionality to target multiple lipases in parallel.

5 **Example 2: Effect of artificial miRNA following transient transfection**

Transient transfection of artificial miRNA against lipases into CHO host cells

[231] As described above, lipases were identified in several DPs as critical CHO host cell impurities responsible for PS degradation. Additionally, some difficult-to-remove lipases were found to be immunogenic in patients. To reduce difficult-to-remove lipases in an early step of biopharmaceutical manufacturing, amiRNAs were designed with the objective to simultaneously knock down multiple lipases. In a long-term perspective, stable co-expression of amiRNAs in CHO production cell lines is targeted for multilipase gene regulation. However, the process of stable cell line generation is very time-consuming and tedious. Therefore, functionality of *de novo* designed amiRNAs against multiple lipases was elucidated via transient transfection of amiRNA mimics. Thus, amiRNAs against LPL and its homologs and LPL and PLBL2 were transiently transfected into CHO host cells to evaluate their post-transcriptional gene regulation properties. Subsequently, CHO cell pellets and cell culture supernatant were harvested to assess the effect of the amiRNA transfection on cell growth, viability, lipase KD and PS degradation.

20 Analysis of cell growth and viability

[232] *De novo* designed amiRNAs against LH and LP were ordered as mirVana mimics from ThermoFisher and transiently transfected into the CHO-K1 GS host cell line. After transfection, cells were cultivated in 96-half-DW microplates in batch culture. Three days post transfection, cell growth and viability of amiRNA transfected CHO host cells were determined via flow cytometry (Figure 5). Furthermore, HCCF and cell pellet samples were harvested for lipase concentration analysis and determination of PS degradation.

[233] Figure 5 presents the impact of amiRNA transfections on cell growth and viability in CHO host cells. In average, VCCs of $6.5 - 8.0 \times 10^6$ cells/ml were achieved for most transfections except for LH 8mer and Tox5 which led to average VCCs of 5.0×10^6 cells/ml and 0.1×10^6 cells/ml, respectively. Tox5 represents a functional positive control miRNA inducing cell death after transfection. Regarding viability, values $> 97\%$ were measured for all amiRNA and control transfections except for Tox5. Cells transfected with Tox5 resulted in an average viability of 16% as expected. In summary, a minor impact of multilipase amiRNAs on cell growth was observed especially with respect to LH 8mer. However, reduced VCC values of multilipase amiRNA transfected samples were not significant.

35 [234] In a follow-up experiment (data not shown) using more duplicates ($n = 4$) significant differences in viability were observed for LH 8mer and LH non-canonical. However, since the absolute differences were within a range of 1% these changes are neglectable.

Determination of lipase concentrations

[235] In order to assess the LPL KD properties of the amiRNAs, LPL (Lipase 1) concentrations in HCCF samples of transfected CHO host cells were determined via a sandwich ELISA (Figure 6). All amiRNAs were designed with 100% sequence complementarity against LPL.

5 [236] All multilipase targeting amiRNA transfections led to average normalized LPL concentrations < 3.0 fg/cell except for LP 8mer, which resulted in an average normalized LPL concentration of 4.4 fg/cell. These concentrations translated into downregulations of >50% in comparison to the NT siRNA control. The positive control with LPL siRNA led to a significant LPL KD with an average normalized LPL concentration of 0.9 fg/cell relative to 5.6 fg/cell measured for the NT siRNA control. For the Mock
10 control, an average normalized LPL concentration of 5.5 fg/cell was measured, which is in the range of the NT siRNA sample. With most *de novo* designed multilipase amiRNAs a clear trend in LPL post-transcriptional downregulation was detectable.

[237] In a follow-up experiment (data not shown) using more duplicates (n = 4) all miRNAs elicited a significant reduction of >50% in LPL abundance, with an average normalized LPL concentrations <
15 3.0 fg/cell, except for LP 8mer which reduced LPL expression by only 33% in comparison to the NT control (with < 4.0 fg/cell).

[238] To investigate whether the artificial amiRNA mimics were capable of downregulating not only LPL but also PLBL2 (Lipase 2) expression, HCCF samples of amiRNA transfected CHO host cells were subjected to a PLBL2 bio-layer interferometry (BLI) assay for the quantification of PLBL2
20 concentrations (Figure 7). LP amiRNAs were specifically designed to target the PLBL2 mRNA in addition to the LPL mRNA while LH amiRNAs were built to target LPL and its eight homologs. Nevertheless, all amiRNA transfection samples were analyzed in the PLBL2 BLI assay.

[239] LP 8mer, LH 8mer and LH Non-canonical amiRNA did not show a significant reduction in PLBL2 expression, whereby normalized PLBL2 concentrations ranging from 23.5 to 25.9 fg/cell were
25 determined. These measured concentrations were in line with values generated from the NT siRNA reference control, which had normalized PLBL2 concentrations of in average 26.0 fg/cell. Relative to the NT siRNA control, significantly reduced PLBL2 concentrations were determined in HCCF samples of LH 9mer and LP 6mer transfected CHO host cells with 14.6 and 18.2 fg/cell of normalized PLBL2 concentrations, respectively. Hence, transfection of LH 9mer and LP 6mer amiRNAs resulted in 44%
30 and 30% reduced normalized PLBL2 concentration relative to the NT siRNA control.

[240] All amiRNAs were designed with the objective of multilipase targeting. Especially LH amiRNAs were supposed to target LPL and various homologs thereof. In order to investigate amiRNA KD properties with respect to further lipases, Pla1a (Lipase 1a) was selected as a representative homolog of LPL. Consequently, a sandwich ELISA was used to quantify Pla1a. Due to the fact that Pla1a was
35 found to be intracellularly localized into the cells, cell lysis was performed prior Pla1a quantification. Therefore, CHO host cells were harvested 3 days after transfection with the amiRNAs and Pla1a was quantified in the cell lysates. In addition, total protein content of cell lysates was measured enabling

normalization of Pla1a concentrations based on total protein content (Figure 8). Although LP amiRNAs were not designed to target Pla1a, samples transfected with LP amiRNAs were included in the analysis to evaluate potential off-target KD properties of LP amiRNAs with respect to Pla1a.

[241] As shown in Figure 8, normalized Pla1a concentrations were reduced in cell lysate samples of LH amiRNA transfected CHO host cell lines (7.3 - 8.0 ng/mg) in comparison to normalized Pla1a concentration measured in the NT and Mock control (9.4 and 9.7 ng/mg), respectively. Thereby, a significant lower Pla1a concentration of in average 7.4 ng/mg was determined for cell lysate samples from LH Non-canonical transfections relative to the NT control. Notable, LP amiRNA mimics resulted in decreased Pla1a concentration with 8.1 ng/mg for LP 6mer transfected samples and 5.9 ng/mg for LP 8mer amiRNA transfections. In average, 14 - 37% less Pla1a protein concentrations were detected in cell lysate samples transfected with lipase targeting amiRNAs.

[242] A strong downregulation of Pla1a was observed after transfection of the LP 8mer although this amiRNA was not designed to target LPL and its homologs (Figure 8). A similar observation was made for LH 9mer, which was specifically designed to bind transcript sequences of LPL and homologs thereof. Strikingly, transient application of LH 9mer in CHO host cells caused a strong downregulation of PLBL2 in addition to post-transcriptional regulation of LPL and Pla1a (Figure 7). To identify the root cause for these unexpected observations, an off-target analysis was carried out to analyze potential binding sites of LP 8mer in the Pla1a transcript sequence and of LH 9mer amiRNA in the PLBL2 transcript sequence, respectively (Figure 9). Therefore, less stringent settings were applied for amiRNA:mRNA alignments.

[243] For the LP 8mer amiRNA, a theoretical binding site in the transcript sequence of Pla1a was identified with eight mismatches between mRNA and amiRNA. At the 5' end of the amiRNA, partial sequence complementarity was observed with four nucleotides. In addition, three matching nucleotides were identified in the middle of the mRNA:amiRNA alignment separated by a mismatch. Furthermore, a stretch of six complementary nucleotides was found in the 3' end of the amiRNA. For the LH 9mer amiRNA, a theoretical binding site in the transcript sequence of PLBL2 was identified with only seven mismatches between mRNA and amiRNA. Three stretches of four complementary nucleotides were observed at the 5' end and towards the middle of the amiRNA. Moreover, three single complementary nucleotides were found in the 3' end of the amiRNA. In summary, potential off-target binding sites of LP 8mer and LH 6mer amiRNAs in lipase transcript sequences, which were not intended to be targeted by one of the respective amiRNAs, were identified as the root cause for un-specific lipase downregulation. The results were further confirmed in cells stably expressing mAb1 (IgG1) or mAb2 (IgG4), which further showed no effect on volumetric productivity and specific productivity, except for LH 9mer which showed increased specific productivity. A summary of the results is provided in Table 6.

Table 6: Summary of the effects of the *de novo* designed amiRNAs

	LP 6mer	LP 8mer	LH 8mer	LH 9mer	LH Non-canonical
Lipase Knockdown					
Lipase 1	Yes	No to moderate	Yes	Yes	Yes
Lipase 1a	No to moderate	Yes	No to moderate	Moderate	Moderate
Lipase 2	Yes	No	No	Yes	No
Cell Growth					
Viability	Unaffected	Unaffected	Unaffected	Decreased	Unaffected
Viable Cell Concentration	Unaffected	Unaffected	Increased	Decreased	Unaffected
Cell Productivity					
Volumetric Productivity	Unaffected	Unaffected	Unaffected	Unaffected	Unaffected
Specific Productivity	Unaffected	Unaffected	Unaffected	Increased	Unaffected
Reduction of PS degradation on Cell Culture Level					
PS20	Yes	Moderate	Yes	Yes	Yes
PS80	Yes	Moderate	Yes	Yes	Yes

Determination of polysorbate degradation

- [244] To assess the potential of *de novo* designed amiRNAs on multi-lipase downregulation and thus the impact on PS degradation, HCCF samples of amiRNA transfected CHO host cells were subjected to FMA analysis enabling the quantification of PS degradation over time. Therefore, HCCF samples were diluted 1:50 in PS20 (c = 0.4 mg/ml) and in PS80 (c = 0.2 mg/ml) spiked medium and incubated at room temperature (about 22°C) in the dark for up to 14 days. Subsequently, PS spiked samples were stored 0, 1, 3, 7 and 14 days after addition of the surfactants. PS concentrations were measured after incubation periods via a micelle intercalating fluorescence molecule, which can be bound in PS micelles. PS degradation in amiRNA transfected CHO host cell samples were evaluated in comparison to the NT siRNA control. Furthermore, a Mock transfection control with buffer and a LPL targeting siRNA control were included in the FMA study. Resulting PS concentrations at different time points are depicted in Figure 10.
- [245] The negative controls transfected with the NT siRNA or nuclease-free water (Mock) showed strong PS20 and PS80 degradation over 14 days of incubation. After 14 days of incubation post PS spiking, approx. 0.13 mg/ml PS20 and 0.005 mg/ml PS80 were measured in both samples, respectively. From day 3 onwards, significantly less PS20 and PS80 degradation was detected in amiRNA transfected CHO samples in comparison to the NT siRNA control. In spiked HCCF samples of transfections with LP 6mer, LH 8mer, LH 9mer and LH Non-canonical amiRNAs comparable PS degradation was observed for PS20 and PS80, respectively. Thereby, PS20 concentrations between 0.31 and 0.34 mg/ml and PS80 concentrations ranging from 0.062 and 0.081 mg/ml were determined in these samples incubated for 14 days. Thus, 21-30% of PS20 was degraded over time in these samples, whereas 68% of PS20 was degraded in NT samples relative to the initial PS concentration.

In PS80 spiked HCCF samples of LP 6mer, LH 8mer, LH 9mer and LH Non-canonical amiRNA transfections, 63-71% of the initial PS80 concentration was reduced over time versus a PS80 reduction of 98% in NT siRNA control samples over an incubation period of 14 days. Furthermore, lower PS concentrations were determined in spiked HCCF samples of LP 8mer transfections in comparison to samples of the remaining amiRNAs, which indicated a stronger PS degradation. Therefore, 0.21 mg/ml PS20 and 0.009 mg/ml PS80 were determined in spiked LP 8mer HCCF samples after 14 days of incubation, respectively. However, the measured PS concentrations were significantly higher at the end of the FMA incubation as PS concentrations detected in the NT siRNA control. HCCF samples from CHO host cells transfected with LPL siRNA showed the least PS20 and PS80 degradation compared to all remaining samples. For PS20, a concentration of 0.39 mg/ml was measured on day 14 of the FMA incubation time point, hence, only 7% of the initial PS20 concentration was degraded. A PS80 concentration of 0.168 mg/ml was determined in these sample, which indicates a percentage reduction of initial PS concentration by 21%.

[246] Additionally, average degradation rates of PS20 and PS80 in HCCF samples were calculated (Figure 11). Therefore, the difference between initial (day 0) and remaining PS concentration (day 14) was divided by 14 days to obtain the daily PS degradation rate in mg/ml*day.

[247] In general, all HCCF samples, except for the Mock control, showed a significant reduction of the PS degradation rate relative to the NT siRNA control. Thereby, 0.020 mg/ml PS20 and 0.015 mg/ml PS80 was degraded per day in NT siRNA samples. No change in both PS degradation rates relative to the Scramble control was determined in Mock HCCF samples. LP 6mer and all LH transfections resulted in similar degradation rates in spiked HCCF samples of 0.007 – 0.009 mg/ml*day observed for PS20 and 0.009 – 0.011 mg/ml*day observed for PS80. Moreover, higher PS degradation rates were determined in spiked HCCF samples of LP 8mer transfections in comparison to samples of the remaining amiRNAs. Therefore, 0.016 mg/ml PS20 and 0.015 mg/ml PS80 were degraded per day in spiked LP 8mer HCCF samples. The lowest PS degradation rate was observed in HCCF samples of the LPL siRNA control. For PS20, a degradation rate of 0.002 mg/ml*day was calculated and for PS80, a degradation rate of 0.003 mg/ml*day was calculated. Generally, PS80 degradation rates were higher than PS20 degradation rates in HCCF samples in which a LPL KD > 50% was observed (Figure 11).

Example 3: Functionality of amiRNAs in IgG producing CHO cell lines

[248] Considering a stable overexpression of amiRNAs in CHO production cell lines, it is critical to ensure that the designed amiRNAs do not compromise cellular growth, viability or productivity of protein therapeutics. Therefore, representative IgG1 and IgG4 CHO-K1 GS^{-/-} production cell lines were transiently transfected with the anti-lipase amiRNAs and the impact of *de novo* amiRNAs on cell growth, viability and productivity was analyzed. Following transfection, IgG1 and IgG4 production cells were cultivated in a batch process for 5 days. Viability and viable cell concentration (VCC) was

measured from day 3 to day 5 and the integral of viable cell concentration (IVC) was calculated on day 5. Growth performance data are summarized in Figure 12.

[249] Introduction of LH 9mer amiRNA led to a significant decrease in viability to 80.3 and 87.3% on day 5 in IgG1 and IgG4 production cells, respectively. All other amiRNA transfected cells including the NT siRNA transfected cells showed constantly high viabilities of > 96% in both IgG producing CHO cell lines. With regards to IVC, LH 9mer amiRNA transfections resulted in a significant decrease in IVC in both IgG producing CHO cell lines while cell growth was increased for cells transfected with LH 8mer amiRNA. In both IgG expressing CHO cell lines, cell growth was not significantly affected by LP 6mer and LP 8mer amiRNAs compared to the NT control. LH Non-canonical transfections in IgG1 production cells did not impact cell growth, whereas the IVC of LH Non-canonical transfected IgG4 producing CHO cells was significantly increased compared to the control.

[250] Finally, the effect of amiRNA transfection on volumetric and cell-specific IgG productivity was assessed (Fig. 13). In IgG1 expressing cell lines the previously observed growth inhibitory effect of LH 9mer was in line with a significantly increased specific productivity. However, volumetric titers were significantly decreased. Transfection of LH Non-canonical resulted in a significantly improved final product concentration in IgG1 producing CHO cell lines. All other transfected amiRNAs did not significantly change volumetric or specific IgG1 productivity. No impact on protein production was observed for all transfected amiRNAs in the IgG4 producing CHO cell lines.

Discussion

[251] To this day, the concept of multiple target knock-down (KD) applying rationally designed amiRNAs is largely unexplored. The few studies that have addressed this field, focused on seed binding in the 3'-UTR and left the remaining conserved regions in 5'-UTR and coding sequence unnoticed (Arroyo et al., 2014; Guire et al., 2010) or altered single nucleotides in native miRNAs to reduce off-target effects (Klingler et al., 2023).

[252] Applying this technology for the first time in CHO cells, we were able to identify multi-lipase targeting amiRNAs reducing lipase expression levels of LPL and PLBL2 or PLA1A simultaneously. Since the conceptualization of this work, PLBL2 has been proven not to be responsible for PS degradation despite its high abundance in the drug product (S. Zhang et al., 2020, Journal of Pharmaceutical Sciences, 109(11), 3300-3307). Nevertheless, as a difficult-to-remove lipase that has been shown to induce immune responses in a mAb drug product, it is a valid model protein that should be removed (S. K. Fischer et al., 2017, The AAPS Journal, 19(1), 254-263). Besides strong evidence for the multiple lipase KD on protein level, which rendered proof on transcript level redundant, an FMA verified the KD on a functional level. PS spiked into the supernatant of amiRNA transfected CHO cells degraded the PS substrate significantly slower than in control samples. Furthermore, a stronger PS degradation was observed for PS80 pointing towards a preferential PS20 degradation by LPL/PLA1A.

[253] By careful evaluation of different amiRNAs it might even be possible to identify additional pro-productive effects upon amiRNA transfection (see LH Non-canonical in IgG1 producing cells), which has already been harnessed in the past for CHO cell line engineering using endogenous miRNAs (Barron et al., 2011, Journal of Biotechnology 151(2), 204-211; S. Fischer et al., 2017, Biotechnology and Bioengineering, 114(7), 1495-1510; S. Fischer et al., 2015, Journal of Biotechnology, 212, 32-43; 5 Loh et al., 2014, Biotechnology Journal, 9(9), 1140-1151; Loh et al., 2017, Biotechnology Journal, 12(4), <https://doi.org/10.1002/biot.201600488>).

[254] The present Examples provide for the first time a proof-of-concept that multi-target regulating amiRANs can be rationally designed and generated and represent a powerful and flexible tool for CHO 10 cell line engineering. It was successfully applied to mitigate PS degradation in CHO cell culture supernatant by reducing the expression of critical HCPs. Considering that all experiments were conducted transiently, it is expected that stable expression of amiRNAs has even more pronounced effects on PS degradation, since transient transfection may not effectively reduce expression of HCPs with a long half-life and amiRNAs will be degraded relatively quickly over time. A careful amiRNA 15 design will enable to target even larger groups of mammalian hydrolases acting on ester bonds exhibiting "micro-conserved" regions in their mRNA sequence in the future. Conceivable other applications in order to improve product quality of a therapeutic protein of interest would be the reduction of immunogenic HCPs, such as PLBL2.

CLAIMS

1. An artificial microRNA (miRNA) comprising an artificial miRNA guide strand sequence
5 targeting two or more mammalian hydrolases acting on ester bonds.
2. The artificial miRNA of claim 1, wherein
- (a) the two or more hydrolases are endogenous hydrolases of a mammalian cell, preferably
of a human or a rodent cell, more preferably of a CHO cell; and/or
- 10 (b) wherein the artificial miRNA guide strand sequence has perfect or imperfect
complementarity to target sequences of at least two mRNAs encoding the two or more
mammalian hydrolases; and/or
- (c) wherein the target sequences are located in the 3' untranslated regions or in the coding
regions of the at least two mRNAs encoding the two or more mammalian hydrolases.
- 15 3. The artificial miRNA of claim 1 or 2, wherein the artificial miRNA targeting two or more
mammalian hydrolases reduces activity of said two or more hydrolases in a mammalian cell,
preferably a rodent or human cell, more preferably a CHO cell.
- 20 4. The artificial miRNA of any one of claims 1 to 3, wherein
- (a) the artificial miRNA guide strand sequence has 19 to 25 nucleotides, preferably 21 to 23
nucleotides; and/or
- (b) the artificial miRNA guide strand sequence hybridizes to the target sequences of the at
least two mRNAs encoding the two or more mammalian hydrolases independently without
25 the formation of a bulge loop and/or with formation of a maximum of two bulge loops;
and/or
- (c) the artificial miRNA guide strand sequence has at least 55% sequence complementarity
with the target sequence(s) of the at least two mRNAs encoding the two or more
mammalian hydrolases; and/or wherein the miRNA guide strand sequence has at least
30 13 Watson-Crick base pairings with the target sequence(s) of the at least two mRNAs
encoding the two or more mammalian hydrolases.
5. The artificial miRNA of any one of claims 1 to 4, wherein
- (a) one of the two or more mammalian hydrolases is lipoprotein lipase (LPL); and/or
- 35 (b) one of the two or more mammalian hydrolases is LPL and the target sequence(s) in the
mRNA encoding LPL is within nucleotides 560-590, 700-745, 930-960 or 1330-1360 of
SEQ ID NOs: 6 or 7, preferably within nucleotides 560-590, 705-745, or 930-960 of SEQ
ID NOs: 6 or 7; and/or
- (c) the two or more mammalian hydrolases are selected from the group consisting of
40 lipoprotein lipase (LPL), phospholipase B-like 2 (PLBL2), phospholipase A1 (Pla1a),

- inactive pancreatic lipase-related protein 1 (PNLIPRP1), inactive pancreatic lipase-related protein 2 (PNLIPRP2), pancreatic triacylglycerol lipase precursor (PNLIP), lipase member I precursor (LipI), lipase member H precursor (LipH), lipase member G precursor (LipG), lipase member C precursor (LipC) and isoforms thereof, and/or
- 5 (d) wherein the artificial miRNA guide strand sequence has the nucleotide sequence of SEQ ID NO: 1 (LP 8mer), SEQ ID NO: 2 (LP 6mer), SEQ ID NO: 3 (LH 8mer), SEQ ID NO: 4 (LH 9mer) or SEQ ID NO: 5 (LH Non-canonical).
6. The artificial miRNA of any one of the preceding claims, wherein the artificial miRNA guide strand sequence has imperfect complementarity to the target sequence(s) of at least one of the at least two mRNAs encoding the two or more mammalian hydrolases and wherein said miRNA guide strand sequence comprises a 5' seed region starting at nucleotide 2 (nt 2) and a 3' complementary region that undergo base pairing with said target sequence(s), wherein the 5' seed region comprises
- 10 (i) a sequence of 8 nucleotides (nt 2-9) complementary to the target sequence(s) and having 0, 1, 2 or 3 mismatches with the target sequence(s), preferably 0, 1 or 2 mismatches, and
- (ii) a GC-content of 55-70% wherein all or all but one or all but two guanine (G) or cytosine (C) are complementary to the target sequence(s); and
- 15 wherein the 3' complementary region comprises
- (i) a sequence of the last 6 nucleotides complementary to the target sequence(s) and having 1, 2 or 3 mismatches with the target sequence(s), preferably wherein the mismatches are not consecutive mismatches, and
- (ii) a GC-content of $\leq 50\%$ in the last 6 nucleotides, wherein 1 or 2 of the complementary nucleotides are G or C.
- 20 7. The artificial miRNA of claim 6, wherein the artificial miRNA guide strand sequence has the nucleotide sequence of SEQ ID NO: 2 (LP 6mer), SEQ ID NO: 4 (LH 9mer) or SEQ ID NO: 5 (LH Non-canonical).
- 30 8. The artificial miRNA of any one of claims 1 to 5, wherein the artificial miRNA guide strand sequence has imperfect complementarity to the target sequence(s) of at least one of the at least two mRNAs encoding the two or more mammalian hydrolases and wherein said miRNA guide strand sequence comprises a 5' seed region starting at nucleotide 2 (nt 2) and a 3' complementary region that undergoes base pairing with said target sequence(s), wherein the 5' seed region comprises
- 35 (i) A sequence of 8 nucleotides (nt 2-9), wherein at least the first 4 consecutive nucleotides (at least nt 2-5) are complementary to the target sequence(s), wherein

the sequence of 8 nucleotides comprises at least one mismatch with the target sequence(s), and

- (ii) a GC-content of 55-75%, wherein at least 4 of the consecutive nucleotides are complementary to the target sequence(s) are G or C; and

5 wherein the 3' complementary region comprises

- (i) a sequence of the last 6 nucleotides complementary to the target sequence(s) and having 0 or 1 mismatches with the target sequence(s), preferably wherein the last nucleotide is complementary to the target sequence(s) and/or the penultimate nucleotide is a mismatch, and
- 10 (ii) a GC-content of 30-50% in the last 6 nucleotides, preferably wherein at least 2 of the complementary nucleotides are G or C.

9. The artificial miRNA of claims 8, wherein the artificial miRNA guide strand sequence has the nucleotide sequence of SEQ ID NO: 1 (LP 8mer) or SEQ ID NO: 2 (LP 6mer).

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10. The artificial miRNA of any one of claims 1 to 9, wherein the artificial miRNA is a pri-miRNA, a pre-miRNA, a double stranded mature miRNA (comprising the passenger strand and the guide strand) a single stranded miRNA guide strand or a single or double stranded miRNA mimic.

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11. A mammalian expression vector comprising a polynucleotide sequence encoding the artificial miRNA of any of claims 1 to 10.

12. The mammalian expression vector of claim 11, wherein

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- (a) the mammalian expression vector further comprises at least one gene of interest encoding a recombinant protein of interest; and/or
- (b) the mammalian expression vector comprises a polynucleotide sequence encoding two or more of the artificial miRNAs of any of claims 1 to 10 wherein the artificial miRNAs are identical or different.

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13. A mammalian cell comprising the artificial miRNAs of any one of claims 1 to 11 or the mammalian expression vector of claims 11 or 12.

14. The mammalian cell of claim 13, wherein

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- (a) the mammalian cell is a human or a rodent cell, preferably a CHO cell; and/or
- (b) the mammalian cell further expresses a recombinant protein of interest.

15. Use of the artificial miRNA of any one of claims 1 to 10, in a mammalian cell for preventing expression of at least two endogenous hydrolases in the mammalian cell and/or preventing

hydrolase activity and/or polysorbate degrading activity associated with a recombinant protein of interest produced in the mammalian cell.

16. A method for manufacturing a protein of interest comprising the steps of:
- 5 (a) introducing an expression vector comprising a gene of interest encoding a recombinant protein of interest into a mammalian cell;
 - (b) introducing an artificial miRNA comprising an artificial miRNA guide strand sequence targeting two or more mammalian hydrolases acting on ester bonds into the mammalian cell, wherein the artificial miRNA is introduced before, simultaneously or
10 after step (a) into the mammalian cell, wherein the artificial miRNA is introduced as RNA or as a mammalian expression vector comprising a polynucleotide sequence encoding said artificial miRNA;
 - (c) cultivating said mammalian cell under conditions that allow expression of the recombinant protein of interest; and
 - 15 (d) harvesting the recombinant protein of interest in the harvested cell culture fluid; and optionally further comprises the following steps:
 - (e) purifying the recombinant protein of interest; and
 - (f) formulating the purified recombinant protein of interest into a composition.
- 20 17. The method of claim 16, wherein
- (a) the harvested cell culture fluid has reduced hydrolase activity and/or reduced polysorbate degrading activity; and/or
 - (b) a composition comprising the purified recombinant protein of interest of step (e) or the composition of step (f) has reduced hydrolase activity and/or reduced polysorbate
25 degrading activity; and/or
 - (c) the artificial miRNA prevents expression of at least two endogenous hydrolases in the mammalian cell and/or prevents hydrolase activity and/or polysorbate degrading activity associated with a recombinant protein of interest produced in the mammalian cell.
- 30 18. The method of claim 16 or 17, wherein
- (a) the artificial miRNA is the artificial miRNA of any one of claims 1 to 10; and/or
 - (b) the expression vector comprising a polynucleotide sequence encoding the artificial miRNA is the mammalian expression vector of claim 11 or 12; and/or
 - (c) the two or more mammalian hydrolases are endogenous hydrolases of the mammalian
35 cell, preferably the mammalian cell is a human or a rodent cell, more preferably a CHO cell; and/or
 - (d) wherein the artificial miRNA is introduced as RNA by transient transfection or as expression vector comprising a polynucleotide sequence encoding said artificial miRNA by transfection or transduction.