The translation of clinically important proteins is post-trancriptionally regulated. Understanding this field of research has led to the development of novel drug discovery approaches. One such approach, GEMS, opens the door to the discovery of small molecules targeting post-transcriptional control processes.

Mining the GEMS – a novel platform technology targeting post-transcriptional control mechanisms

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The physiological levels of many clinically important proteins are regulated through cellular mechanisms that control the stability and translational efficiency of mRNA. These post-transcriptional processes, which play a critical role in the regulation of gene expression, depend on interactions of specific *trans*-acting factors with sequence elements located within the 5′- and 3′-untranslated regions (UTRs) of an mRNA. A technology platform called GEMS (Gene Expression Modulation by Small-molecules) exploits the interactions of UTR elements with the *trans*-acting factors, thereby specifically targeting mechanisms of post-transcriptional control. In this review we describe how this technology enables the identification of small-molecules that modulate the levels of proteins involved in disease pathogenesis.

Introduction: the rational for targeting post-transcriptional control mechanisms

RNA participates in a wide variety of biological processes in all living organisms. It transfers genetic information from DNA to protein and for some viruses, serves as its founding genetic material. In addition, it also plays structural roles in protein synthesis (as ribosomal RNAs and tRNA adaptors) and several post-transcriptional processes [1,2]. There is also evidence that RNA is involved in the control of gene expression [3]. RNA, like protein, can fold into complex three-dimensional structures, creating unique binding interactions between the structured RNAs and effector proteins. The field of post-transcriptional control seeks to understand how RNA–protein interactions are responsible for controlling diverse biological functions that are key events in the regulation of gene expression [4–6]. Our understanding of these mechanisms and how they control gene expression has increased dramatically over the past fifteen years. Experimental findings clearly demonstrate that the three dimensional RNA structures as well as the RNA–protein complexes are a novel set of potential targets for small-molecule drugs [2,7].

Multiple control points in the flow of genetic information from DNA to RNA to protein can be targeted for therapeutic intervention (Figure 1). Genomic companies have focused on DNA and how this information can be used to discover and develop drugs. Pharmaceutical and biotech companies have focused on inhibition of protein enzymatic action or modulation via monoclonal antibodies or protein products. A new area of drug discovery, targeting post-transcriptional control processes, has emerged as an alternative to these traditional approaches. We have developed multiple platform technologies that systematically target post-transcriptional control processes to modulate gene expression. These technologies allow us to focus on the discovery and development of small molecules to target these processes. Importantly, by targeting gene expression there is the prospect to not only downregulate or inhibit protein production but also to increase or upregulate the production of targeted proteins. A pharmaceutical approach to targeting post-transcriptional control processes can address a large number of medically important but previously intractable targets in multiple therapeutic areas.

The biology of post-transcriptional control processes – RNA as a key mediator in regulating gene expression

For a large number of genes, post-transcriptional control of their expression is a key determinant for modulating protein levels in the body. Post-transcriptional control processes include mRNA splicing and editing, transport, subcellular localization, mRNA

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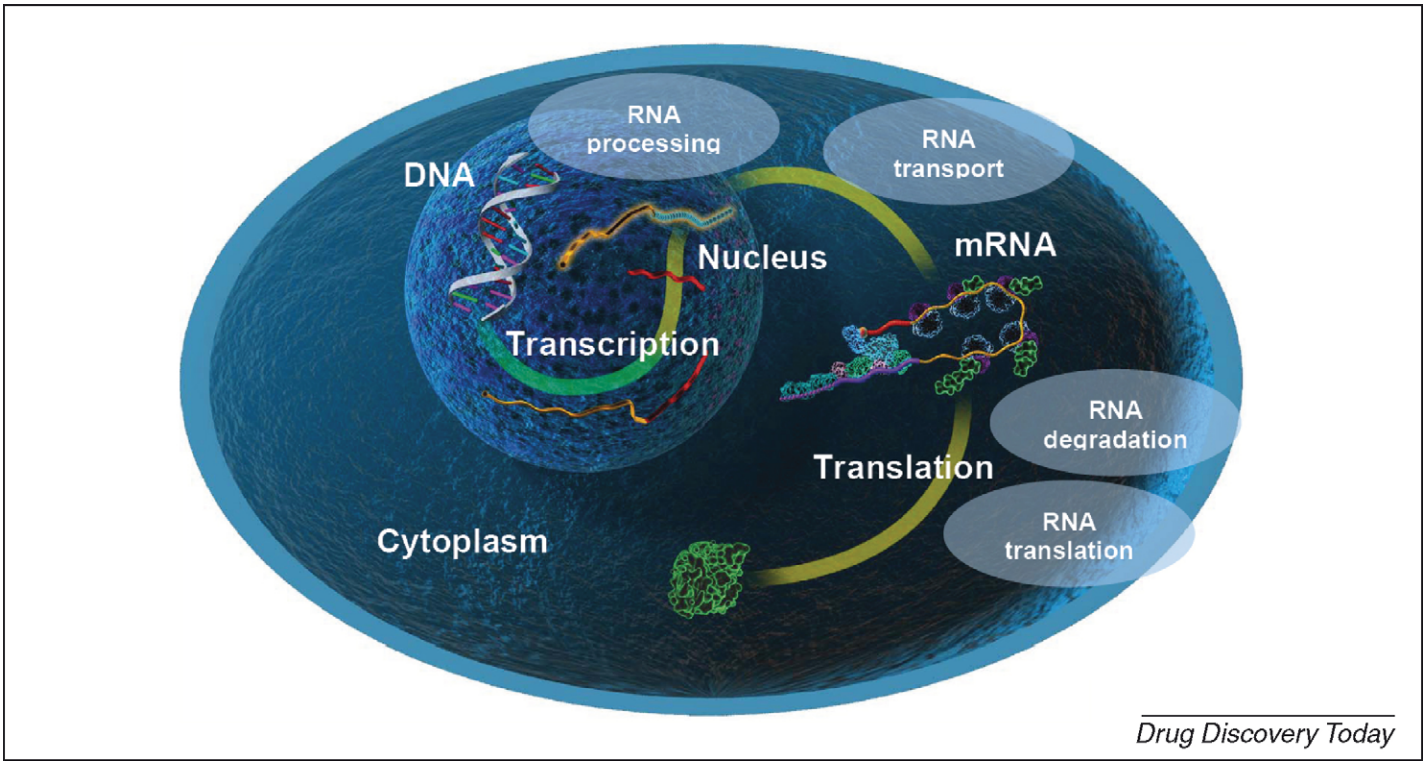

FIGURE 1

Post-transcriptional control of gene expression. After transcription of a gene from DNA to an RNA copy, the molecule, messenger RNA (mRNA) is processed further to a mature molecule, which is transported to the cytoplasm. Within the cytoplasm, the mRNA molecule associates with a number of proteins including ribosomes that function as machines to translate the mRNA into a protein. Post-transcriptional control processes modulate the length of time an mRNA molecule survives within the cell and how efficiently it is used to make its protein. These processes are governed by the constellation of *trans*-acting proteins that are bound to the mRNAs 5′ and 3′ untranslated regions (UTRs).

surveillance and turnover, and translation efficiency (Figure 1). Each mRNA harbors a 5′-cap structure and 3′ terminal poly-A tail that is not encoded by the gene but is added subsequently by post-transcriptional mechanisms. The 5′-most segment of the transcribed mRNA contains the 5′-untranslated region (UTR), followed by an open reading frame (ORF) and then the 3′ UTR (Figure 2). *Cis*-acting elements that map to the 5′ and 3′ UTRs are important determinants of post-transcriptional interactions [5,6,8,9]. These interactions have biological significance and play a critical role in regulation as described in the next section [6,8–10].

Although DNA and proteins have long been drug targets for the pharmaceutical industry, RNA, in its structural role or in its role as a key mediator in controlling gene expression, has been significantly underutilized as a biological target for small-molecule drug design. Only recently has the concept of targeting RNAs or the regulation of RNA utilization in controlling gene expression captured the interest of pharmaceutical companies. There is growing evidence for the involvement of deregulated RNA–protein interactions that lead to increased or decreased protein expression and subsequently disease pathogenesis. Defects in post-transcriptional control mechanisms have been linked to several diseases such as cancer and neurodegenerative and autoimmune, disorders [9–16]. The targeting of specific RNA structures, regulatory elements, and RNA–protein complexes with small molecules to increase or decrease gene expression is anticipated to result in our ability to treat a vast array of diseases that currently have no treatments.

Post-transcriptional control mechanisms modulate expression of many medically relevant genes

Post-transcriptional control of gene expression plays an important role in the regulation of mRNA transcripts that encode protein products such as growth factors, receptors, cell cycle regulators, oncogenes, and cytokines. These proteins are key determinants in important regulatory pathways and contribute to the control of diverse pathological processes like cancer, autoimmune and other degenerative disorders [9–16]. Structural and sequence motifs located within the UTRs of these mRNAs are involved in specific RNA–protein interactions and lead to a dynamic mRNA–protein complex (mRNP) that involves the cooperation between these *cis*-acting elements and *trans*-acting factors [8]. It has become clear that it is the constellation of proteins bound to the *cis*-acting elements that dictates post-transcriptional control of mRNAs within a eukaryotic cell. Importantly, several of these *cis*- and *trans*-acting factors are well conserved across species, underscoring the critical role for these elements in gene regulation [17].

Two of the first characterized transcripts regulated by post-transcriptional control mechanisms encode the proteins ferritin and the transferrin receptor. Ferritin stores excess iron in the cell whereas the transferrin receptor transports iron into the cell when its levels are low. An iron-responsive element (IRE) is present in the

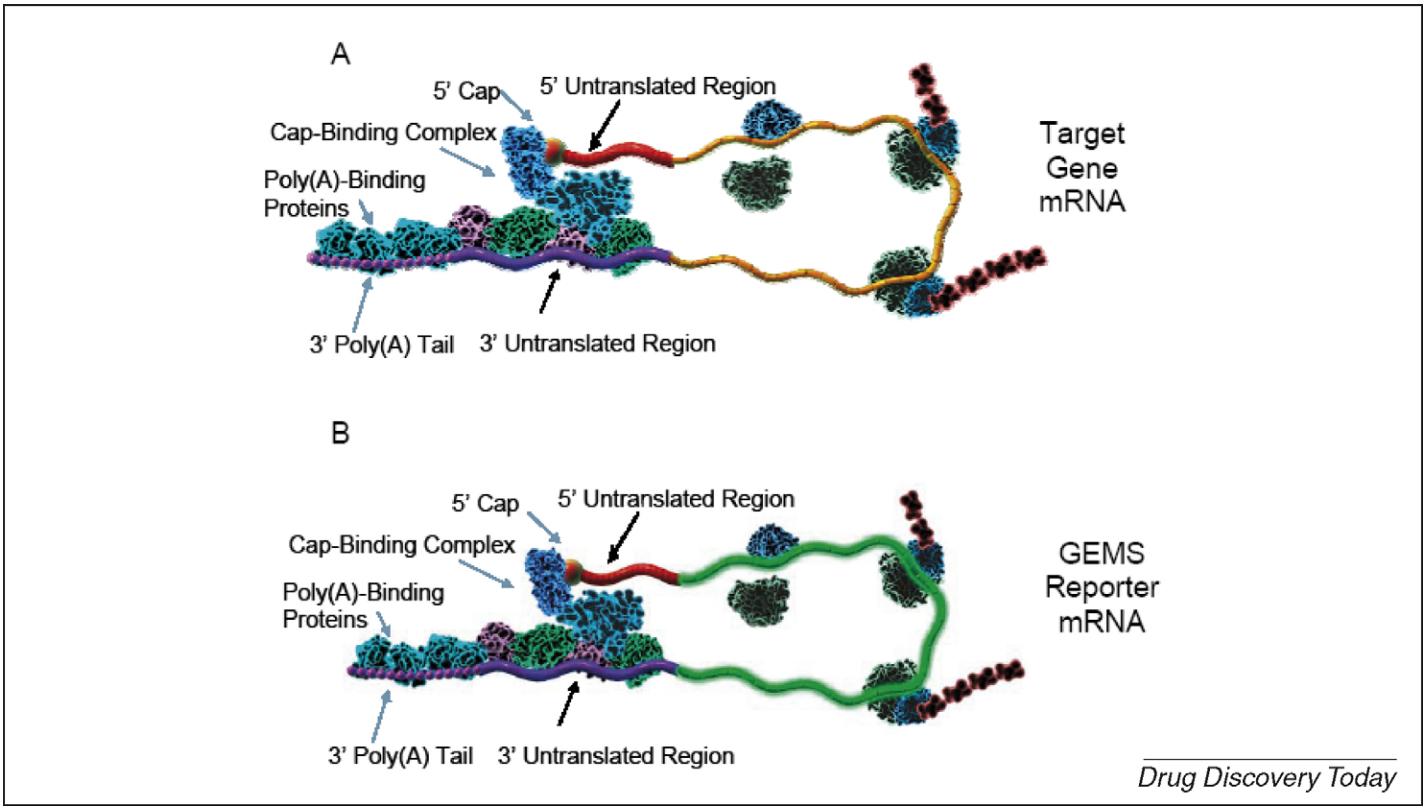

FIGURE 2

The 5′ and 3′ untranslated regions interact with proteins important for post-transcriptional control. (A) Target gene mRNA: A typical mRNA contains a 5′ cap, a 5′ untranslated region (5′ UTR) upstream of a start codon, an open reading frame encoding a protein, a 3′ untranslated region (3′ UTR) downstream of the termination codon and a polyadenylated (poly(A)) tail. In the closed-loop model, linkage between the 5′ and 3′ ends of the transcript is facilitated via interaction of the poly (A)-binding protein (PABP), bound to the poly(A) tail, with the cap-binding complex at the 5′ end of the mRNA. These proteins promote interactions between the 5′ and 3′ ends of the RNA and contribute to the levels of protein expression from a given mRNA. Additional *trans*-acting proteins associate with the 5′ UTR and 3′ UTR through interactions of specific sequence elements located in the UTR. These interactions regulate translation initiation, translation efficiency, and translation re-initiation. (B) GEMS reporter mRNA: A reporter mRNA is engineered by inserting the 5′ and 3′ UTRs of a therapeutically relevant mRNA upstream and downstream of the reporter open reading frame (green). The UTRs of the target gene endow the reporter gene with the post-transcriptional control mechanisms that govern the target gene.

5′-UTR of the Ferritin RNA while the same element is present in the 3′ UTR of the transferrin receptor transcript [10,18]. The iron-regulatory proteins (IRP) interact with the *cis*-element IRE to maintain the intracellular iron levels, and it is this interaction that directs the post-transcriptional expression of proteins involved in iron metabolism. An IRP-IRE interaction in the Ferritin IRE leads to translation repression, whereas binding of the IRPs to the IRE in the 3′-UTR of the transferrin receptor stabilizes its mRNA, leading to increased levels of the transferrin receptor protein and thus increased iron transport. The counterbalance of post-transcriptional regulation of ferritin and transferrin receptor transcripts ensures that free iron levels will be in the appropriate concentrations in cells. It should be noted that this is an elegant example of how a small molecule, in this case iron, selectively and specifically regulates post-transcriptional control mechanisms to monitor protein levels.

Sequence elements in the 5′-UTR

An increasingly large number of transcripts have been shown to be post-transcriptionally regulated and their sequence elements have been characterized. For example, there are several examples of structural elements within the 5′ UTR that play a major role in translational regulation. Although the majority of 5′ UTRs are relatively short, unstructured, and contain a single start AUG codon, there is a growing number of mRNAs that encode growth factors, cellular receptors, and oncogenes that have long 5′-UTRs with secondary structures and upstream open reading frames (uORFs) [10]. Important to this discussion, these elements have been shown to regulate gene expression at the post-transcriptional level.

There are a number of examples in which uORFs regulate translational efficiency. An uORF consists of an upstream AUG (uAUG) initiation codon followed by a stop codon upstream of the protein coding region (Figure 2). The function of uORFs has been studied in the context of both physiological and pathologic processes. The oncogenes mdm2 and Her-2 carry uORFs in their 5′ UTR that have an inhibitory effect on their translation [19–22].

Translational regulation can also be mediated by an internal ribosome entry site (IRES) located in the 5′ UTR. An IRES is a *cis*-acting element that is utilized for the direct recruitment of ribosomes to an mRNA in order to initiate internal translation [23,24]. A large number of pathogenic viruses including poliovirus, hepatitis C (HCV), rhinoviruses, and coxsackieviruses have IRES elements within their 5′ UTRs that support efficient internal translation and viral protein synthesis during infection [23,25–27]. In

addition to viral IRESs, there are several examples of cellular IRES-like elements in the mRNAs whose protein products are involved in cell growth, proliferation, differentiation, apoptotic regulation, and angiogenesis [28].

Sequence elements in the 3′-UTR

Sequence elements present in the 3′-UTRs of transcripts have also been shown to regulate post-transcriptional control processes. A recent comparative genomic analysis has uncovered common regulatory motifs in the 3′ UTRs of human, mouse, rat, and dog genomes [29]. Over 170 sequence motifs were identified that were hypothesized to be important in post-transcriptional regulation. The conserved motifs found in mRNAs may function by directly interacting with various RNA-binding proteins [29].

One of the first and best studied 3′-UTR sequence motif is the AU-rich element (ARE). AREs have been shown to regulate not only mRNA turnover but also translation efficiencies [5,30–33]. AREs vary in length and sequence, and often contain one or more AUUUA pentanucleotides. The complex network of RNA–protein interactions is responsible for mediating their effects. AREs have been found in many important regulators including interferons, cytokines, and proto-oncogenes, indicating that ARE-mediated post-transcriptional control plays a critical role in the regulation of gene expression during cell proliferation, differentiation, and apoptosis [12,34–37]. VEGF, a mitogen for vascular endothelial cells, has been implicated in angiogenesis under both physiological and pathological conditions. The 3′ UTR of VEGF contains an ARE that specifically interacts with the RNA-binding protein HuR to mediate hypoxia-induced stabilization of the transcript [38,39].

Deregulation of post-transcriptional mechanisms in various disorders

Post-transcriptional control processes regulate how the mRNA is used to produce therapeutically important proteins. Much of our knowledge of post-transcriptional control comes from the study of diseases where the consequence of aberrant regulation of gene expression results from alteration or mutation of the UTR regulatory elements or the *trans*-factors with which these elements interact [9,12]. Protein levels of several oncogenes that include BCL2, mdm-2, and c-myc are regulated through UTR–protein interactions, and deregulated expression of these genes promotes cancer [12].

The IREs and IRPs that were first identified to regulate cellular iron levels have also been implicated in regulating several transcripts and their deregulation has been associated with different pathologies. For example, IRE-IRP regulation has been implicated in Alzheimer's disease (AD) [40]. The amyloid precursor protein (APP), which is proteolytically cleaved into the neurotoxic β amyloid peptides, contains a functional IRE within the 5′ UTR [40,41]. Iron levels were shown to stimulate translation of APP in neuroblastoma cells, possibly through a direct interaction between the IRE and the *trans*-activators IRP-1/IRP-2. Because APP levels and iron metabolism have been linked to the pathogenesis of AD, targeting this critical post-transcriptional mechanism mediated through the 5′-UTR of the APP mRNA is a therapeutic approach that can be considered to reduce APP levels in AD patients [10,40].

A number of diseases have been linked to modulating the translational efficiency of uORFs. Mutations and deletions in the uORFs or changes in splicing patterns of the 5′ UTR result in the modulation of translation efficiency. In certain tumors the mdm2 protein is overexpressed because of enhanced translation efficiency resulting from an mdm2 transcript lacking the inhibitory uORFs [19]. In other cases a point mutation in the 5′ UTR of *CDKN2A* that inserts a novel uORF results in a dramatic decrease in translation from the main start codon [42]. Normally CDKN2A is a tumor suppressor that induces a G1 cell cycle arrest; inhibiting its action is implicated in multiple cancers, including melanoma [43].

Oncogenes and growth factors (c-myc, Apaf-1, XIAP, Bcl-2) are expressed at low levels and have been shown to be post-transcriptionally regulated. Under pathological conditions their translational efficiency is increased, possibly through cellular IRESs in their 5′-UTRs [44]. For example, the c-myc transcript has a large and highly structured 5′ UTR harboring an IRES that is capable of initiating translation in a cap-independent manner [45–48]. Deregulation of c-myc expression has been shown to be associated with a wide range of cancers. A gain-of-function mutation in the c-myc IRES has been identified in multiple myeloma that results in increased translation initiation and c-Myc expression [49]. In addition to c-myc, many oncogenes that contain IRESs mediate internal translation during oncogenic transformation [50,51]. Furthermore, there are a number of tumor suppressors that are translated via an IRES and are downregulated in cancer cells [12]. Collectively these observations make it evident that the cellular IRES-mediated deregulation of oncogenes and tumor suppressors is an attractive target for cancer therapeutics.

Many cytokines play an essential role in different pathological processes in humans and their modulation in expression leads to several immune and inflammatory disorders. Most of these cytokines are post-transcriptionally regulated. This regulation determines the levels and timing of their expression during inflammatory processes. For example, tumor necrosis factor-alpha (TNF-α), a cytokine that is involved in various inflammatory and immune responses, is regulated at multiple levels and the AREs located in the 3′ UTR of the TNF-α transcript mediate its post-transcriptional regulation [52,53]. Deregulation of the rate of TNF-α mRNA decay results in the overexpression of the protein and development of diseases such as chronic inflammatory arthritis, inflammatory bowel disease, and other autoimmune disorders [52–56].

Deregulation of ARE-mediated post-transcriptional regulation has been shown to cause lymphomas, carcinomas, and neuroblastomas [12,31,37,57–59]. A more advanced stage and grade of tumors has been correlated with alterations in AREs or altered expression of the ARE-binding proteins [12]. Many genes known to regulate gene expression or be involved in tumorgenesis contain AREs in their 3′-UTRs and are post-transcriptionally regulated. These include the c-fos c-jun, c-myc, and COX-2 factors [12]. The ARE-mediated post-transcriptional control mechanism represents an attractive target for the development of therapeutics for the treatment of several immune and inflammatory disorders and cancers.

Targeting RNA and post-transcriptional control processes

Post-transcriptional control mechanisms play essential roles in many biological processes, and the sequence-specific binding of

RNA to many disease-related genes makes it both an important tool for target identification and a potential therapeutic approach in drug discovery and development [2]. The therapeutic potential of several anti-sense oligonucleotides (ASO) that selectively knock-down gene expression has been demonstrated both *in vitro* and *in vivo* disease models such as cancer, cardiovascular, and infectious diseases. These sequence-specific agents interfere with their mRNA targets and cause specific post-transcriptional inhibition of gene expression [60].

ASOs hybridize to the complementary target mRNA, which causes destruction of the transcript and/or translational inhibition of the targeted protein. Although using ASOs is a very efficient and specific way of targeting gene regulation, there are several obstacles in the development of their therapeutic applications like cellular uptake and delivery [61]. Small interfering RNA molecules (siRNA) also interfere with the mRNA and block the production of the target protein within cells. Ribozymes are another attractive antisense therapeutic agent because these catalytic molecules bind and cleave RNAs in a highly sequence-specific manner. Several ribozymes (Angiozyme, Herzyme, Heptazyme) have been reported to be in clinical trials.

The biggest challenge of these applications is the delivery of these agents into targeted diseased cells within the body. These agents have to retain biological activity inside the cell and interact with the target. They face several other challenges like unwanted off-target biologic effects and the activation of the interferon response [61–65].

Systematic targeting of post-transcriptional control mechanisms with small molecular weight compounds is a new approach that creates unique opportunities for drug discovery that can overcome the hurdles associated with the delivery of ASO and siRNA. Commercially available drugs that target post-transcriptional control processes are not without precedent. Although not discovered as molecules that affect post-transcriptional control process, subsequent to their development, several Food and Drug Administration (FDA)-approved drugs have been found to act through post-transcriptional mechanisms providing proof of principle that this approach can yield important drugs. For example, ribosome inhibitors such as macrolides, aminoglycosides, and oxazolidinones have been used to treat human bacterial infections for decades [66–68]. These antibiotics target different regions of the ribosomal RNA (rRNA) molecules in the ribosome and demonstrate a clear precedence of RNA as the target of small molecule drugs [67,69].

Identification of small molecules that target post-transcriptional control processes

As described above, it has become clear that the expression of a large number of medically important genes is controlled post-transcriptionally, specifically by mRNA stability and translational control mechanisms. Recent results have identified small-molecule modulators that target these post-transcriptional processes. These include a eukaryotic translation initiation eIF4A [70–72] and eIF4E/eIF4G inhibitors [73]. Furthermore, the kinase mTOR (mammalian target of rapamycin) regulates translation initiation by altering the phosphorylation status of 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1) and S6K (ribosomal p70 S6 kinase) [74]. The macrolide antibiotic rapamycin and its analogs are highly specific inhibitors of mTOR, and several of these small molecules are currently in clinical development as anti-cancer therapeutics [75,76].

GEMS – gene expression modulation by small molecules

Although the reports above are examples that constitute a significant advancement in the development of small-molecule modulators of post-transcriptional mechanisms, we have focused our efforts on development of platform technologies that can be utilized against targets in many therapeutic areas. An example of one platform technology is the GEMS (gene expression modulation by small molecules), a phenotypic approach that specifically targets the mechanisms of post-transcriptional control that functions through the 5′- and 3′-UTRs. The paradigm of the GEMS screening protocol is the construction of a reporter gene, luciferase, flanked by the UTRs of medically important genes (Figure 2). The goal is to construct a reporter gene whose expression is controlled by the regulatory elements embedded within the 5′ and 3′ UTRs of the target gene. The reporter gene for a given target is used to generate a stable cell line that expresses the target reporter gene in a cell type where post-transcriptional control of the target gene is known to be operative. The stable cell line harboring the GEMS reporter construct is challenged in a high-throughput screen by a small-molecule chemical library (Figure 3).

We applied the GEMS screening paradigm to investigate the role of Her-2 UTRs in modulating post-transcriptional regulatory processes under pathologic conditions. Overexpression of the Her-2 oncogene is a result of deregulation at both transcriptional and post-transcriptional levels. This overexpression is associated with the progression of various types of carcinomas and poorer prognosis. Reporter constructs containing the luciferase reporter gene flanked by the Her-2 or control UTRs and a panel of stable cell lines that express varying levels of Her2 were generated. We found that the Her-2 UTR reporter construct modulates luciferase expression in a tumor cell-line-specific manner. We found that in Her-2-overexpressing breast cancer cells, *cis*-acting sequence element within the 3′ UTR was able to override the inhibitory effect of the Her-2 uORF in the 5′ UTR, leading to increased reporter expression [20]. These data strongly suggest that the GEMS reporter construct with the flanking target UTRs mimics post-transcriptional regulation of the endogenous protein and validate the fact that the target-specific UTR reporter constructs can be used for the identification and development of new small molecules that modulate the post-transcriptional control processes.

The goal of a GEMS HTS effort is to identify chemical scaffolds that specifically modulate the expression of the reporter gene by influencing the 5′ and 3′ UTR-dependent post-transcriptional control processes that govern the target gene expression. Chemical scaffolds are identified from a library of diverse small molecules (~200 000 molecules). This library has been designed for reduced redundancy and rapid analog generation and prescreened for pharmaceutical potential [77–79]. The majority of the compounds in the collection possess favorable characteristics of known oral drugs currently on the market [77–79].

The screening of this library represents a chemical genomics approach in which the specificity of the small molecule modulators for the target gene is demonstrated by screening these com-

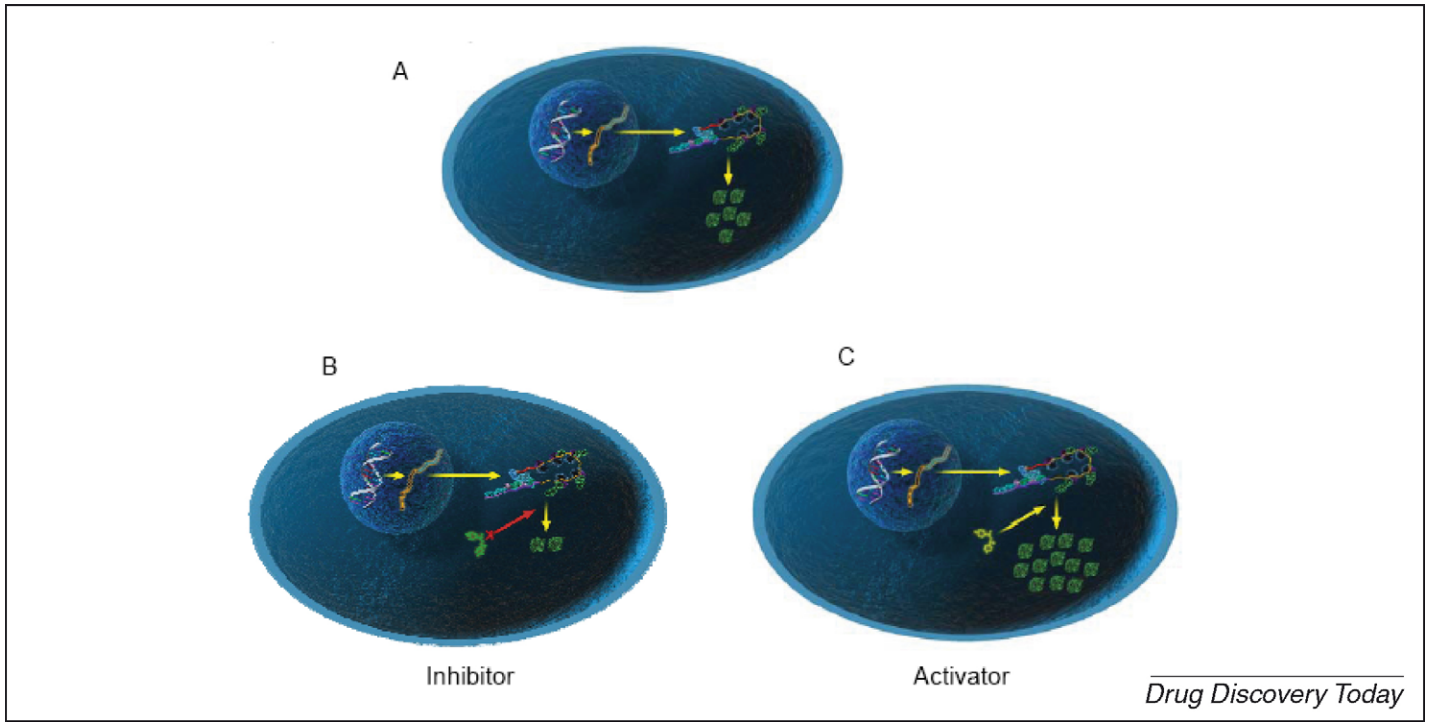

FIGURE 3

Schematic representation of the GEMS HTS assay. GEMS reporter constructs are transfected into an appropriate cell line, and a stable clone is generated. (A) A cell line stably expressing the reporter protein under the control of the 5′ and 3′ UTRs of the target gene is then incubated in the presence of compounds, and modulation of reporter gene expression is monitored for either: (B) gene expression inhibition by a small-molecule inhibitor; or (C) gene expression activation by a small-molecule activator.

pounds against luciferase reporter genes harboring different 5′ and 3′ UTRs from multiple unrelated transcripts. This panel is referred to as a 'UTR-specificity panel' (USP). A comparison of compound activity utilizing a USP identifies those molecules with specificity for the target gene 5′ and 3′ UTR pair.

Once a UTR-specific chemical scaffold is identified, the effect of these molecules on endogenous target gene expression is tested in a biologically relevant cell line. Typically an enzyme-linked immunosorbent assay (ELISA) measuring the level of the target protein allows demonstration of activity of a selected chemical scaffold on endogenous protein expression. In order to demonstrate specificity at the level of endogenous protein expression, secondary screening is then utilized to characterize each of these chemical scaffolds for specificity as compared with other targets. Depending on the type of post-transcriptional control elements present in the UTRs of each target, different assays are performed. These experiments lead to the identification of chemical scaffolds that exhibit UTR specificity and selectively modulate the expression of the target gene through a post-transcriptional control mechanism controlled by their untranslated regions. Selected molecules are analyzed for their pharmaceutical properties and their activities in an animal model. Lead compounds are chemically optimized to improve efficacy, potency, and pharmaceutical properties through medicinal chemistry efforts. Structure–activity relationship (SAR) studies are performed, and a pharmacophore is defined. Although the molecular targets are initially unknown at the molecular level, the compounds are known to function through the UTRs. Mechanism of action studies can then be employed to define the post-transcriptional control processes that are affected, the precise sequence element that can be defined and the proteins that are involved in the process can be subsequently identified. The compounds identified through this process are not only new potential drugs but are also tools to identify and characterize novel post-transcriptional pathways.

Utilizing our GEMS technology we have also identified small molecules that specifically modulate post-transcriptional control of gene expression of medically important genes in multiple therapeutic areas. These small-molecule drugs have the potential of being much easier to produce, less costly, and orally bioavailable. Using this drug discovery platform, we are able to target genes that are implicated in cancer, central nervous system disorders, infection, inflammation, metabolic disorders, and cardiovascular disease. It is an attractive approach because it allows both proteins and RNA to be drug targets. Our most advanced compound identified from the GEMS technology is a VEGF inhibitor that has recently completed phase 1 trials in healthy volunteers and will be moving into trials in cancer patients.

The GEMS approach is a novel technology platform that allows us to tackle a large number of medically important targets in multiple therapeutic areas that are critical in different pathologies. An important advantage of the GEMS technology is that it allows the identification of small, pharmaceutical-like compounds that upregulate or downregulate protein synthesis by altering the post-transcriptional regulation of a target mRNA. The GEMS technology can be used in drug discovery programs against drug targets that are known to be medically important but were thought to be previously intractable. Furthermore, the technology can identify orally bioavailable compounds that have the potential to replace

conventional protein therapeutics with small molecules. A wide range of orally available, small-molecule drugs that target both RNA and proteins are currently being developed through the application of this novel screening platform.

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