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MEETING REPORT

EPIGENETICS

HIGHLIGHTS OF THE SOCIETY FOR MEDICINES RESEARCH SYMPOSIUM, HELD ON SEPTEMBER 22, 2010, LONDON, UK

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SUMMARY

On September 22, 2010, the Society for Medicines Research (SMR) held a 1-day meeting entitled Epigenetics. The first generation of epigenetic-modifying drugs (histone deacetylase inhibitors and DNA methyltransferase inhibitors) have now reached the marketplace and are rapidly proving to be useful agents for a range of oncology indications. An explosion in the amount of research being carried out in this field is under way, with a plethora of new histone- and DNA-modifying drug targets being pursued for a range of therapeutic indications, such as cancer, inflammatory disorders and metabolic diseases. This SMR symposium brought together a panel of international speakers across the breadth of research and development in the field of epigenetic drug discovery, to present and discuss new advances and results from early research to clinical data. Topics included the fundamental biology of epigenetics, therapeutic opportunities and approaches, first-generation epigenetic drug case histories in oncology and the next generation of epigenetic drugs.

FUNDAMENTAL BIOLOGY OF EPIGENETICS

Dr. Karl Nightingale (Institute of Biomedical Research, Birmingham, U.K.) gave the opening presentation on the fundamental biology of epigenetics and began by illustrating the field with the simple question – why are identical twins really not identical? In simple genetic terms, gene output reflects changes in DNA sequence, such as mutations and polymorphisms, whereas in epigenetics the gene output is changed, but without a change in the DNA sequence. In reality it is epigenetic mechanisms that are essential for gene regulation, and these mechanisms are under the influence of environmental factors, such as nutrition, toxins, metabolites, etc. This offers an explanation of why twins with identical DNA and genotype can have differing gene activity and phenotype. Epigenetic regulation is driven by chromatin proteins such as histones, chromatin-binding proteins and enzymes acting on chromatin, and also by effects on DNA methylation. Chromatin is the structure of DNA assembled onto proteins (such as histones), which allows layers of regulation and prevents adventitious transcription. Although the chromatin structure is well understood at the DNA and nucleosome level, the structure beyond this size is poorly understood.

Post-translational modifications on core histone *N*- and *C*-terminal tails, such as lysine acetylation, lysine/arginine methylation and serine/threonine phosphorylation, are known as histone marks. These modifications act as regulatory marks, and associate with different regions of chromatin and determine (or reflect) their functional status in an incredibly specific fashion. The histone tails are packed with these post-translational modifications in a highly complex and dynamic manner. This is achieved by a range of histone-modulating enzymes that can either put a histone mark in place, e.g., histone acetyltransferases, histone methyltransferases and kinases, or remove a histone mark, e.g., histone deacetylases, histone demethylases and phosphatases. In general, there is more process specificity in the transferase enzymes than in the removal enzymes. Effector proteins, such as bromo and chromo domains, are then able to read these complex marks and progress or terminate a gene modification. Examples of this are the activating transcription factors p300/CBP and PCAF, which have histone acetyltransferase activity-activating genes, and hence, in this case, histone deacetylase activity will turn gene activity off: the histone methyltransferase G9a,

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which methylates the histone H3 tail on lysine residue 9 (H3K9) read by HP1 to deactivate the gene; or the histone methyltransferase MLL, which methylates histone H3 on lysine residue 4 (H3K4) read by CHD-1 to activate the gene. In summary, histone modification is central to gene regulation, although it is highly complex. Exquisite histone modification specificity exists via multiple modification and removal enzymes, and when coupled with the multiple effector proteins and "cross talk" in these processes, gene regulation is tightly controlled, dependent on the impact of the intra- and extracellular environment.

THERAPEUTIC OPPORTUNITIES IN EPIGENETICS

Dr. Nessa Carey (CellCentric Ltd., Cambridge, U.K.) presented an overview on the possible therapeutic opportunities for epigenetic-derived drugs. To date, marketed epigenetic target-based drugs, such as nonselective histone deacetylase inhibitors and DNA methyltransferase inhibitors, have focused on oncology indications. Both of these classes of compounds have found particular use in the treatment of blood-borne cancers, such as cutaneous T-cell lymphoma; however, their use has been limited due to a lack of understanding of which cancers and patients are likely to respond best, and with what drug combination. Undoubtedly, there are further oncology applications to be found from epigenetic drug targets, such as solid tumors, and the second generation of epigenetic drugs should be more specific in their action compared to the earlier promiscuous enzyme classes and inhibitors.

There is also great promise that epigenetic drug targets can provide disease intervention in a range of therapeutic areas, such as inflammation, hemoglobinopathies, metabolic diseases, diabetic complications, pain, addiction, psychiatry, mental retardation and neurodegeneration. An example of a single gene disorder has been discovered in mental retardation, where mutations in the histone demethylase *PHF8* gene are associated with X-linked mental retardation, while the *MECP2* gene has been implicated in X-linked Rett syndrome using elegant conditional gene modification experiments in mice. In the field of neurodegenerative disease, there is recent hope that existing histone deacetylase inhibitors can be repositioned from oncology to Huntington's disease, as SAHA (vorinostat) has been shown to ameliorate motor deficits in a mouse model of this disease, and there is now a growing body of evidence implicating *MECP2* and *DNMT3A* genes in the processes involved in addiction.

There is clearly a growing literature implicating epigenetics in a range of diseases; however, there is still much to understand. With regard to drug discovery, key questions still need to be answered on the choice and "drugability" of targets, target selectivity and off-target effects.

CHEMICAL PROTEOMICS TOOLS FOR EPIGENETIC DRUG DISCOVERY

Dr. Gerard Drewes (Cellzome AG, Germany) described a new approach to target identification and screening in epigenetics. Traditional cell-based studies of drug action assess activity in settings that employ a mechanistic or phenotypic readout far downstream of the drug's molecular target, for instance, by means of a transcriptional reporter or by the secretion of a specific gene product. While useful, such studies do not provide direct insight into the molecular mechanism of action. Cellzome has employed recent methodological advances in chemical biology tools and in mass spectrometry-based analysis of proteins to enable a quantitative analysis of protein–small molecule interactions directly in cell lysates and in intact cells. This has particular relevance to epigenetic targets, as many of them reside in large protein complexes and do not possess correct biological activity in recombinant purified form.

Dr. Drewes demonstrated the usefulness of this approach through a histone deacetylase example. Histone deacetylases are the catalytic subunits of megadalton protein complexes such as the NuRD complex, the NCoR complex, the Sin3 complex and the CoREST complex. The profile of a panel of known histone deacetylase inhibitors was described in a competition binding assay using the Episphere™ Technology. The inhibitors were incubated with appropriate cells, e.g., human leukemic cell lines, over a range of concentrations, and the Episphere™ beads were then added. The competition with the beads was subsequently quantified by tandem mass spectrometry or antibody-based approaches, and the IC_{50} determined. Dose–response binding data for the approximately 500 proteins in the human leukemia cell extract can be grouped into 3 types of proteins: histone deacetylases, proteins known to be in complex with histone deacetylases and other proteins. The analysis also demonstrated that some of the histone deacetylase inhibitors have subtly different selectivity profiles for the complexes NCoR and Sin3. Bidirectional clustering of the dose–response data for > 300 proteins for 16 known histone deacetylase inhibitors demonstrated the potential to delineate drug–target complexes.

Dr. Drewes finished his presentation showing the potential to discover new inhibitors by library screening against HD1, HD2, HD3 and HD6 using the Episphere™ approach and a chemoproteomic binding assay. From the data presented, it was clear that compounds with selectivity for HD3 or HD6 with respect to HD1 could be identified.

ZOLINZA™ (VORINOSTAT, SAHA) – A CASE HISTORY

Dr. Thomas Miller (Merck, U.S.) presented on the discovery of Zolinza™ (vorinostat, SAHA; Fig. 1), a novel hydroxamic acid-derived histone deacetylase inhibitor, along with recent advances in histone deacetylase inhibitor design which led to the identification and characterization of highly subtype-selective inhibitors. Zolinza™ is Merck's first-in-class oral HD1, HD2, HD3 (class I) and HD6 (class IIb) inhibitor, approved to treat refractory cutaneous manifestations in patients with T-cell lymphoma (CTCL). In some cancer cells, histone deacetylases are often overexpressed, creating a condensed chromatin structure that inhibits gene transcription. It is believed that histone deacetylase inhibition opens chromatin to allow for gene transcription and inhibition of cell division and proliferation.

Dr. Miller described how the discovery in 1975 that dimethyl sulfoxide (DMSO) could cause growth arrest and terminal differentiation of transformed cells ultimately led to the discovery of Zolinza™ in 1990. Extensive structure–activity studies were performed in the discovery of Zolinza™ from DMSO (1). With DMSO as a starting point (inhibition of urine erythroleukemia cell growth at 280 mM), simple amides were identified as being more potent than DMSO, and linking two amides with an optimal six-methylene chain length led to increased potency (HMBA: 5 mM inhibitory potency; Fig. 2). Substitution of the amide end groups with bis-hydroxamic acid

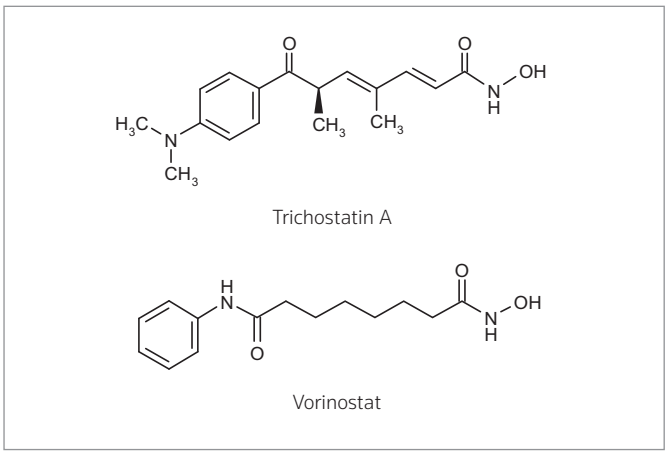

Figure 1. Structures of trichostatin A and vorinostat.

increased potency by two orders of magnitude (SBHA: 30 μM inhibitory potency; Fig. 2). Further optimization via hydrophobic substitution of one of the hydroxamic acid groups led to the discovery of Zolinza™, which proved to be about sixfold more potent than SBHA in causing transformed cell growth and cell death. It was the similarity in structure of Zolinza™ to trichostatin A (Fig. 1), a natural hydroxamic acid compound that had been shown to inhibit histone deacetylases in 1990, which led to the discovery in 1996 that class I and class II histone deacetylases were the targets of Zolinza™.

Dr. Miller went on to describe that histone deacetylase inhibitors are characterized by a common pharmacophore, comprised of metal binder, linker and surface recognition domains (Fig. 3). Analogues of Zolinza™ employing structural variations at each of the three domains have been tested. Addition of methyl on the hydroxamic acid obliterated activity. Shorter methylene linkers were active, but more promiscuous. Longer methylene linkers were tolerated, but the 6-methylene linker was optimal. The surface recognition domain was more tolerant to substitution at the *meta* and *para* positions, but *ortho* substitution on the benzamide was not tolerated. This did, however, identify the opportunity to modulate the physicochemical

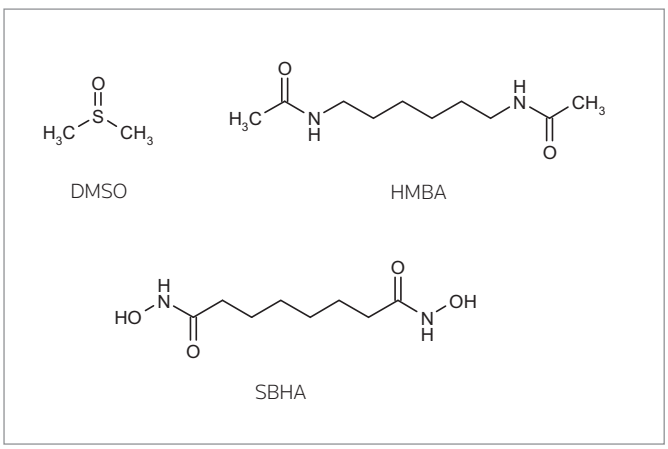

Figure 2. Structure of histone deacetylase inhibitors.

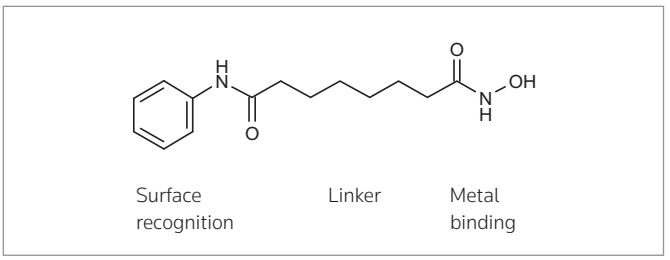

Figure 3. Pharmacophore for histone deacetylase inhibitors.

properties of Zolinza™ and further investigate opportunities to improve potency and selectivity.

Consideration of substrate mimetics as a mode of rational design led to the overlay of Zolinza™ with lysine, resulting in identification of the amino-suberate analogues, which ultimately led to more potent histone deacetylase inhibitors. Further data presented indicated that the next generation of histone deacetylase inhibitors displayed low nanomolar potency and selectivity towards HD1. A refined pharmacophore model containing an internal binding domain in association with the metal binding domain has been proposed.

Dr. Miller finished his presentation by describing that Zolinza™ resulted in durable histone acetylation in lymphoma patients and pharmacokinetic/pharmacodynamic relationship in man following oral administration. He presented data from the CTCL phase II study with Zolinza™ (400 mg p.o. once daily), the primary objective of which was to determine the response rate in the treatment of skin disease in patients with advanced CTCL who have progressive, persistent or recurrent disease. The 400-mg dose of Zolinza™ was well tolerated, and clinically meaningful long-lasting responses were observed. Approximately 30% of patients had at least a partial response. The median time to response was less than 2 months. Phase III studies with Zolinza™ in advanced malignant pleural mesothelioma (single agent) and multiple myeloma (in combination with bortezomib) are ongoing.

DACOGEN® (DECITABINE) – A CASE HISTORY

Dr. Pierre Wijermans (Haga Hospital, The Netherlands) described the use of Dacogen® (decitabine) as an epigenetic therapy for myelodysplastic syndrome (MDS), and described a series of case histories in patients undergoing Dacogen® treatment.

Dacogen® is a cytosine nucleoside (cytidine) analogue that is currently delivered as an intravenous medication for the treatment of MDS. It is believed to exert its antineoplastic effects after phosphorylation and direct incorporation into DNA and inhibition of DNA methyltransferase, causing hypomethylation of DNA and cellular differentiation or apoptosis. It inhibits DNA methylation in vitro at concentrations that do not cause major suppression of DNA synthesis. Dacogen®-induced hypomethylation in neoplastic cells may restore normal function to genes that are critical for the control of cellular differentiation and proliferation. In rapidly dividing cells, the cytotoxicity of Dacogen® may also be attributed to the formation of covalent adducts between DNA methyltransferase and Dacogen® incorporated into DNA. Nonproliferating cells are relatively insensitive to Dacogen® (Patient Information, www.dacogen.com).

MDS is a potentially life-threatening group of bone marrow diseases that alter the production of functional blood cells. MDS is not a rare disease in the elderly, and it is estimated that MDS affects 15-50 people per 100,000 over the age of 70. The incidence of MDS in children is around 5-7% of all pediatric malignancies. Patients with MDS may experience bone marrow dysfunction (anemia, neutropenia and/or thrombocytopenia), hypercellular marrow and show evidence of dysplasia by bone marrow examination. They also have a risk of the disease progressing to acute myeloid leukemia (AML), which is a bone marrow malignancy. MDS is the first malignancy that can be treated with epigenetic therapy (currently two potential drugs of choice: azacitidine and its deoxy derivative Dacogen®).

Dr. Wijermans described the classification systems used to determine the extent or severity of the disease in MDS patients. They include the French-American-British (FAB) classification system, the World Health Organization (WHO) system and the International Prognostic Scoring System (IPSS). Within the IPSS scoring system, subcategories include: low risk, intermediate 1, intermediate 2 and high risk. Prognosis is poor (about 6 months) in high-risk category patients. Dacogen® is only registered for intermediate- and high-risk patients.

Treatment of MDS is generally individualized for each patient, taking into consideration factors such as the severity of low blood count, the risk of progression to AML, the patient's age and overall health. After diagnosis is made, a risk analysis is performed and discussed with the patient. Treatment in younger patients generally involves intensive chemotherapy with allogeneic stem cell transplant. If such treatment is, however, unsuccessful, Dacogen® can be prescribed until a matched unrelated donor transplant is possible. In elderly patients, there are no curative options at present, but alternatives include supportive care (blood transfusion, platelet transfusion, growth factors and antibiotics), therapy that does not influence the natural course of the disease and experimental therapy attempting to influence the course of the disease.

There are two regimens for Dacogen® administration. With either regimen, it is recommended that patients be treated for a minimum of four cycles; however, a complete or partial response may take longer than four cycles. However, Dr. Wijermans provided a case history of an 81-year-old patient with high-risk MDS not able to receive intensive chemotherapy. Both his neutrophil and platelet counts were very low. Following just one cycle of Dacogen®, complete remission in the bone marrow and complete normalization of the blood cell count were observed. However, Dr. Wijermans went on to describe how this type of response is not observed in other patients, and how most patients require several cycles of Dacogen® therapy in order to respond. The number of cycles required for a response is often very patient-specific. Early predictions of which patients will respond and which will not is also undefined, although Dr. Wijermans presented data that could indicate that an early rise in platelet count could be the first sign of a positive response. In addition, initial lactate dehydrogenase (LDH) levels may be an important prognostic factor to predict the outcomes for patients, with those having higher than normal LDH levels typically showing a poorer survival rate. Dr. Wijermans went on to hypothesize that intraindividual differences in the metabolism of Dacogen® may be important for understanding why some patients respond to therapy while others do not.

SECOND-GENERATION HISTONE DEACETYLASE INHIBITORS

Dr. Stephen Shuttleworth (Karus Therapeutics Ltd., U.K.) outlined progress on their second-generation histone deacetylase programs. Histone acetyltransferases and histone deacetylases were initially identified as regulators of chromatin remodeling and gene transcription. Acetylation of specific lysine side chains on histones by histone acetyltransferases or inhibition of deacetylation by histone deacetylases initiates chromatin remodeling. Conversely, histone deacetylation by histone deacetylases would prevent chromatin remodeling and therefore silence gene transcription. Modulation of gene transcription is an attractive drug development opportunity for a number of diseases, the most advanced area being cancer treatment, where the loss of transcriptional control over certain oncogenes and cellular functions results in tumorigenesis. In addition, Dr. Shuttleworth highlighted the emerging precedent of utilizing histone deacetylases in the treatment of immunoinflammatory disorders such as rheumatoid arthritis, psoriasis and systemic lupus erythematosus. Emerging data support the rationale for histone deacetylase inhibition via the nuclear factor NF-κB pathway. Suppression of proinflammatory mediators can be achieved by inhibiting NF-κB-regulated gene expression by reducing the expression, recruitment and activation of the cofactors required for NF-κB transcription.

Presenting data on the pan-histone deacetylase inhibitor program at Karus, Dr. Shuttleworth highlighted progress in this area. Initial results based on in vitro biomarker data using human peripheral blood mononuclear cells (PBMCs) and human breast adenocarcinoma MCF7 cells measuring acetylation on histone H4 (one of the five main histone proteins associated with chromatin structure) indicated that the compounds showed improved activity compared with vorinostat and JNJ-26481585, an oral histone deacetylase inhibiter currently undergoing clinical trials. Furthermore, in vivo antirheumatic efficacy in a mouse collagen-induced arthritis model for the compounds dosed every 4 days for 4 days was superior to the gold standard Enbrel® (etanercept) dosed daily over 15 days. In vitro data confirmed the gradual upregulation of acetylated histone H4, supporting the extended pharmacodynamic response and the potential for achieving sustained histone H4 acetylation in the clinic using low and infrequent dosing. It is hoped that clinical studies on a compound from this series will be initiated by the end of 2011.

The 18 histone deacetylases are classified structurally into class I, IIa, IIb, III and IV. HD6 is a class IIb histone deacetylase, and is characterized as having more limited cellular expression and control of regulatory processes in a more gradual and subtle manner than its class I counterparts. HD6 plays a key role in immune cell function, catalyzing the removal of acetyl groups from the histone tails, but also from many other non-histone proteins. One such protein is the transcription factor forkhead box protein P3, a key regulator in the development and function of regulatory T cells (Tregs). The ability of HD6 inhibitors to control the function of forkhead box protein P3 Tregs could have therapeutic utility not only in oncology, but also in other autoimmune diseases, such as rheumatoid arthritis, transplantation and neurodegeneration. Karus has an HD6 inhibitor program and Dr. Shuttleworth presented data on two compounds that are potent (IC_{50} < 10 nM) and selective (a minimum of 40-fold over 10 other histone deacetylases) inhibitors. The compounds have

excellent development potential, with no potential cytochrome P450-mediated drug–drug interaction liabilities, and good permeability (human colon adenocarcinoma Caco-2 cells), with minimal active efflux. Activity in vivo, similar to Enbrel®, has been confirmed in the mouse collagen-induced arthritis model, with a significant reduction (≥ 55%) in histological scores. In vitro inhibition of proinflammatory cytokines from lipopolysaccharide-, phytohemagglutinin- and anti-CD3- stimulated human PBMCs demonstrated inhibition (≥ 60%) of TNF-α, interferon-γ, IL-1β, IL-21 and IL-23.

BROMODOMAINS – A TRACTABLE EPIGENETIC TARGET FOR DRUG DISCOVERY

The final lecture of the day was delivered by Dr. Jason Witherington (GlaxoSmithKline's EpiNova Discovery Performance Unit [DPU]), and looked beyond histone deacetylase inhibitors to a new area ripe for small-molecule epigenetic drug discovery. Dr. Witherington outlined a journey that started with a black box assay and led to the discovery of inhibitors of bromodomains. Scientists within GSK successfully identified compounds that upregulated apolipoprotein A-I (ApoA-I) using a high-throughput phenotypic screen. These molecules were subsequently optimized to a candidate compound for the treatment of dyslipidemia. Initial attempts to identify the molecular target responsible for the phenotype using conventional cross-screening activities were unsuccessful.

Employing some elegant chemoproteomic techniques, scientists tagged two enantiomers from a series of benzodiazepines to a matrix gel, and demonstrated that the active enantiomer bound to the bromodomain and extraterminal proteins (BET), which comprise the bromodomain-containing proteins BRD2, 3 and 4. There are 58 bromodomains present in 40 mammalian proteins, and one of their biological functions is to regulate gene transcription through binding to acetylated lysine residues on histones. Having identified the molecular target, the scientists went on to employ a range of biophysical techniques (thermal shift, Biacore and Isothermal calorimetry [ITC]) in order to determine the binding site for the compounds. Importantly, this work demonstrated that the compounds bound to the *N*-terminal domain, which contained the tandem bromodomains, and not the *C*-terminal domain, which expressed the extraterminal domain. Using ITC, it was then shown that the compounds bound with roughly equal affinity to both bromodomains in the stoichiometry of two ligands to one full-length protein. Having established the binding site, they went on to perform a fluorescence polarization assay and show a remarkable correlation between BRD4 binding and ApoA-I upregulation. The final confirmation of the binding mode was achieved when the GSK scientists became the first to solve the structure of a small molecule cocrystallized within the acetyl lysine pocket of the bromodomain.

This structure clearly identified the key interactions for recognition and affinity, and Dr. Witherington indicated that EpiNova now has in excess of 100 X-ray structures across the bromodomain family. Dr. Witherington suggested that analysis of these data, and that available in the public domain, indicates that the heterogeneity outside of the acetylated lysine pocket could be targeted in order to identify selective inhibitors across the bromodomain phylogenetic tree. One challenge the EpiNova group foresaw early on was the potential limitations of working with recombinant proteins. As discussed by Dr. Gerard Drewes from Cellzome in an earlier talk, epigenetic targets can exist in large complexes, and the scale of establishing protein expression and assays for a large panel of proteins of interest can be both difficult and resource-intensive. To address this, the EpiNova DPU has formed a successful alliance with the Cellzome group in order to extend the KinoBead technology into epigenetics. This collaborative alliance appears to have made remarkable progress within the bromodomain arena, as evident from the platform's ability to bind to over 80% of the endogenous proteins that contain bromodomains.

Having discussed the chemoproteomic work and the wider opportunity for small-molecule drug discovery across the bromodomain space, Dr. Witherington presented two areas of potential therapeutic utility for the BET family of proteins. The nuclear protein in testis (protein NUT) midline carcinoma (NMC) is an extremely rare and lethal tumor that presents in young individuals and has a median survival time of 6 months. NMC arises from the fusion of the *BRD3/4* and *NUT* genes, and results in the fusion protein remaining strictly in the nucleus via interactions with chromatin. Dr. Witherington highlighted the work of Chris French from Harvard, who demonstrated that silencing of these proteins using siRNA to the BET proteins in NMC cell lines resulted in squamous differentiation and cell cycle arrest, giving hope that inhibition of BRD3/4 binding to chromatin with a BET inhibitor may ultimately lead to a treatment for this incurable disease.

In presenting early emerging preclinical BET biology suggesting the potential for clinical utility in inflammation, Dr. Witherington showed that, following stimulation of macrophages with either lipopolysaccharide (LPS) or polyinosinic:polycytidylic acid (pIC), the BET inhibitor inhibited both IL-6 and interferon-β (IFN-β) but had no effect on TNF-α. Interestingly, the inactive enantiomer of the BET inhibitor had no effect on the above cytokine profile, suggesting the phenotype to be due to BET inhibition. Using chromatin immunoprecipitation (CHIP) experiments, it was shown that the BET inhibitor displaced BRD4 from IFN-β and IL-6 gene promoters. A genome-wide analysis demonstrated that the BET inhibitor selectively inhibited a series of secondary response genes, while having no effect on primary response genes. Dr. Witherington then highlighted the in vivo utility of these new compounds, based on data from an LPS-driven lethal model of endotoxemia, where a dramatic protective effect of the BET inhibitor was shown when the compound was dosed both prophylactically (i.e., pre-LPS challenge) and, most interestingly, therapeutically (i.e., post-LPS challenge).

DISCLOSURES

R. Armer is an employee of Lectus Therapeutics, D. Coe and P. Jeffrey are employees of GlaxoSmithKline, and R. Lock is an employee of Novartis.

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