Conference Scene

2nd Cancer Epigenetics Conference

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Global Technology Community (GTC), Boston, MA, USA, 8–9 November 2012

The GTC Cancer Summit: Novel Approaches to Drug Discovery was divided into two parallel tracks: the 2nd Cancer Epigenetics Conference, and the Protein Kinases and Drug Design Conference. The 2nd Cancer Epigenetics Conference focused on exciting changes in drug discovery that include an unprecedented private and public collaboration on drug discovery in epigenetics through the Structural Genomics Consortium (SGC), which has led to several major breakthroughs including: the development of small-molecule inhibitors that interfere with protein interactions, especially bromodomain-containing protein acetylation readers; the indirect but successful targeting of the elusive *MYC* oncogene; and the identification of epigenetic drugs that are disease-specific. Also reported were the development of clinically useful DNA methylation assays; cell, peptide and protein arrays for testing antibody- and protein-binding specificity; and tools for chromatin capture and DNA modification analysis. Several groups reported on the lack of specificity of some commercial, but unnamed, antibodies used for epigenetic studies.

This meeting summary is divided into two parts. Part 1 is focused on histone writing, reading and erasing; and Part 2 is focused on DNA writing, reading and erasing; RNA epigenomics; and cancer and schizophrenia.

Part 1: histone writing, reading & erasing

■ Structural Genomics Consortium

Dafydd Owen (Pfizer, Canterbury, UK) reported on the both publically and privately funded Structural Genomics Consortium (SGC) that places unencumbered small molecules of potential use to the epigenetics community into the public domain [10]. A goal is to provide initial structure relationship activity (SAR) analysis on small molecules that can be further developed by multiple groups simultaneously. The SGC has made considerable progress recently in the area of bromodomain (BRD) inhibitors that block protein binding to acetylated lysine residues [1–4], and this is discussed further below.

■ Changing strategies for epidrug development

Rabinder Prinjha (Epinova Epigenetics DPU, Stevenage, UK and GlaxoSmith-Kline, Canterbury, UK), a keynote speaker, reviewed the state of epigenetic drug development. Prinjha pointed out that future progress depends on

understanding the histone code and the dynamics of how the code changes [5].

A new drug research area, based on SAR, focuses on inhibiting interactions between proteins – for instance, the BRD and extra C-terminal (BET) domain family of transcriptional regulators, carrying tandem bromodomains and an extra C-terminal bromodomain [6,7]. A number of cancers, including multiple myelogenous leukemia (MLL) and NUT-midline tumors, have fusion proteins with BRD4 domains [8,9], and are blocked by BRD4 inhibitors.

The BRD4 drugs may be useful as anti-inflammatory agents. For instance, a BRD4 inhibitor was shown to reduce a lipopolysaccharide-induced inflammatory lethal response in mice [10], and inhibit transcription of HIV [11]. Some viruses code for BET inhibitors that interfere with the host's response to infection [12]. The epidrugs under development include BET inhibitors (IBET762, IBET151 and IBET726), and non-BET inhibitors that target protein methyl transferases (PMTs), *Ezh2* and protein demethylases against *Jmjd3* (e.g., *GKS1*, *Pad14* and so on).

■ Bromodomain inhibitors

Jun Qi (from the Bradner group, Dana-Farber Cancer Institute, MA, USA) reported on the small molecule, JQ1, developed by the SGC, which interferes with *MYC* activity. In the past, direct drug targeting of *MYC* has not been possible.

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Bradner and colleagues proposed an alternative targeting approach, focused on inhibiting acetylation of lysine residues in histones and transcription activation by MYC [13].

Thienodiazepines were first noted by Mitsubishi Tanabe Pharma Corporation [201] to bind BRD4 motifs, while other research indicated that these simple compounds had anti-inflammatory activity. JQ1 was developed to inhibit downstream MYC effects, although recent data (see below summary of Peter Sandy's report) show that the BET4 inhibitors interfere with MYC expression directly.

Successful tumor targeting by JQ1 inhibition of histone readers was validated *in vitro* on NUT-midline tumor cells [14]. The *NUT1* gene is a nuclear protein expressed in the testes. NUT-midline tumors have a chromosome translocation t(15;19) that creates a BRD4–NUT fusion protein. BRD4 inhibitors are also useful against acute myelogenous leukemia (AML) [15]. As toxicity issues with JQ1 have prevented treatment of patients, a number of groups have developed derivatives as potential drugs.

Qi used a time-resolved fluorescence resonance energy transfer (FRET) temperature-based competition assay to isolate derivatives of JQ1 that interfered with BRD4 binding to the acetylated peptide. A JQ1 derivative with an improved half-life, solubility and potency was isolated by this group. Recently, one JQ1 inhibitor that bound BRD4 protein expressed in the testes reversibly decreased sperm production, and is being explored as a nonhormone male contraceptive [16].

Anastasia Wyce (GlaxoSmithKline) described the preclinical efficacy of a pan-histone deacetylase (HDAC) inhibitor IBET726 against a solid tumor, neuroblastoma. Approximately 40% of neuroblastomas have elevated N-MYC levels, the strongest negative marker of disease outcome. All neuroblastoma cell lines tested had increased cell death when treated with IBET726 [16]. Neuroblastoma cells (13 out of 17) with N-MYC amplification showed a decrease in N-MYC transcription. In addition, IBET726 appears to decrease BCL2 expression partially. In rodent xenograph models, significant inhibition of neuroblastoma tumor growth was seen for a N-MYC-amplified (CHP212) cell line and

in a C-MYC-expressing cell line. The *in vivo* effects are likely due to the silencing of both *MYC* and *BCL2* genes.

Hella Kohlhof (4SC AG, Planegg-Martinsried, Germany) reported on results with a novel class I HDAC inhibitor, 4SC-202. An analysis of gene expression revealed shared and unique changes when three class I inhibitors (entinostat, mocetinostat and 4SC-202) were compared in a mouse xenograph model of colon cancer. The inhibitor 4SC-202 decreased the WNT pathway genes, and affected three characteristics of cancer (stemness, proliferation and epigenetics), and was the only class I inhibitor to induce a strong G2/mitosis arrest. Currently, dose escalation studies are being carried out in monotherapeutic testing with advanced hematological disorders (e.g., Hodgkin's lymphoma [HL], non-Hodgkin's lymphoma, chronic myelogenous leukemia, myelodysplastic syndromes, MLL and AML) patients not previously treated with HDAC inhibitors. Thus far, there appears to be a good response, with a reduction of HDAC activity and re-establishment of lysine acetylation levels in peripheral blood, as well as stabilization of the majority of patients (14 out of 15). Gene-expression analysis is ongoing.

Stefan Henning (4SC AG) reported that resminostat (an oral pan-HDAC inhibitor with a good safety record) induced growth arrest and apoptosis in solid and hematological tumors, and sensitized tumors to other cancer therapies. Drug resensitization is believed to occur through modification of the epigenome that re-establishes the 'stem-cell-like' phenotype of cancer cells until, and unless, irreversible genetic resistance mutations develop [17]. The drug is in stage II clinical testing for hepatocellular carcinoma (HCC), HL and colorectal carcinoma. Resminostat alone or in combination with sorafenib (a small-molecule inhibitor of several tyrosine kinase-binding proteins, e.g., VEGFR and PDGFR) and RAF kinases, has increased progression-free survival of patients, and medium overall survival in HCC and HL versus colorectal carcinoma patients, respectively. Resminostat is covered by a composition-of-matter patent for the USA and EU until 2030, and has orphan drug status by the US FDA and EMA for HCC and HL.

Peter Sandy (Constellation Pharmaceuticals, MA, USA) reported on the observations that both thienodiazepin (JQ1) and isoxazole inhibitors of BET domains inhibit MYC expression directly [18]. A new BRD4 inhibitor, CP1–456, was found to be more effective at tumor inhibition in a nude mouse model of cancer than earlier drugs. Currently, a FRET screen is being used to develop and improve BET-specific inhibitors of the NF- κ B pathway.

Carsten Hopf (Cellzone GmbH, a GSK company, Heidelberg, Germany) reported on a global proteomic strategy to identify intracellular protein complex targets that bind to small-molecule HDAC inhibitors [19]. The assay incubates cell extracts with inhibitors in solutions, then inhibitor–protein complexes are captured on an affinity matrix, and the immobilized proteins identified by mass spectrometry [18]. The assay was used to characterize protein complexes bound by 16 small-molecular-weight HDAC inhibitors. Multiple HDAC complexes were identified, including a novel mitotic deacetylase complex, and several non-HDAC targets. The HDAC inhibitors cluster into distinct groups based on their target profiles with the aminobenzamides having preferential binding to the NCoR-containing complexes, and little or no binding to the Sin3-containing complexes.

■ Sirtuins HDACs

David Sinclair (Harvard Medical School, MA, USA) reviewed work on sirtuins (SIRT; which make up NAD⁺-dependent deacetylase Class III HDACs) implicated in mediating health benefits including increasing life expectancy of caloric restriction diets. Three sirtuins (SIRT1, 2 and 3) are important for cancer growth, and inhibitors have antitumor activity [20].

■ Histone methylation modulation

Robert Copeland (Epizyme, Inc., MA, USA), a keynote speaker, reviewed protein methylation at lysine (K) and arginine (R) residues, and drug targeting of PMTs [21,22]. Protein methylation is important in a broad spectrum of biological responses, and diseases and a large number of PMTs are altered in cancer.

Known protein lysine methyltransferases, including the MLL protein, have

a SET domain and target histone tails. MLL, WRAD and other family members are responsible for mono-, di- and tri-methylation of H3K4, respectively, a modification seen in 3' gene regions, and enhancer regions, open reading frames and promoters, respectively. The not-SET domain histone methyltransferase, DOT1 methyltransferase (MT), uniquely mono-, di- and tri-methylates a nontail residue H3K79 – a change associated with active transcriptions, and a driver for MLL [23].

The first HMT inhibitors were competitive S-adenosyl methionine (SAM) inhibitors. Today, SAR analysis has led to the development of other types of inhibitors. For instance, the inhibitor EpZ5676 is selective for the DOT1 MT and only kills cells with an MLL rearrangement. DOT1 MT, unlike other lysine methyl transferases, does not have a SET domain and instead, is more structurally similar to RMTs. Inhibitor EpZ5676 is in Phase I treatment trials. Another drug, EPZ568, targets the MT EZH2 (PRC2), the only enzyme that mono-, di-, and tri- methylates H2K27. EZH2 mutations have been identified in non-Hodgkin's lymphoma.

Michael Cosgrove (SUNY Upstate Medical University, NY, USA) reported on the development of MLL inhibitors. The MLL genes encode a PMT enzyme with SET1 domains. Structural analysis of the core MLL1 complex (containing MLL-N, MLL-C, WDR5, RbBP5, Ash21 and Dpy-30 proteins) demonstrated that the WIN motif in the N-SET (not the SET domain) was required for complex formation by specifically binding to the WDR5 protein [24]. Peptides encoding this Win motif bound to MDR5 protein, and prevented complex formation. Translocations and amplification of the *MLL1* gene are linked to aggressive myeloid and lymphocytic leukemias. The N-SET domain is conserved in translocation; hence, the WIN peptides may prove useful in these diseases [25].

Akane Kawamura (University of Oxford, Oxford, UK), starting with a SGC small molecule, developed a broad-spectrum JmjC-domain-containing lysine demethylase inhibitor, 1RX1, using high-throughput AlphaScreen technology where FRET between fluorescent beads detects interaction between molecules

immobilized to their surface [26]. Here, a donor streptavidin-coated bead with immobilized biotinylated histone tails was mixed with antily sine demethylase antibody immobilized to a Protein A-coated bead. The proteins were purified and identified and a broad-spectrum 2-oxoglutarate-dependent oxygenase was identified [27].

Tamara Maes (Oryzon Genomics, Barcelona, Spain) spoke of translation of KDM1A (LSD1s) inhibitor use in the clinic. Overexpression of LSD1 protein in different cancers is linked to poor prognosis. All-*trans* retinoic acid [28] therapy of patients with the acute promyelocytic leukemia subtype of AML has been successful, although resistance can develop. Nonacute promyelocytic leukemia AML patients responded to all-*trans* retinoic acid therapies when combined with the LSD1 inhibitor tranylcypromine [29]. Maes reported that SAR data were used to design and synthesize approximately 800 LSD1 inhibitors. One inhibitor, approximately 1000-fold more effective than tranylcypromine and with excellent pharmacological properties, is being further developed and tested in mouse xenograft models.

Yujun Zheng (Georgia State University, GA, USA) discussed novel compounds based on carbocyanine (a near infrared dye) that inhibits PRMT enzymes [30] responsible for mono-, di- and trimethylation of R residues in histone and other proteins. Carbocyanine derivatives having indolium, benz(e)indolium or benzo(c,d)indolium heterocyclic moieties were developed as PRMT inhibitors and cell imaging reagents. Compound 11, a benzo(c,d)indolium derivative, is a non-competitive (with SAM and histone 4), reversible pan inhibitor for PRMTs including several mutant enzymes, with potencies in a therapeutic range, that arrested HELA cells in S phase.

Wei Xu (University of Wisconsin, WI, USA) reported on a cell-based high-throughput screen for PMRT4 (CARM1) inhibitors. PMRT4 knock-out mice die at birth and defects are seen in cartilage, aveolar and muscle and so on. This enzyme is linked to activation of relevant transcription factors including NF- κ B, p53, E2F1 and steroid receptors such as ER α that are overexpressed in the breast cancer cell line

MCF7 [31]. The developed cell-based, time-resolved FRET uses PBBP1 methylation to monitor cellular activity of CARM1.

■ Drug efficacy screening

Yvonne Heubach (University of Tübingen, Tübingen, Germany) used cultured spheroid-resembling microtumors from patient tumors for *ex vivo* analyses of drug efficacy. Lysates containing 3–5 μ g protein from individual spheroids made up of approximately 2000 cells are analyzed in a reverse-phase protein microarray consisting of 10,000 microdots. This system analyzed the molecular dose responses for different drugs on resected human colon carcinomas and on cell lines. This approach allows screening of hundreds of extracts in parallel for specific protein levels using antibodies.

■ What do antibodies & proteins target?

Heubach also reported on a histone peptide bead array for assaying antibody specificity. The library has 379 different peptides with 1–4 modifications (acetylation, methylation, phosphorylation and citrullination). Testing 60 commercially available antibodies from six providers showed that 20% had high specificity, 40% acceptable specificity and 40% had high cross- or no-reactivity. This assay was used to examine the role of nearby modification on antibody binding. For instance, H3K8m interfered with anti-H3K9ac(2) but not anti-H3K9ac(1) antibody binding. Also, the presence of a H3S10p interfered with binding of both antibodies. By contrast, the presence of H3K9acS10p did not interfere with binding of the same antibodies. Also described was a bead array assay that had 135 native and modified proteins that were involved with chromatin structure apoptosis, proliferation, mTOR signaling, cell cycle and MARK signaling.

Bing Zhu (University of Houston, TX, USA) reported on profiling of >60 PMTs (>50 protein lysine methyltransferases and nine PRMTs) in cancer cells using histone peptide microarrays produced *in situ* using a μ ParaFlo[®] PepArray system (LC Sciences, TX, USA). In this assay, nuclear extracts and SAM are incubated with the peptide array that is subsequently scanned with antihistone methyl antibody. Antibody binding was affected by mutations

in adjacent amino acid residues. Future experiments will profile PMT inhibitors.

Ulf Reimer (JPT Peptide Technologies GmbH, Berlin, Germany) described a 4000-member peptide library with all histone (H1, H2, H2B, H3 and H4) modifications that was used to study antibody specificity [32].

Part 2: DNA writing, reading & erasing

■ DNA modification

Taiping Chen (University of Texas, MD Anderson Cancer Center, TX, USA) reviewed the link between DNA methylation and disease. In mice, knockouts of the *DNMT1* and *DNMT3b* genes are embryonic lethal mutations. DNMT3a knockout mutations lead to postnatal death. Knockouts of the *DNMT3L* gene lead to an absence of imprinting and sperm. In humans, mutations in the *DNMT1* gene are linked to sensory defects, neuropathology and dementia. Mutations in the *DNMT3A* gene are linked to AML, chronic myelogenous leukemia and T lymphoma. Mutations in DNMT3B are linked to patients with immunodeficiency, centromeric instability and facial anomalies syndrome. The *DNMT3A* mutations R882H, R88C and R88S are associated with poor prognosis in AML. These mutations occur at the dimer interface, but do not interfere with dimer formation.

■ Translation of DNA methylation monitoring to the clinic

Several groups developed clinical bisulfite-based quantitative PCR (qPCR) DNA methylation assays. PCR-based assays ease sample handling and were used to monitor immune cell populations, disease states and drug efficacy. The approach was validated through correlations with mock mixtures, comparisons to the literature, and cell-sorting results in the case of blood cells.

Thomas-Oliver Kleen (Epiontis GmbH, Berlin, Germany) reported on a developed clinical platform that monitors responders and nonresponders to cancer vaccines and other immunotherapeutics. Single-target qPCR tests are used to distinguish between circulating cells including: T-reg, Th17, overall T, overall B, CCRs, granulysin, neutrophils, granulocytes, naive CD8 T and CD4 monocytes. For instance, the

demethylated *Foxp3* gene target could detect levels of T-reg cells down to 30% of total blood cell concentration.

William Accomando (Brown University, RI, USA) reported on global [33] and locus-specific methylation monitoring. qPCR methylation assays at individual loci, such as *CD3Z*, *FOXP3* and *NKp46*, can be used to measure T cell, Treg cells and NK cells, respectively. In glioblastoma multiforme patients, T cells and Treg cells were decreased in blood, and poor prognosis was linked to increased infiltration of these cells into tumor tissues [34]. Decreased NK cells were found in archival head and neck cancer tumors [35].

Andrew Sledziewski (Epigenomics AG, Berlin, Germany) reported on three gene DNA methylation markers: *SEPT9*, *SHOX2* and *PITX2*. A methylated *SEPT9* marker detects circulating colorectal cancer cells with specificity and sensitivity of 95 and 81%, respectively [36], and is proposed to replace screening colonoscopies. Methylated *SHOX2* is used to detect lung cancer in bronchial lavages. Epigenomics has put global methylation data available online for the NCI160 cancer cell line panel versus drug treatments. For instance, global methylation monitoring was used to test the sensitivity of different cells to EGFR inhibitors.

■ Analyzing DNA modification

Sriharsa Pradhan (New England Biolabs, MA, USA) described types and importance of DNA modifications [37]. The enzymes TET1, TET2 and TET3 demethylate 5-methyl cytosine (5mC) through an oxidation reaction to form 5-hydroxymethyl cytosine (5hmC) [38], then a proposed series of reactions mediated by TET and other enzymes (e.g., deaminase, base excision repair proteins and TDG) to reform unmethylated C.

DNMT1 is stabilized by AKT1 kinase phosphorylation of SER143, which in mammals peaks in early S phase [28]. DNMT1 SER143 phosphorylation and LYS142 monomethylation (by SET7 lysine methyltransferases) are mutually exclusive modifications [39,40]. Similar methylation–phosphorylation switches can be found on chromatin.

5hmC is found in promotor and gene bodies primarily in genes expressed both

in nuclear and mitochondrial DNA. Growing evidence indicates that 5hmC is important for brain function, embryonic cell development and in diseases such as cancer and diabetes [38].

Analytical tools for DNA studies use enzymes that differentially modify 5mC and 5hmC. The enzyme β -glucosyltransferase (T4-BGT) transfers the glucose moiety of uridine diphosphoglucose to 5hmC. The restriction enzymes MspI and HpaII can be used to distinguish between 5hmC and 5mC. For instance, the MspI enzyme recognizes and cleaves sequences with 5-glycosyl hmC, but not methylated or unmethylated cytosine residues. The Hpa II enzyme only cleaves unmethylated DNA. These reactions can be used in endpoint or real-time PCR assays. The use of methylation-binding proteins can replace antibodies for affinity enrichment and identification of mC-containing DNA, and was used in affinity enrichment experiments to identify enhancer sequences with 5hmC.

The novel group of MspJ1 family restriction enzymes require a central 5mC or 5hmC and generates a 22-base double-stranded fragment [41]. Experiments with MSPJ1 enzyme were used to demonstrate that 98% of mC are in the dinucleotide sequence CpG, while 1.2 and 1.3% are in the trinucleotide sequences CHH and CHG, respectively, with H = A, T, or C. The PvuRTsII enzyme requires two central Cs on both strands, with one being 5hC or 5-glycosylhmC, to generate a double-stranded fragment 9–13 bp in length.

■ Chromosome conformation analysis

Shoulian Dong (in place of Vasiliki Anest, Life Technologies, MA, USA) focused on describing the newly developed chromosome capture assay based on methods described by Dekker [42,43], which allows physical proximity measurement of different chromatin regions *in vivo*. In this assay, DNA is crosslinked *in vivo*, then cell lysates are treated with a restriction enzyme to generate a small fragment, followed by a DNA ligation reaction that promotes linking between nearby DNA ends. The DNAs that make up the chromosome capture library can be analyzed by PCR, or by sequencing to generate locus-specific or global information.

■ RNA epigenetics & chromatin

Claes Wahlestedt (University of Miami, Miller School of Medicine, FL, USA) reviewed ncRNAs and their importance in disease [44]. Recent finding revealed that 95% of the genome is transcribed (ENCODE project [102]), and that 70% of genes have natural antisense transcripts (FANTOM project [103]). lncRNAs (>200 nucleotides in length) include natural antisense transcripts derived from the complementary strand of the protein-coding or noncoding region, and may overlap with exons, regulatory and 3' regions of genes. In humans, there are approximately 5000 lincRNAs, and one out of three of these are from introns. The lncRNA can interact with transcriptional or chromatin complexes and represent potential drugable targets. For instance, recently a *BDNF*-antisense antagonist consisting of linked nucleic acid gapmers was used to increase transcription.

■ Repurposing cancer drugs

Cassandra Smith (Boston University, MA, USA) discussed the relationship between schizophrenia and cancer. Schizophrenia is a complex lifelong illness of epidemic proportions, affecting approximately 1% of the population and costing the USA approximately US\$160 billion/year. This illness is linked to both genetic and environmental factors that overlap those linked to cancer, including genomic and epigenomic instability [45,104]. Furthermore, in some cases there are anticorrelations between cancer and schizophrenia occurrence. Multiple, variable and widespread genomic instabilities are detected that include aneuploidy, multiple rearrangements involving (rare) fragile sites, small but elevated rates of copy number variations, and heritable and *de novo* mutations. Environmental components linked to disease occur early in life and include malnutrition, viral infection, inflammation and oxidative stress.

Although genetic results are disappointing, many studies detect an elevated frequency of disease in close relatives of ill individuals, and this has led to a misconception that schizophrenia is a genetic disease. Complexity modeling of factors linked to schizophrenia suggests that the frequency of disease is largely due to *de novo* mutations, rather than inheritance of disease

mutations. This observation reminds us that the term ‘heritability’ refers to the amount of variation in phenotype due to genetics, and not the number of individuals in the population with the genetic form of disease. These results further align schizophrenia with other common diseases, such as cancer, with strong environmental components, and argue that anticancer drugs may be repurposed for antischizophrenia drugs.

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The author has no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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