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Development of live-cell imaging probes for monitoring histone modifications

Kazuki Sasaki [a,b], Akihiro Ito [b,c,d,*], Minoru Yoshida [b,c,d]

[a] Japan Science and Technology Agency, PRESTO, 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan
[b] Chemical Genetics Laboratory, RIKEN Advanced Science Institute, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan
[c] Chemical Genomics Research Group, RIKEN Advanced Science Institute, Wako, Saitama 351-0198, Japan
[d] Japan Science and Technology Agency, CREST Research Project, Kawaguchi, Saitama 332-0012, Japan

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ABSTRACT

The combination of histone posttranslational modifications occurring in nucleosomal histones determines the epigenetic code. Histone modifications such as acetylation are dynamically controlled in response to a variety of signals during the cell cycle and differentiation, but they are paradoxically maintained through cell division to impart tissue specific gene expression patterns to progeny. The dynamics of histone modifications in living cells are poorly understood, because of the lack of experimental tools to monitor them in a real-time fashion. Recently, FRET-based imaging probes for histone H4 acetylation have been developed, which enabled monitoring of changes in histone acetylation during the cell cycle and drug treatment. Further development of this type of fluorescent probes for other modifications will make it possible to visualize complicated epigenetic regulation in living cells.

1. Introduction

Despite identical genome sequences, cells acquire and maintain unique tissue-specific gene expression pattern during differentiation. The concept of epigenetics, initially described by Waddington in 1942, has shed new light on the developmental phenomena above the level of genome, the gap between genotype and phenotype.[1] Because transcriptionally active chromatin was tightly associated with histone acetylation, histone acetylation was proposed as an epigenetic code.[2] Furthermore, Strahl and Allis proposed histone code hypothesis; combinatorial histone modifications regulate the adequate gene expression in appropriate phase.[3] These histone modifications, mainly phosphorylation, acetylation, and methylation of N-terminal tails of histones, are reversibly and dynamically controlled by modifying and demodifying enzymes. Although histone methylation had been considered a stable modification, it became clearer that methylation is also dynamically modulated when lysine-specific demethylase, LSD1, was discovered in 2004.[4] Although it is generally accepted that histone modifications serve as epigenetic marks to determine the cell fate, it remains unclear when, where, and how histone modifications are induced or removed during cellular events such as cell division, differentiation, and reprogramming, mainly due to the lack of imaging tools that allow monitoring the histone modifications in living cells.

Herein, we briefly introduce general imaging techniques for epigenetics focusing on histone modifications; phosphorylation, methylation, and acetylation. We include a method using an antigen binding fragment (Fab)-conjugated chemical fluorescent dye.[5,6] Finally we discuss recent advances in imaging histone modifications via Förster/fluorescence resonance energy transfer (FRET).[7–10] FRET has previously been used for detecting the intracellular dynamics[11,12] of Ca^{2+} and protein phosphorylation[13–20]; but recently FRET-based probes have successfully visualized histone acetylation, providing an application to drug screening and evaluation.[9,10]

2. Probing with an antigen-binding fragment (Fab) labeled with a fluorescent dye

An imaging tool using a Fab of IgG for endogenous histone modification in living cells has been reported in 2009.[6] Two Fab fragments, Fab311 or Fab313, were prepared from monoclonal antibodies that can recognize phosphorylated histone H3 at S10 adjacent to un-, mono-, and dimethylated H3K9 or di- and trimethylated H3K9, respectively. The Fab fragments were conjugated with fluorescent dyes, then were loaded into cultured cells using glass beads or were injected into mouse embryos. Because a Fab fragment (~50 kDa) is much smaller than whole IgG, it is able to pass through nuclear pore complex. These fluorescent dye-labeled Fab fragments allowed monitoring the phosphorylation of histone H3 at S10 in cultured cells and mouse embryos. A different spatiotemporal pattern of phosphorylation of histone H3S10 between normal cells and cancer cells was also observed using the Fab311 fragment. In addition, it was revealed that the Fab313 fragment preferentially concentrated at maternal chromosomes, while the Fab311 fragment distributed in both maternal and paternal

* Corresponding author. Tel.: +81 48 467 9518; fax: +81 48 462 4676.
E-mail address: akihiro-i@riken.jp (A. Ito).

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chromosomes during one- and two-cell stage embryos. Recently, researchers developed Fab fragments for other modifications of histone H3: H3K27me3, H3K27ac, H3K9ac.[5] All the reported Fab fragments can be used in living cells. H3K9ac and H3K27ac were visualized by the fluorescently labeled Fab fragments in mouse preimplantation embryos, but the Fab fragments for H3K4me and H3K9me failed to stain the in vivo modifications in cells due to low affinities. Importantly, the fluorescently labeled Fab fragments for monitoring endogenous histone modifications do not affect cell cycle progression. Therefore, it is also possible to develop imaging probes using genetically encoded antibodies (e.g. single chain antibody), which are stably expressed in a variety of cell types. In this case, it is not necessary to directly introduce the fluorescent probes into cells, because they can be expressed in cells by conventional transfection or infection using viral vectors, which may allow development of transgenic mice.

3. Probes based on Förster/fluorescence resonance energy transfer (FRET) for epigenetics

Another approach for monitoring epigenetic alternations in living cells has been designed based on the principle of FRET, which uses two distinct fluorescent molecules. FRET is the transfer of the excited-state energy from the initially excited donor to acceptor only when the two fluorophores are close together with an appropriate orientation. FRET-based probes are typically tandem fusion proteins consisting of a substrate, a flexible linker, a domain recognized post-translational modifications, and the two different-colored mutants of GFP, for example, CFP and Venus (Fig. 1a). Two groups have reported FRET-based probes for detecting epigenetic histone modifications. One group developed FRET-based probes for methylation of histone H3K9 and at H3K27[7] and for phosphorylation of histone H3S28.[8] The H3K9 methylation probe utilized HP1 chromodomain as a modification recognition domain and a short peptide of histone H3 (residues 1–13) as a substrate domain, whereas the H3K27 methylation probe used the Polycomb chromodomain and residues 24–35 of histone H3. A nuclear localization sequence was added for their nuclear localization, due to the lack of the histone globular domain responsible for the nuclear import and chromatin association in these probes. Both H3K9 and H3K27 probes exhibited an increase in FRET efficiency in vitro, when vSET, a histone H3 methyltransferase from *Paramecium bursaria* chlorella virus, and *S*-adenosyl methionine, a methyl donor, were provided. Furthermore, using the H3K9 probe, it was shown that a methylation level at K9 of histone H3 in MEF lacking Suv39h1 and Suv39h2, methyltransferases for histone H3K9, was lower than that in wild-type MEF. Although histone methylation had been considered enzymatically irreversible, it became accepted that histone methylation is dynamically regulated by histone methyltransferases and demethylases in living cells, as several demethylases such as LSD1 and Jumonji C family have been discovered.[21] However, these methylation probes have not been shown to detect the dynamic change in histone methylation in living cells.

A probe for phosphorylation of histone H3 at serine 28 also utilized a short peptide sequence as a substrate domain, which contains two potential phosphorylation sites (H3S10 and H3S28). The phosphoserine binding domain in the probe was 14-3-3τ, which naturally binds to the phosphoserines of Cbl. The in vitro response and reversibility of the phosphorylation probe, consisting of CFP, 14-3-3τ, linker, an H3 peptide, and YFP, was demonstrated using Msk-1, a kinase for both S10 and S28 of histone H3 and PP1, a serine/threonine phosphatase. This suggests that it is possible to develop a modification probe even if its endogenous recognition domain does not exist. The mutagenesis study revealed that it specifically recognized the phosphorylation at H3S28. On the other hand, another phosphorylation probe, consisting of CFP, the H3 peptide, linker, 14-3-3τ, and YFP, was able to respond to phosphorylation at both H3S10 and H3S28. These results suggest that the site specificity of the former phosphorylation probe does not reflect an inherent selectivity of 14-3-3τ, but rather the reporter's geometry. An NLS-fused probe for phosphorylation at H3S28 exhibited the dynamic increase and decrease in phosphorylation at H3S28 during mitosis in living cells. However, the NLS-fused phosphorylation probe was distributed in a whole cell even after mitosis, where the nuclear-envelope is broken down and endogenous core histones are in condensed chromatin, suggesting that the FRET signal does not always reflect the level of phosphorylation in chromatin. In addition, because the phosphorylation probe may exist as a dimer due to the dimerization potential of 14-3-3τ, the possibility that intermolecular FRET rather than intramolecular conformational change upon phosphorylation occurs in the probe cannot be ruled out.

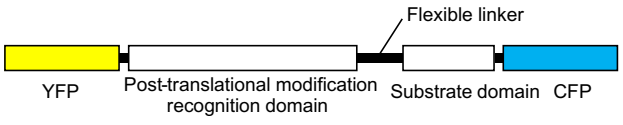

Figure 1. FRET-based epigenetic sensor. Schematic representation of the domain structures of FRET-based epigenetic sensor.

In 2009, a FRET-based probe for histone H4 hyperacetylation has been reported, named Histac.[9] Histac is a tandem fusion protein, consisting of Venus (one of the GFP variants with yellow fluorescence), a bromodomain region of BRDT, a flexible linker, full-length histone H4, and CFP (Fig. 1). BRDT, a testis-specific BET (bromodomain and extra-terminal domain) protein, contains two bromodomains, one of which binds to acetylated histone H4 at both K5 and K8. To effectively incorporate the probe into chromatin, a full-length histone H4 was used as the substrate domain. Histac is acetylated in response to treatment with trichostatin A (TSA)[22] (Fig. 2), a specific histone deacetylase (HDAC) inhibitor. The acetylation-dependent intramolecular conformational change in the probe induces a significant decrease in the FRET efficiency between Venus and CFP. When Venus in Histac was replaced by mVenus, a monomeric form of Venus, it reduced the ability to change the emission ratio in response to TSA compared with Histac. The increase in the FRET efficiency by heterodimerization between Venus and CFP in the non-acetylated state of Histac may contribute to the ability to induce a large emission ratio change upon acetylation of the substrate domain. A mutant lacking the C-terminal globular region of histone H4 was not efficiently acetylated in response to TSA, because it was not incorporated into chromatin, but was located in both the nucleoplasm and the cytoplasm. These results suggest that incorporation of the probe into chromatin is required for correctly monitoring histone acetylation. As was the reversible histone hyperacetylation induced by TSA, the response of Histac to TSA treatment was reversible in a similar time-dependent manner.

The success in the first FRET-based imaging probe recognizing acetylated histone H4K5 and H4K8 prompted the researchers to further develop new probes specific for other acetylation sites. Histac-K12 is such a new probe visualizing in vivo histone H4K12 acetylation.[10] To recognize the acetylation of histone H4K12, the bromodomain of BRD2 was used as the acetylated histone-binding domain. The ability of Histac-K12 to respond specifically to H4K12 acetylation was verified by introducing mutations in each acetylation site; only the mutant probe containing H4K12R lost the response to TSA-induced acetylation. Using these probes, the dynamic changes in histone H4 acetylation during mitosis was investigated, because the dynamic behavior of histone H4

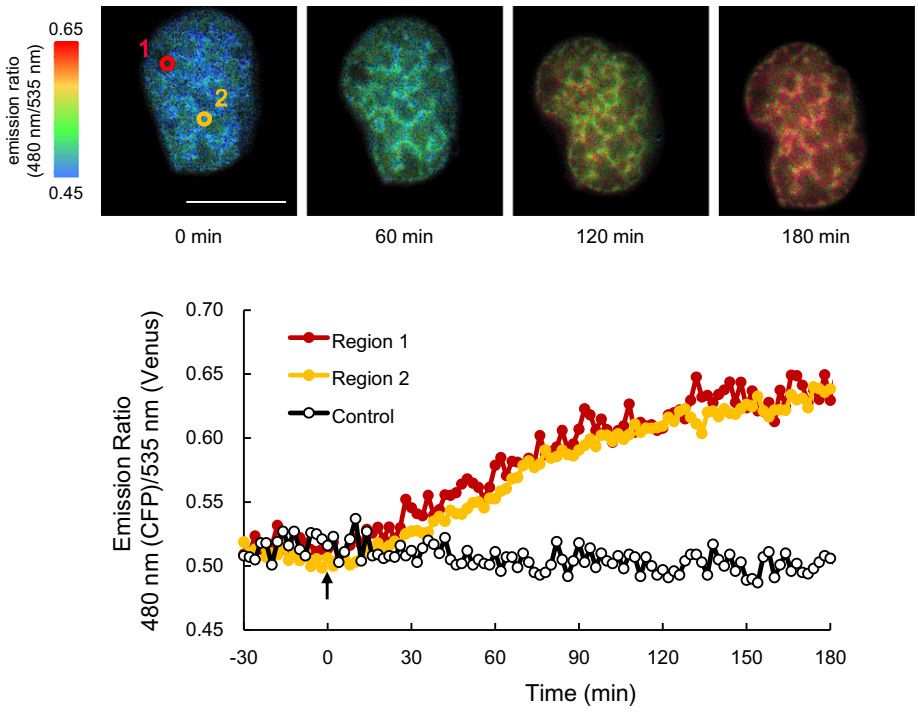

Figure 2. Visualization of dynamic change in the histone acetylation level in response to TSA using Histac in living cells. Pseudo-color images of the 480/535 nm emission ratio at the indicated times in the nucleus of a COS7 cell expressing Histac. The COS7 cell was treated with 1 μM TSA at 0 min or 0.1% EtOH as a control. Scale bar; 10 μm.

acetylation in mitosis was controversial. Histac visualized that the acetylation state of histone H4 at K5 and K8 was decreased during mitosis, and immediately recovered after progression into the G1 phase. In contrast, acetylation of histone H4K12 was maintained throughout mitosis. As histone H4K12 is important for expression of genes required for the G1 progression, it should be kept during mitosis for the immediate onset of gene expression in G1. Thus, site-specific histone modifications play important roles as epigenetic marks, and the development of imaging tools for each specific site allows extensive analysis of epigenetic processes for development, differentiation, and reprogramming.

4. Evaluation of HDAC inhibitors in living cells using Histac probes

Epigenetic gene regulation by histone modifications is involved in diseases such as cancer. Indeed, two HDAC inhibitors, suberoylanilide hydroxamic acid (SAHA) and FK228 (Fig. 3), were recently approved as anti-cutaneous T-cell lymphoma drugs and several HDAC inhibitors are currently evaluated in clinical trials for anticancer therapeutics.[23] Therefore, observation of a dynamic behavior of histone acetylation upon treatment with HDAC inhibitors in living cells is not only important for understanding mode of action of HDAC inhibitors in cells but also beneficial for a drug development. Indeed, cellular response to FK228 in living cells has been unveiled using a series of Histac probes.[9,10] FK228 is a cyclic depsipeptide with an intramolecular disulfide bond,[24] which is reduced in cells, giving an active sulfhydryl group that can interact with the active-site zinc in the catalytic pocket of HDAC enzymes.[25] However, it is still unclear how much time is necessary for this activation process in cells. Analysis using Histac revealed that FK228 induces histone acetylation in living cells within minutes, suggesting that the intramolecular disulfide is reduced as soon as FK228 is introduced into cells. Importantly, an analysis using Histac-K12 revealed that the effect of FK228 was apparently irreversible in living cells,[10] although previous study showed that FK228 reversibly interacts with the HDAC active site in vitro[25] (Fig. 4). This observation is consistent with a recent report that FK228-treated cells maintained high histone acetylation levels even after the compound removal.[26] This is probably due to the lower efflux of the compound than its influx rate once it was incorporated and reduced in cells. In addition, dynamic changes in histone acetylation upon treatment with various other HDAC inhibitors were also evaluated in living cells by Histac series of the probes.[9,10] Thus, the FRET-based imaging probes appear to be powerful tools for investigating in vivo action of HDAC inhibitors.

5. Evaluation of bromodomain inhibitors in living cells using Histac probes

Histone code hypothesis consists of three steps, 'writing' by histone modifying enzymes, 'erasing' by histone demodifying enzymes, and 'reading' by proteins interacting with specific histone modifications.[2,27] Both writing and erasing steps have been shown to be promising targets for antitumor drug development.[28] Accordingly, inhibitors of epigenetic writers and erasers have been extensively investigated. On the other hand, small molecules that target the reading step have not been explored until recently. Epigenetic output by histone modifications regulated by histone modifying and demodifying enzymes is believed to be mediated by epigenetic readers. In the case of histone acetylation, bromodomain-containing proteins such as transcriptional coactivators and chromatin remodeling factors act as readers through a bromodomain that specifically recognizes acetylated lysine residues. Theoretically, small molecules that can bind the acetyl-lysine-binding site in bromodomain may interrupt histone acetylation-mediated chromatin regulation by inhibiting the interaction between a bromodomain-containing protein and histone acetylation (Fig. 5). Filippakopoulos et al. reported (+)-JQ1, a BRD4 bromodomain inhibitor, developed by using the biochemical platform and co-crystalization (Fig. 6 and Table 1).[29] They demonstrated that (+)-JQ1 exhibited potent antitumor activity against NUT midline

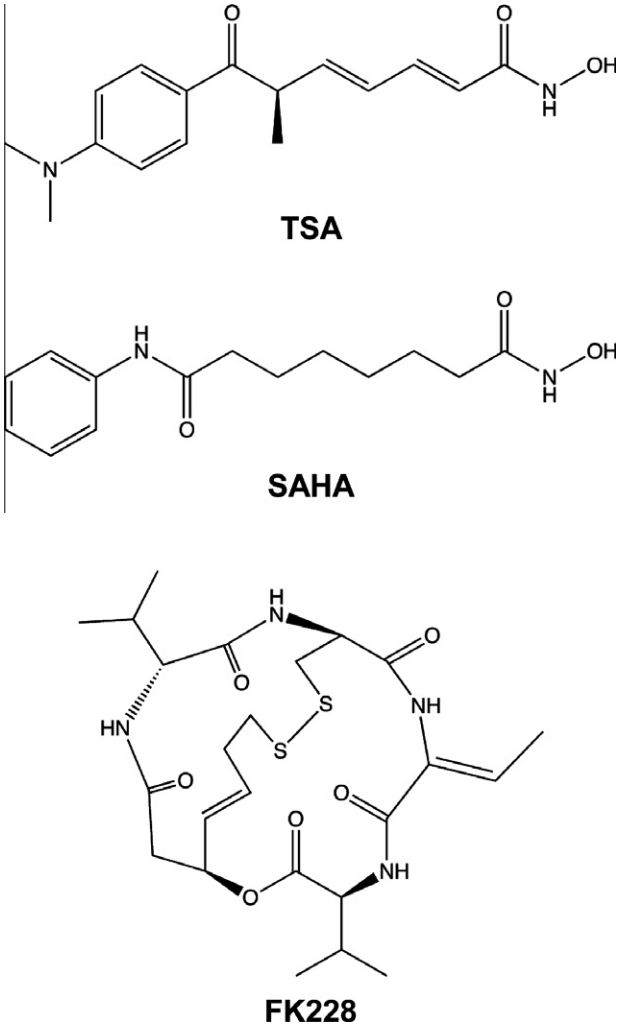

Figure 3. Structure of HDAC inhibitors.

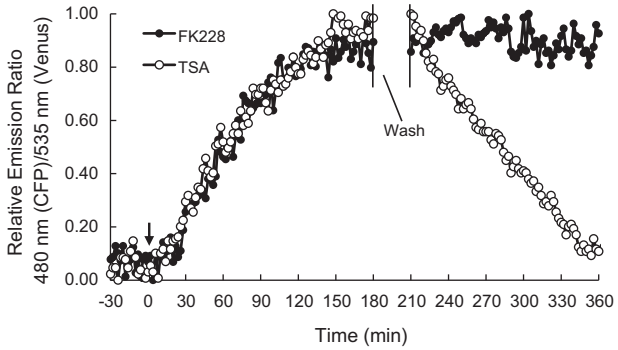

Figure 4. Dynamics analysis of HDAC inhibitors. Time courses of relative emission ratio of COS7 cells expressing Histac-K12. TSA or FK228 was added to culture at 0 min and then was washed with growth medium at 180 min and further cultured.

carcinoma (NMC), in which the t(15;19) chromosomal translocation occurs thereby producing an in-flame fusion protein consisting of N-terminal bromodomains of BRD4 and NUT, a nuclear protein in testis.[29] The stereoisomer (−)-JQ1 showed no significant interaction with bromodomains and was inactive to inhibit NMC. Furthermore, RNAi screening recently revealed that BRD4 is critically required for maintenance of acute myeloid leukemia,[30] an aggressive hematopoietic malignancy that is frequently associated with aberrant chromatin state. Suppression of BRD4 by either shRNA knockdown or (+)-JQ1 had potent antitumor activity in vitro and in vivo.[30] Thus, the BRD4 bromodomains are crucial for the pathogenesis of various types of aggressive cancers and its inhibitors will be promising for therapy. Conversely, Nicodeme et al. reported that I-BET (GSK525762A) synthesized as an acetylated histone mimic, which shares benzodiazepine ring as a common structural skeleton with (+)-JQ1 (Fig. 6), acts as an inhibitor of the BET family of proteins (Table 1).[31] The authors demonstrated that I-BET disrupts chromatin complex responsible for the expression of inflammatory genes in activated macrophages and suppressed LPS-mediated inflammation in vivo. In addition, a combination of phenotype screening and structural optimization of benzodiazepine as a main scaffold recently allowed to synthesize another BET bromodomain inhibitor, named GW841819 (Fig. 6 and Table 1), which shows potent activity to induce apolipoprotein A1 (ApoA1) gene expression in HepG2 cells.[32] Interestingly, ApoA1 upregulation is associated with protection from an inflammatory effect.[33] These observations suggest that small molecules inhibiting BET bromodomains serve as anti-inflammatory drugs.

A small molecule that bind the BRD2 bromodomain was also identified through in silico screening and SPR analysis, and named BIC1 (BRD2-interacting compound-1) (Fig. 6 and Table 1).[10] Consistent with a critical role of BRD2 in the efficient expression of G1 cyclin genes, BIC1 inhibited the SV40 promoter-driven gene expression.[10] Most recently, Heightman and co-workers developed a high-throughput screening (HTS) system in which the binding of bromodomains to acetylated histone peptides can be measured in a high-throughput manner.[34] This combination of HTS and structure-based design led to the identification of 3,5-dimethylisoxazole derivatives as novel BET domain inhibitors.[35]

Because the FRET-based imaging probes for histone acetylation depends on the physical interaction between acetylated histone and its binding molecule bromodomains, Histac imaging probes

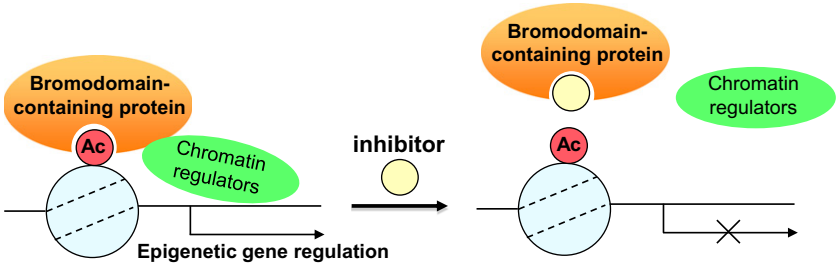

Figure 5. Inhibition of histone acetylation-mediated epigenetic regulation by bromodomain inhibitors. Bromodomain-containing proteins regulate chromatin function via recruitment of chromatin regulators by recognizing specific histone acetylation sites on targeted chromatin through their bromodomains. Small molecules that bind the acetyl-lysine-binding site in the bromodomain can compete with acetylated histone, thereby inhibiting the interaction between a bromodomain-containing protein and acetylated histone, which leads to interference with histone acetylation-mediated epigenetic regulation by a bromodomain-containing protein.

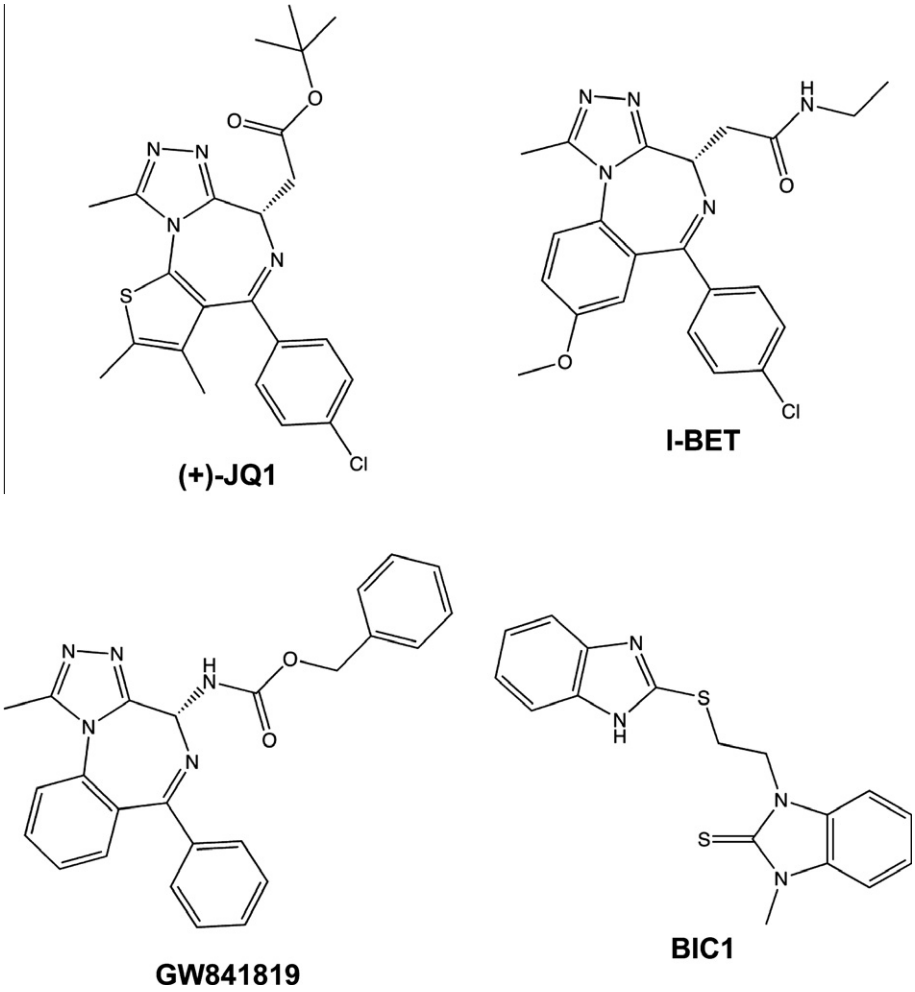

Figure 6. Structure of bromodomain inhibitors.

theoretically allow measurement of not only HDAC and HAT inhibitors but also small molecules that interfere with the interaction between acetylated histone and the bromodomain. Indeed, live imaging analysis using Histac-K12 showed that BIC1 alters the steady-state emission ratio without affecting histone H4K12 acetylation and inhibits the FRET change induced by TSA.[10] These results indicate that BIC1 can truly inhibit the interaction between acetylated histone H4K12 and the BRD2 bromodomain in cells. Further development of the novel technology including Histac derivatives will be essential for evaluating the in vivo activity and efficacy of drugs that regulate epigenetic enzymes and readers.

Table 1
Summary of activities of bromodomain inhibitors

Compound	Protein	K_D (nM)	IC_{50} (nM)
(+)-JQ1	BRD2-BD1	128.4	ND
	BRD3-BD1	59.5	ND
	BRD3-BD2	82	ND
	BRD4-BD1	49	77
	BRD4-BD2	90.1	33
	BRDT-BD1	190.1	ND
	CREBBP	ND	>10,000
I-BET	BRD2-BD1,2	61.3	32.5
	BRD3-BD1,2	50.5	42.4
	BRD4-BD1,2	55.2	36.1
GW841819	BRD2-BD1,2	52	29.9
	BRD3-BD1,2	46	28.4
	BRD4-BD1,2	52.5	15.5
BIC1	BRD2-BD1	28,000	ND

ND: not determined.
Dissociation constants between each compound except for BIC1 and bromodomains were measured by isothermal titration calorimetry while those of BIC1 was measured by SPR spectroscopy. The ability of compounds to displace acetylated histone peptides from bromodomains was measured by either a luminescence proximity homogenous assay (JQ1) or FRET assays (I-BET and GW841819).

6. Prospects

Histac is the first live cell imaging probe that allows detection of dynamic change in histone acetylation by FRET in the living cell chromatin, which enabled monitoring of cellular activity of small molecules inhibiting HDAC, HAT, and interaction between bromodomains and acetylated histones. The FRET-based approach has a number of advantages over the conventional immunological methods (e.g. immunoblotting, immunofluorescence, chromatin immunoprecipitation), which are essentially inapplicable to living cells. Therefore, further applications to detecting acetylation at other sites as well as other modifications. However, there are several issues in the current FRET-based probes to be solved in future. A main issue is that the cells with an appropriate level of expression should be selected for the imaging analysis among the transiently transfected cells in culture, because overexpression sometimes induces deleterious effect on the cell cycle and morphology, from potential cytotoxicity of histones fused with large tags at the both N- and C-termini. We cannot exclude the possibility that

Histac-nucleosomes may not behave the same as the endogenous ones and could cause aberrant gene expression and/or genome instability, despite the fact that Histac can be assembled into nucleosomes. A cell line stably expressing Histac will be useful for the routine analysis and drug screening, but such a tool has remained elusive. The increase in the throughput in the FRET measurement is another issue to apply Histac for drug screening. In addition, a drawback compared with fine methods using antibodies such as ChIP would be its lower spatial resolution. It may be improved by combining bright-enhanced fluorescent proteins and novel high resolution microscopic technologies like structured illumination microscopy.[36] Thus, Histac is a prototype of the histone acetylation probe, which will be further developed as rapid, sensitive sensors for epigenetic histone modifications.

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