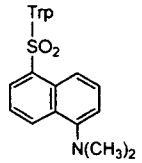
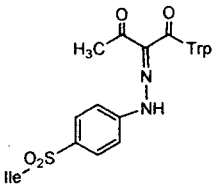
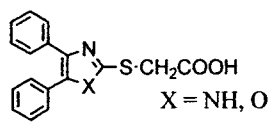
7）トリプトファンと塩化ダンシルから、ダンシルトリプトファンを合成した。



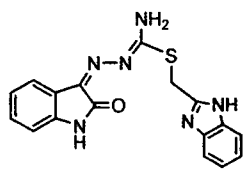
8）塩化N—アセチルスルファニリル酸とイソロイシンを反応させてN—アセチルスルファニリルイソロイシンを合成した後、加水分解してジアゾ化し、別途に合成したアセトアセチルトリプトファンとの反応でヒドラジノアセトアセチルトリプトファンを合成した。



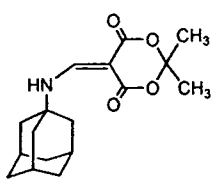
9）ジフェニルメルカプトイミダゾールおよびジフェニルメルカプトオキサゾールとクロロ酢酸から、対応するチオ酢酸誘導体２種類を合成した。

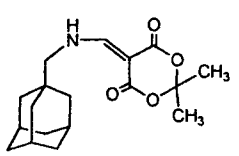


10）イサチンからイサチンチオセミカルバジドを合成した後、クロロメチルベンズイミダゾールと反応させて、ベンズイミダゾールメチルチオエーテル誘導体を合成した。

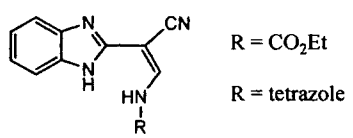


11）アダマンタナミン及びアダマンタンメチルアミンと別途に合成したメトキシメチレンメルドラム酸から、それぞれ対応するN－ジケト-1,3-ジオキシノメチレンアミノアダマンタンとN－ジケト-1,3-ジオキシノメチレンアミノメチルアダマンタンを合成した。





12）別途に合成したベンズイミダゾールアセトニトリルとオルトギ酸トリメチルとを反応させてメトキシメチレンベンズイミダゾールアセトニトリルとした後、ウレタン及びアミノテトラゾールと反応させて、それぞれ対応するエトキシカルバモイルメチレンベンズイミダゾールアセトニトリルとテトラゾイルアミノメチレンベンズイミダゾールアセトニトリルを合成した。



C　研究結果

1）フェニル－４－ピリジルケトキシムと２，２‘－ジピリジルケトキシムはそれぞれCC_{50} 46μg/ml およびCC_{50} 26μg/mlで細胞毒性を示し、フェニル－３－ピリジルケトキシムを含めいずれも抗HIV活性は見られなかった。

2）２－ベンゾイルベンズイミダゾールとフェニル－２－ベンズイミダゾールケトキシムはそれぞれ CC_{50} 85μg/ml および CC_{50} 31μg/ml で細胞毒性を示し、抗 HIV 活性は見られなかった。

3）キサンテノールおよびキサントンはそれぞれ CC_{50} 2.3μg/ml および CC_{50} 3.9μg/ml で細胞毒性を示し、キサンテンカルボン酸を含めいずれも抗 HIV 活性は見られなかった。

4）N―ミリスチルフェニルグリシンは CC_{50} 29μg/ml で細胞毒性を示し、抗 HIV 活性は見られなかった。

5）アセトアセチルトリプトファンには細胞毒性もなく全く活性を示さなかった。

6）Ｎ－ジケト-1,3-ジオキシノメチレントリプトファンには細胞毒性もなく全く活性を示さなかった。

7）ダンシルトリプトファンには細胞毒性もなく全く活性を示さなかった。

8）ヒドラジノアセトアセチルトリプトファンに活性が予想されたが、細胞毒性もなく全く活性を示さなかった。

9）ジフェニルイミダゾールチオ酢酸は EC_{50} 10μg/ml, ジフェニルオキサゾールチオ酢酸は EC_{50} 10μg/ml と活性を示したが、ともに CC_{50} 100μg/ml で細胞毒性があった。

10）現在、スクリーニング中である。

11）現在、スクリーニング中である。

12）エトキシカルバモイルメチレンベンズイミダゾールアセトニトリルは CC_{50} 33μg/ml で、テトラゾイルアミノメチレンベンズイミダゾールアセトニトリルは CC_{50} 3.3μg/ml で細胞毒性を示した。

D 考察

抗菌活性を示すピリジン環を有するケトキシム；抗 RS ウイルス活性を示すベンズイミダゾール環を有するケトンおよびケトキシム；キサンテン誘導体；長鎖脂肪酸を持つ N―ミリスチルフェニルグリシンはいずれも細胞毒性を持ち、抗 HIV 活性を示さなかった。また、トリプトファン誘導体には全く活性が見られなかった。チオ酢酸誘導体には活性が若干見られるが、細胞毒性があるため薬剤としての応用には向かないと考えられる。アミノメチレンベンズイミダゾールアセトニトリル誘導体は３種類のウイルス（RSV, PFLUV-3, Measles）に対して、活性を示す薬剤であったが、HIV に対しては全く活性を示さないことが判明した。

E 結論

Ｄ－フェニルアラニンとイソロイシンを有し、スルファニリル酸を内在するヒドラジノアセトアセチルアミノ酸に活性が見られ、構造上芳香族疎水性アミノ酸の存在が必須と考えられることから、活性の向上を目的として芳香族アミノ酸である D-フェニルアラニンを L-トリプトファンに変えて、スクリーニングにかけたが、全く活性が消失することが判った。従って、フェニルアラニンの場合と同様トリプトファンもＤ－体を導入することが必要と推定している。平成１７年度は疎水性相互作用を持つベンゼン環を含む薬剤を合成してスクリーニングにかけたが、いずれも期待した活性が見られなかった。ベンズイミダゾール誘導体には、同じ RNA ウイルスの仲間である RSV や FLUV に活性を示すものが多いこと、また、骨格的に核酸塩基成分であるプリン塩基のデアザアナログであることに着目し、今後もこれらの誘導体から活性のある薬剤を探索する予定である。

F 健康危険情報

特になし。

G 研究発表

1 論文発表

2 学会発表

千葉卓男、児玉栄一、松岡雅雄「アマンタジン誘導体の抗 HIV 活性について」
平成１７年度化学系学協会　東北大会（仙台）にて発表。

H 知的財産権の出願・登録状況

なし

厚生労働科学研究費補助金（創薬等ヒューマンサイエンス総合研究事業）
分担研究報告書

HIV 融合過程を標的とする耐性克服型新規治療薬の開発に関する研究

主任研究者：京都大学ウイルス研究所　　松岡　雅雄
分担研究者：徳島大学大学院ヘルスバイオサイエンス研究部　大高　章

研究要旨
HIV と標的細胞の膜融合段階を阻害するペプチド性化合物の開発を進めた。HIV はこの膜融合段階で自身の表面蛋白質を構造変化させることで、細胞への侵入を果たしている。そこで、この動的蛋白質構造体の形成阻害を中心にペプチドの開発研究を行った。その結果、未だ活性は低いものの、従来になく残基数を低減させたペプチド性化合物を見いだすに至った。

A．研究目的

HIV と標的細胞の膜融合段階には、ウイルス、標的細胞側の様々な蛋白質分子の動的相互作用が大きく関与している。この中で、CD4（標的細胞側）と gp120（ウイルス側）およびケモカインレセプター（ウイルス側）の蛋白質相互作用に引き続く、ウイルス側 gp41 蛋白質の超分子複合体形成が膜融合に大きく関与することが知られている。この gp41 に由来する超分子複合体は、gp41 の N および C 領域と呼ばれるヘリックス性の高い部分の相互作用に伴う 6-helix bundle 形成であり、この bundle 形成が膜融合に必須である。逆に言えば、この 6-helix bundle 形成を阻害することが出来れば抗 HIV 剤開発に繋がる。そのような 6-helix bundle 形成阻害剤の一つとして gp41 の C 領域由来の 34 残基ペプチド C34 がある。我々は、C34 を基盤構造とした耐性が発現しにくい膜融合阻害性抗 HIV 剤開発を行うことを研究目的とした。

B．研究方法

前述の C 領域由来ペプチドが抗 HIV 活性を示すためには次のような条件が必要である。まず、複合体が 6-helix bundle という名前からも判るように、C 領域ペプチドは、helix 構造を形成することにより、N 領域由来部分（N36）に相互作用し、抗ウイルス活性を発現する。

そこで C34 ペプチドを基盤構造とし、N36 との相互作用面を維持したまま helix 性を増強させることとした。増強法としては、適当なアミノ酸置換を導入することによる手法と人工 helix 形成ユニットの開発とペプチドへの導入の 2 つの方法論が考えられる。

前者の手法として、相互作用面のアミノ酸残基は保持し、一方 bundle を形成した際の溶媒接触面には helix 構造を安定化すると共に水溶性を増強するアミノ酸としてグルタミン酸（Glu, E）およびリジン（Lys, K）を i と i＋4 の関係で導入することとした。すなわち、X-EE-XX-KK（X: 相互作用に必須の残基）スキャンを C34 ペプチドに施すことを基本戦略とし、ここから低分子化などについても検討することとした。

Helix 構造形成ユニットとしての E, K 置換による戦略は確かに蛋白質化学的には有用な方法論を提供するものと考えられる。しかしながら、例えば Lys-Lys 配列などは、トリプシン様酵素の認識配列となり生体内ではこのようなペプチドは速やかに分解されてしまうことが予想される。そこで、これら Lys-Lys, Glu-Glu 配列などのペプチド結合の非ペプチド化研究についても併せて合成化学的側面から検討を加えることとした。具体的には、ペプチド結合が二重結合性を有することに着目し、これをアルケンで置換しようとする方法である。さらに helix 構造の重要性を考慮し、アルケン部分への水素結合形成能力を付与する目的でフルオロアルケン型ペプチド等価体（ペプチドイソスター）の合成化学的展開についても検討を加えることとした。

（倫理面への配慮）
特に問題となる事項はございません

C．研究結果

今回の研究における基盤分子とした C34 は、

34 残基ペプチドであり、すでに強力な抗 HIV 活性が有ることが知られていた化合物である。しかし、その溶解性あるいは体内動態に問題があるためか実用化には移されていなかった。今回、この C34 に X-EE-XX-KK 置換を導入することで、水溶性に優れかつ抗 HIV 活性も上昇したペプチドとして SC34EK および SC35EK を得るに至った。さて、C34 は極めて強力な抗 HIV 活性を示すものの、例えばその C 端領域を除き 28-29 残基程度にすると極端に抗 HIV 活性が低下することが知られていた。この原因として、C34 ペプチドの helix 誘起部位は主にその N 端および C 端側に存在し、C34 について C 端部位を除くと helix 形成能力が極端に低下すると考えた。一方、SC34EK, SC35EK 分子は、X-EE-XX-KK 置換を施すことで、分子全体が helix 形成能力を有するものと考えられる。従って、C 端領域を一部削除しても充分な抗 HIV 活性を持つのではないかと考えた。予想通り、SC35EK の C 端 6 残基を除去した SC29EK も SC35EK に全く劣らない抗 HIV 活性を示した。これは、従来の天然配列の結果とは大きく異なる点である。さらに、7 残基を除いた SC22EK も、活性は低下するもののかなり強力な抗 HIV 活性を維持していることが明らかとなった。

次に、この SC22EK 分子を基盤とし、分解を受けやすい Lys-Lys 配列の非ペプチド化について検討を加えた。オルニチンを利用したアルカン置換体、および前述のアルケン置換体について検討を加えた。しかし、いずれの場合もその活性はかなり低下した。

D．考察

従来 C 領域ペプチドである C34 は、ほとんどその低分子化研究が不可能であると考えられてきた。今回、C34 の helix 誘起サイトを N および C 末側に存在するとし、低分子化かに伴う活性低下は、helix 誘起サイトの減少に起因するものであると仮定した。そこで、X-EE-XX-KK 置換によって helix 誘起サイトを分子全体とすれば、短鎖誘導体においても充分な活性を維持するのではないかと考えた。予想通り、helix 誘起サイトが分子全体に分散していると考えられる SC35EK を親化合物とした場合、低分子化が可能であることが明らかとなった。これは、従来にない新たな知見であり、ペプチド性膜融合阻害剤のデザイン・合成を遂行する上で重要な指針を提供するものと考えている。

ペプチド結合の一部非ペプチド化については現状では未だ充分な成果が得られていない。前述の研究からも明らかなように helix 構造の活性発現に与える影響は大きなものがある。一般に helix 構造はペプチド結合間の水素結合により安定化されている。今回提案したペプチド等価体では、その水素結合形成能力が維持されないことが問題点として挙げられる。今後、水素結合形成能力を有するペプチド等価体あるいは helix 誘起ユニットのデザイン・合成が必要なものと考えている。

E．結論

HIV の gp41 由来 C34 ペプチドは、helix 構造を形成し、N36 領域に結合して抗 HIV 活性を発現する。今回、C34 を基盤分子として、これに X-EE-XX-KK 置換を導入して、helix 誘起サイトを分子全体にすることでその低分子化が可能であることを明らかにした。従来、C34 の低分子化はほとんど不可能であると考えられていた点を考慮すれば、この知見は膜融合阻害性抗 HIV 剤開発に向けた重要なステップと考えられる。

ペプチド等価体の導入を通じては高活性誘導体を得るには至っていない。これは今回導入した等価体が基本的にそのユニット内に水素結合形成能力を有する部分を持たないためと考えられる。今後、水素結合形成能力の付与あるいは立体的な関与による helix 形成能力をもつ構造体のデザイン・合成が必要と考えられる。

F．健康危険情報

なし

G．研究発表

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H．知的財産権の出願・登録状況

発明の名称：抗 HIV 剤；出願番号：特願 2001-297963 発明者:藤井信孝、大高 章、松岡雅雄 出願人：関西 TLO 株式会社；出願日：2001 年 9 月 27 日（国際 PCT 出願番号:PCT/JP02 10119 出願:2002 年 9 月 27 日 国際公開番号 WO03/029284）国内特許のみ京都大学で継承

研究成果の刊行に関する一覧表

主任研究者：京都大学ウイルス研究所　　松岡　雅雄
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書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
	なし						

雑誌

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Novel HIV-1 Integrase Inhibitors Derived from Quinolone Antibiotics

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Abstract: The viral enzyme integrase is essential for the replication of human immunodeficiency virus type 1 (HIV-1) and represents a remaining target for antiretroviral drugs. Here, we describe the modification of a quinolone antibiotic to produce the novel integrase inhibitor JTK-303 (GS 9137) that blocks strand transfer by the viral enzyme. It shares the core structure of quinolone antibiotics, exhibits an IC_{50} of 7.2 nM in the strand transfer assay, and shows an EC_{50} of 0.9 nM in an acute HIV-1 infection assay.

Human immunodeficiency virus type 1 (HIV-1) integrase, along with HIV-1 reverse transcriptase and HIV-1 protease, is an essential enzyme for retroviral replication and represents an important target for interrupting the viral replication cycle.[1] HIV-1 integrase first catalyzes removal of the terminal dinucleotide from each 3′-end of viral DNA (3′-processing) and subsequently mediates joining of the 3′-end of the viral DNA to host DNA (strand transfer).[2] Reverse transcriptase inhibitors and protease inhibitors have already made significant advances in antiretroviral therapy but cannot achieve complete suppression and risk producing resistant HIV-1.[3,4] On the other hand, despite numerous attempts to develop integrase inhibitors, only the diketo acid class of compounds is at an advanced stage of development and no integrase inhibitors have yet been approved for therapeutic use.[1,5−7] Here, we report that the core structure of quinolone antibiotics can be used as an alternative to the diketo acid class of HIV-1 integrase inhibitors and how this finding led to a novel quinolone integrase inhibitor, JTK-303 (GS 9137).

The diketo acid moiety (γ-ketone, enolizable α-ketone, and carboxylic acid) was believed to be essential for the inhibitory activity of this series of integrase inhibitors,[8] and the structures of diketotriazole **2**,[6] diketotetrazole **3**,[9] diketopyridine **4**,[10] and 7-carbonyl-8-hydroxy-(1,6)-naphthyridine **5** [7,11] were reported to be bioisosters of the diketo acid pharmacophore (Figure 1). The carboxylic acid could be replaced with not only acidic bioisosters, such as tetrazole and triazole, but also by a basic heterocycle bearing a lone pair donor atom, such as a pyridine ring. It has been reported that the heteroaromatic nitrogen in the pyridine ring mimics the corresponding carboxyl oxygen in the diketo acid as a Lewis base equivalent.[11] The enolizable ketone at the α-position of diketo acids can be replaced with a phenolic hydroxyl group, indicating that the α-enol form of each diketo acid is its biologically active coplanar conformation.[11]

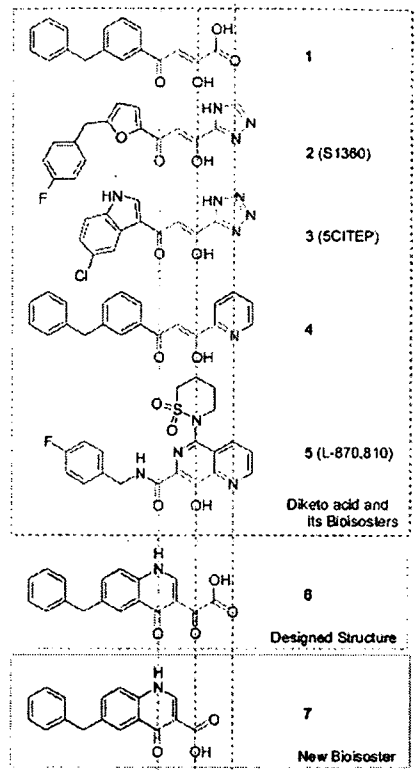

Figure 1. Structures of the diketo acid family and its new bioisoster.

All bioisosters of the diketo acid motif have the three functional groups that mimic a ketone, enolizable ketone, and carboxyl oxygen and can have a coplanar conformation (Figure 1). Therefore, we designed the structure of 4-quinolone-3-glyoxylic acid **6** as a new scaffold that maintained the coplanarity of diketo acid functional groups (Figure 1). Interestingly, not the 4-quinolone-3-glyoxlic acid **6** but its precursor 4-quinolone-3-carboxylic acid **7** showed integrase inhibitory activity. The 4-quinolone-3-carboxylic acid **7** only had two functional groups, a β-ketone and a carboxylic acid, which were coplanar. This result showed that the coplanar monoketo acid motif in 4-quinolone-3-carboxylic acid **7** could be an alternative to the diketo acid motif and provided novel insight into the structural requirements and the binding mode of this type of inhibitor. Quinolone **7** had an IC_{50} of 1.6 μM in the strand transfer assay, and structural modification of **7** led to a far more potent integrase inhibitor **12** with stronger antiviral activity (Table 1). Introduction of 2-fluoro and 3-chloro substituents into the distal benzene ring of **7** (**8**) led to a significant improvement of its inhibition of strand transfer (IC_{50} = 44 nM) and to the appearance of antiviral activity (EC_{50} = 0.81 μM). Compound **9**, bearing a hydroxyethyl group at the 1-position of the quinolone ring, was 1.8-fold more potent at inhibiting strand transfer (IC_{50} = 24 nM) and displayed about 11-fold stronger antiviral activity (EC_{50} = 76 nM) than **8**. Introduction of a methoxy group at the 7-position of the quinolone ring of **9** (**10**) led to a significant improvement of its inhibition of strand transfer (IC_{50} = 9.1 nM) and of antiviral activity (EC_{50} = 17.1 nM). Compound **11**, bearing an isopropyl group at the 1*S*-position of the hydroxyethyl moiety, was about 3-fold more potent at inhibiting strand transfer (IC_{50} = 8.2 nM) and about 10-fold stronger at inhibiting HIV

* To whom correspondence should be addressed. Phone: +81 72 681 9700. Fax: +81 72 681 9725. E-mail: hisashi.shinkai@ims.jti.co.jp.
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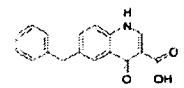
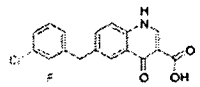
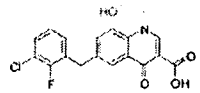
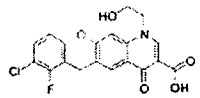
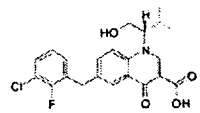
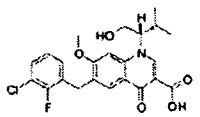
Table 1. Summary of the Structural Optimization Process for Quinolone Integrase Inhibitors[a]

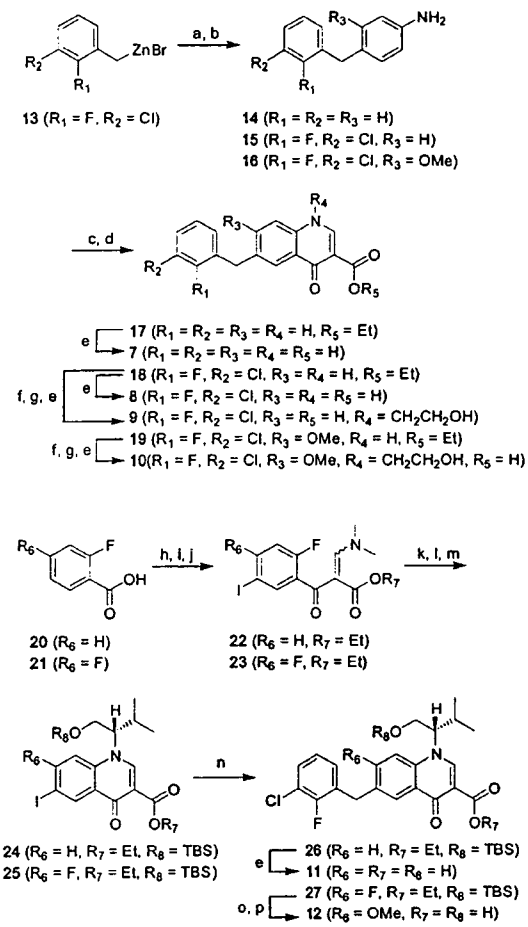
	compd	Inhibition of Strand Transfer[a] IC_{50} (nM)	Antiviral Activity[b] EC_{90} (nM)	Cytotoxicity (μM)
7		1600 ± 300	> 30000	> 30
8		43.5 ± 8.8	805.2 ± 225.0	> 12
9		24.2 ± 11.6	76.3 ± 3.7	> 15
10		9.1 ± 2.1	17.1 ± 2.9	5.3 ± 1.1
11		8.2 ± 1.7	7.5 ± 0.8	14.0 ± 2.0
12		7.2 ± 2.2	0.9 ± 0.4	4.0 ± 0.8
5 (L-870,810)[c]		22.8 ± 4.3	3.6 ± 0.4	0.7 ± 0.06
Ciprofloxacin[d]		> 100000	–	–

[a] The strand transfer assay was performed according to the method of Hazuda[14] with some modifications.[15] [b] Antiviral activity was measured by the acute HIV-1 infection assay[16] with some modifications.[17] [c] Prepared according to the reported method.[18] [d] Available from Wako Pure Chemical [e] Data are given as the mean ± SD (n = 3).

replication (EC_{50} = 7.5 nM) than **9**, although introduction of an isopropyl group at the 1*R*-position of the hydroxyethyl moiety could not enhance inhibitory acitivity. Introduction of both a methoxy group at the 7-position of the quinolone ring and an isopropyl group at the 1*S*-position of the hydroxyethyl moiety of **9** (**12**) led to a synergistic improvement of antiviral acitivity (EC_{50} = 0.9 nM), but there was no additive or synergistic improvement in the inhibition of HIV-1 integrase (IC_{50} = 7.2 nM). This may be due to the condition of the strand transfer assay using 5 nM of target DNA that influences potencies of inhibitors.

Preparation of the quinolone analogues (**7**−**12**) is shown in Scheme 1. Palladium-catalyzed coupling of 3-chloro-2-fluorobenzylzinc bromide **13**, which was derived from the corresponding benzylbromide, with 1-iodo-4-nitrobenzene or 1-iodo-2-methoxy-4-nitrobenzene (Negishi coupling) and subsequent reduction of the nitro group gave the aniline **15** or **16**. Condensation of **15**, **16**, or commercially available **14** with diethyl ethoxymethylenemalonate and subsequent thermal cyclization of the aminoacrylate products in diphenyl ether led to the quinolone esters **17**, **18** and **19**.[19] Hydrolysis of **17** and **18** gave **7** and **8**, respectively. N-Alkylation of **18** or **19** with the *tert*-butyldimethylsilyl (TBS) ether of 2-hydroxyethylbromide and subsequent hydrolysis of the ethyl ester and TBS ether resulted in **9** or **10**. After 5-iodonation of 2-fluorobenzoic acids **20** and **21**, the acid chlorides of **20** and **21** were coupled with ethyl 3-(dimethylamino)acrylate to produce the acrylates **22** and

Scheme 1[a]



[a] Reagents and conditions: (a) 1-iodo-4-nitrobenzene, $PdCl_2(Ph_3P)_2$, THF, reflux; (b) Zn, AcOH; (c) diethyl ethoxymethylenemalonate, toluene, reflux; (d) Ph_2O, 250 °C; (e) NaOH, $EtOH/H_2O$, reflux; (f) $TBSOCH_2CH_2Br$, K_2CO_3, DMF, 80 °C; (g) TBAF, THF; (h) NIS, H_2SO_4; (i) $SOCl_2$, DMF, toluene, reflux; (j) ethyl 3-(dimethylamino)acrylate, THF, 50 °C; (k) (*S*)-valinol, THF; (l) K_2CO_3, DMF, 70 °C; (m) TBSCl, imidazole, DMF; (n) **13**, $Pd(dba)_2$, trifurylphosphine, THF, reflux; (o) NaOH, *i*-PrOH/H_2O, reflux; (p) NaOMe, MeOH, reflux.

23, respectively. Substitution with (*S*)-valinol and subsequent cyclization with potassium carbonate and protection of the alcohol with TBS ether gave the quinolones **24** and **25**, respectively.[20] Negishi coupling of **24** and **25** with **13** led to the quinolone esters **26** and **27**, respectively. Hydrolysis of **26** gave **11**. Hydrolysis of **27** and subsequent methoxylation with sodium methoxide produced **12**.

In summary, modification of quinolone antibiotics, which did not show HIV-1 integrase inhibitory activity (Table 1), led to discovery of the coplanar monoketo acid motif in their scaffold, 4-quinolone-3-carboxylic acid, as an alternative to the diketo acid motif. These novel quinolone integrase inhibitors were structurally optimized in the highly potent **12**, which had little antibacterial activity although it still retained the core structure of quinolone antibiotics. Compound **12** was much more potent at inhibiting integrase-catalyzed strand transfer processes than 3′-processing reactions, as previously reported for compounds of the diketo acid class.[12,13] This indicates that it probably inhibits HIV-1 integrase via a mechanism similar to that of diketo acids, although there is no direct evidence (such as cocrystal data) that the coplanar monoketo acid motif shows the same mode of binding to the enzyme as the diketo acid

motif. Clinical studies of the novel quinolone integrase inhibitor **12** (GS 9137) are currently being conducted by Gilead Sciences.

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Supporting Information Available: Analytical data for **5** and **7**−**12**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Halogenated Thymidine Analogues Restore the Expression of Silenced Genes without Demethylation

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Abstract

Transcriptional silencing of tumor suppressor genes by aberrant DNA methylation is a characteristic frequently observed in cancer cells. Therefore, reversing this process is a therapeutic target against cancer. In this study, we established a screening system for silencing inhibitors with cell lines transfected by a retroviral vector containing a luciferase gene. More than 100 nucleosides were tested for antisilencing activity with a selected clone in which the silenced expression of luciferase could be recovered by 5-aza-2′-deoxycytidine. A group of halogenated thymidine analogues was found to reactivate transcription of not only the reporter retrovirus vector but also endogenous *glutathione-S-transferase 1* gene, without influence to DNA hypermethylation. Gel mobility shift assay showed that 5-bromo-2′-deoxyuridine (BrdUrd) or 5-iodo-2′-deoxyuridine incorporation did not affect the binding of the methyl-CpG binding protein motif to methylated DNA. Finally, in the retroviral promoter, BrdUrd treatment increased the acetylated histone H3 level and decreased methylation of histone H3 Lys^9 in accordance with recovered transcription. This study shows that halogenated thymidines have an antisilencing effect without changing DNA methylation status by interfering with step(s) between DNA methylation and histone acetylation. (Cancer Res 2005; 65(15): 6927-33)

Introduction

CpG methylation, which is established and maintained by DNA methyltransferases, is a common modification in vertebrate genome and is associated with development, differentiation, and transcriptional suppression (1). Aberrant DNA methylation in the promoter region and the subsequent silencing of tumor suppressor genes is frequently observed in various tumors, indicating that DNA methylation plays an important role in tumorigenesis (2–5). Such epigenetic changes in tumors suggest the idea that reversing aberrant hypermethylation and reactivating abnormally silenced tumor suppressor genes should be effective against tumors; this has now been designated as epigenetic therapy (5). Methylation inhibitors, which demethylate and reactivate silenced tumor suppressor genes, have shown their antitumor effects both experimentally and clinically (6). Evidence from either knockout mice or siRNA-based knockdown experiments have shown that a defect in methyl-CpG binding protein 2 (MBD2), a member of the methyl-CpG binding domain proteins that specifically bind with methylated DNA (1, 7), protected tumor-prone mice from developing tumors (8, 9). This observation implies that processes downstream of DNA methylation toward gene silencing could also be good targets for epigenetic therapy.

5-Aza-2′-deoxycytidine (5-aza-dC), first synthesized in 1964, is one of the best known methylation inhibitors (10). 5-Aza-dC is a potent inhibitor of DNA methyltransferases, and is incorporated into DNA by substituting physiologic deoxycytidine during DNA replication and functions by forming covalent complexes with DNA methyltransferases (5). To date, 5-aza-dC has been shown to be clinically effective in treatment of several human tumors (6, 11). Some other nucleoside analogues with demethylation activity have also been developed, including azacytidine, fazarabine, DHAC, and MG98 (12). More recently, another nucleoside analogue, zebularine, that demethylates and reactivates the silenced genes, was identified. Although the mechanism of zebularine is similar to that of 5-aza-dC (13), a characteristic of zebularine is that it can be p.o. administered because of its stability in an aqueous solution (14). In addition, the sequential treatment with 5-aza-dC followed by zebularine has been shown to prevent remethylation, a common obstacle in antimethylation treatments (15).

In this study, we established a screening system for compounds with antisilencing activity using a Moloney murine leukemia virus (MLV)–based retroviral vector, and identified a group of halogenated thymidine analogues that could recover gene expression without influence on DNA methylation.

Materials and Methods

Retroviral vector construction and viral particle production. The MLV-based retroviral vector, pRCV, was constructed by replacement of the neomycin phosphotransferase gene and the SV40 promoter of pLNSX with an oligonucleotide containing a multicloning site. A DNA fragment that has a luciferase gene connected with a neomycin phosphotransferase gene by an internal ribosomal entry site (IRES) was inserted into the blunted *Hin*dIII site in pRCV, yielding the retroviral vector pRCV/LIG (Fig. 1*A*).

The recombinant viral particles were produced by cotransfection of equal amounts of pRCV/LIG plasmids and pcDNA-VSV-G (generous gift from Hiroyuki Miyoshi, RIKEN, Tsukuba, Japan), which encodes vesicular stomatitis virus envelope glycoprotein, into a *gag-pol*–expressing packaging cell line, GP293 (Clontech, Palo Alto, CA) using Fugene reagent (Roche, Indianapolis, IN). Culture supernatants were harvested, aliquoted 48 hours later, and then stored at −80°C.

Cell culture and compounds. A human myeloid leukemia cell line, K562, was grown in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine at 37°C under a 5% CO_2 atmosphere. The human breast cancer cell line MCF7 was grown in DMEM (Sigma) supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine.

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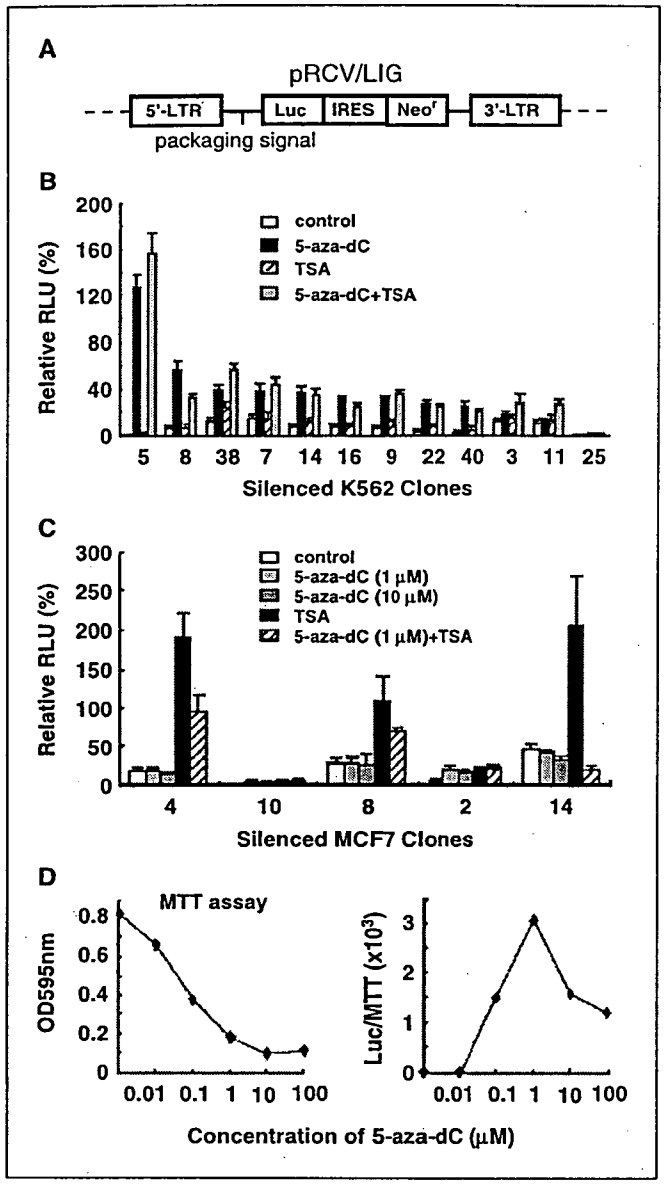

Figure 1. Establishment of a compound screening system. *A*, schematic diagram of the retroviral vector pRCV/LIG used to establish a compound screening system. *B* and *C*, response of silenced clones to 5-aza-dC and/or TSA. Silenced K562 (*B*) or MCF7 (*C*) clones were treated with 5-aza-dC for 3 days or 0.4 μmol/L TSA for 1 day, or 5-aza-dC for 2 days followed by a combination of 5-aza-dC and 0.4 μmol/L TSA for a further 1 day. The dose of 5-aza-dC used for K562 cells was 1 μmol/L. Single-tube luciferase assay was done after drug treatment. Results are described as relative light units (*RLU*) relative to the positive line of each clone. *Columns*, mean from three independent luciferase assays; *bars*, SD. *D*, optimized compound screening system tested with 5-aza-dC. On a 96-well plate, 10^3 K5 cells were exposed to graded concentrations of 5-aza-dC in a total volume of 100 μL for 5 days followed by MTT assay (*left*) or 96-well luciferase assay. Luciferase activities were normalized to the MTT values of corresponding wells and plotted against drug concentration (*right*).

5-Aza-dC, 5-azacytidine (5-aza-C), 5-bromo-2′-deoxyuridine (BrdUrd), 5-chloro-2′-deoxyuridine (CldU), 5-chloro-2′-doxycytidine (CldC), 5-iodo-2′-deoxyuridine (IdU), and trichostatin A (TSA) were purchased from Sigma.

Establishment of clones with a reporter gene. K562 and MCF7 cells were infected with the virus stock for 48 hours in the presence of 8 μg/mL polybrene. Forty-eight hours later, G418 (Nacalai Tesuque, Kyoto, Japan) was added for positive selection (0.4 mg/mL for K562 and 0.8 mg/mL for MCF7). After selection with G418 (~2 weeks), transfected clones were isolated by limiting dilution. Then, each clone was cultured under two conditions; one was cultured in medium with G418 (selection medium) and the other one in medium without G418 (nonselection medium).

Luciferase assays. Two systems, single-tube luciferase assay and 96-well plate luciferase assay, were used in this study. Single-tube luciferase assay was carried out using Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's protocol. Briefly, 2×10^5 cells were collected and lysed, and then luciferase activity was detected by mixing the cell lysate and luciferase assay reagent in a luminometer tube and measured with a LB 9507 luminometer (Berthold, Bad Wildbad, Germany). For the 96-well plate luciferase assay, the Steady-Glo Luciferase Assay System (Promega) was used. Steady-Glo Reagent (100 μL) was added directly to cells growing in 100 μL of medium on a 96-well plate. Luciferase activity was measured using a Wallac 1450 MicroBeta Jet Luminometer (Perkin-Elmer, Wellesley, MA). In this study, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was also done to normalize the results of luciferase assay as described previously (16).

Synergistic effects of compounds. Dose-effect assays were done as reported (17). Briefly, cells were seeded onto 96-well plates and exposed to serial dilutions of each compound individually and concomitantly to both 5-aza-dC and BrdUrd or CldU at a fixed ratio (a ratio of 5-aza-dC to BrdUrd or CldU is 1:100) of doses for 3 days followed by 96-well plate luciferase assay. Doses used for 5-aza-dC were 0.05, 0.1, 0.2, 0.4, and 0.8 μmol/L; doses used for BrdUrd or CldU were 5, 10, 20, 40, and 80 μmol/L. A combination index (CI) value was calculated by the computer-based software Calcusyn developed by Chou et al. (18).

Bisulfite genomic sequencing. Sodium bisulfite treatment of genomic DNA was done as described previously (19). DNA regions were amplified using bisulfite-treated genomic DNA by nested PCR. To amplify 5′-long terminal repeat (5′-LTR) promoter region of RCV/LIG, primers used in the first PCR were 5′-TAGGATATTTGTGGTAAGTAGTTTTTGTT-3′ and 5′-CATAACATCAAACATAAACACTAAACAATC-3′. Primers for the second PCR were 5′-GGTTTAGGGTTAAGAATAGATGGTTTTTAG-3′ and 5′-CACAA-ATAAATTACTAACCAACTTACCTCC-3′. Primer sets target the promoter region of glutathione-S-transferase 1 (*GSTP1*) gene were as follows: For first PCR, the primer set was: 5′-TGAGAGGTGGAGGTTGTAGT-3′ and 5′-TCCTA-AATCCCCTAAACCCC-3′. Primer sets used in second PCR were 5′-TGTG-AAG(T/C)GGGTGTGTAAGT-3′ and 5′-CTAAAAACTCTAAACCCCATCC-3′. PCR products were purified, cloned into pGEM-T Easy vector (Promega), and sequenced using the ABI PRISM DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, CA).

Quantitative real-time reverse transcription-PCR. Total RNA was isolated from MCF7 cells using Trizol reagent (Invitrogen, Carlsbad, CA). RNA was treated with DNase I (Invitrogen) to eliminate the genomic DNA. Reverse transcription was done using random hexamer and SuperScript III reverse transcriptase (Invitrogen). cDNA product was analyzed by real-time PCR using the Taqman Universal PCR Master Mix and ABI Prism 7700 (PE Applied Biosystems, Foster City, CA) sequence detector according to the manufacturer's instruction. Specific primers and Taqman probes for *GSTP1* gene and for 18S internal control gene were used as described previously (20). PCRs were carried out in triplicate. Data was analyzed by comparative C_t method according to manufacturer's protocols (PE Applied Biosystems). The lowest detectable *GSTP1* expression that has been induced in MCF7 cells after compound treatment was set arbitrarily as 1.0, as untreated MCF7 cells are null for *GSTP1* gene expression.

Quantitative chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assay was done according to the protocol recommended by Upstate Biotechnology (Lake Placid, NY). Cells (1.5×10^6) were fixed with 1% formaldehyde for 10 minutes at room temperature, washed twice with ice-cold PBS, treated with SDS-lysis buffer [1% SDS, 50 mmol/L EDTA and 200 mmol/L Tris-HCl] for 10 minutes on ice and then sonicated. Thereafter, the DNA/protein complexes were immunoprecipitated with antibodies specific for acetylated-Histone H3 or anti-dimethyl-Histone H3 (Lys^9) antibodies (Upstate Biotechnology) overnight at 4°C. Immune complexes were collected with salmon sperm DNA-protein A and G Sepharose slurry, washed, and eluted with freshly prepared elution buffer

(1% SDS, 100 mmol/L $NaHCO_3$). Protein-DNA complexes were de-cross-linked at 65°C for 4 hours. DNA was purified and subjected to real-time PCR amplification.

ChIP DNA samples were quantified by Taqman real-time PCR assay according to the method described by Mutskov et al. (21). Briefly, primers and TaqMan probe were designed to specifically target the RCV 5′-LTR retroviral promoter. Sequences for the primer set were 5′-CCTGTGCCTTATTT-GAACTA-3′ and 5′-TCTCCAAATCCCGGACG-3′. Sequence for the FAM-labeled TaqMan probe was 5′-CAGTTCGCTTCTCGCTTCTGTTCGCGC-3′. Individual PCRs were done in triplicate to control for PCR variation and mean C_t values were collected. Fold difference of the antibody bound fraction (*IP*) versus a fixed amount of input (*In*) was calculated as

$$IP/In = 2^{\Delta\Delta C_t} = 2^{-(C_t(IP)-C_t(In))}.$$

Then, the fold difference value for a target antibody (t) was subtracted by the nonspecific value derived from rabbit IgG (t_0):

$$(IP/In)^t - (IP/In)^{t_0}.$$

Finally, the fold difference value from the positive control cell line (K5/+) was arbitrarily set as 1.0 and relative abundance of the analyzed protein from untreated or compound treated K5/− cells was subsequently calculated.

Purification of recombinant proteins used in gel mobility shift assay. Vectors that express the GST-tagged methyl-CpG binding domain of MBD1 [pGEX-2TH-MBD (1-84 amino acids)] or only GST (pGEX-2TH) as control were constructed as described previously (22). *Escherichia coli* BL21 with expression vectors were cultured overnight at 20°C, and then expression of recombinant proteins was induced by isopropyl-β-D-thiogalactopyranoside (a final concentration of 0.4 mmol/L), following further 6-hour incubation. Cells were harvested and resuspended in ice-cold PBS followed by sonication. Recombinant proteins were purified with ReadiPack GST Purification Modules (Amersham, Buckinghamshire, England), aliquoted into small fractions and stored at −20°C.

Gel mobility shift assay. Probes for mobility shift assay are shown in Fig. 5*A*. Probes (20 fmol) labeled with [γ-^{32}P]dATP were mixed with 0.4 μg purified recombinant protein in EMSA binding buffer [10 mmol/L Tris-HCl (pH 8.0), 3 mmol/L $MgCl_2$, 50 mmol/L NaCl, 0.1 mmol/L EDTA, 0.1% NP40, 2 mmol/L DTT, 5% glycerol, and 0.4 mg/mL bovine serum albumin; ref. 23] at room temperature for 30 minutes. For the competition assay, unlabeled probe DNA was added into the reaction mixture at a concentration of 10, 100, or 1,000 fold relative to the [γ-^{32}P]dATP-labeled probe. The reaction mixture (3 μL) was loaded on 8% polyacrylamide gel and run in 0.5× Tris-borate EDTA buffer. Gel was dried and exposed to Medical X-ray film (Kodak, Rochester, NY) overnight at −80°C.

Results

Establishment of a screening system for compounds with antisilencing activity. To establish a screening system for compounds with antisilencing activity, we used an MLV-based retroviral vector to monitor the silencing and recovery of reporter gene expression (24). For this purpose, an MLV-based retroviral vector pRCV/LIG was constructed, which carried a luciferase reporter cassette under the control of the retroviral 5′-LTR promoter (Fig. 1*A*). A neomycin-resistant gene was included in this vector for positive selection of transduced cells. K562 and MCF7 cell lines were infected with recombinant RCV/LIG, subcloned in the presence of G418, and tested for luciferase expression by single-tube luciferase assay. Each clone with detectable luciferase activity was cultured in the presence (positive) or the absence of G418 (negative). Luciferase activity could be maintained in cells cultured with G418, so these cells could be used as a control when the degree of silencing was judged in "negative" cells.

Time-dependent diminishing of luciferase activity was observed at various rates and degrees among the different clones after the removal of G418. Cells with a luciferase activity lower than 30% relative to their "positive" counterpart were arbitrarily considered as being silenced. Then, the silenced clones were tested about responsiveness to 5-aza-dC and TSA, an inhibitor of histone deacetylases (HDAC; Fig. 1*B* and *C*; ref. 25). Among isolated clones, the clone 5 derived from K562 (K5) showed the most prominent responsiveness to 5-aza-dC (Fig. 1*B*), whereas it did not respond to TSA. The 5′-LTR promoter region in this clone was hypermethylated although it was demethylated after exposure to 5-aza-dC (see below), indicating that promoter hypermethylation was directly associated with silencing in K5 cells. Therefore, we selected this clone for further analyses to identify antisilencing compounds. To establish a screening system with K5 cells, data from 96-well luciferase assay was normalized by values from MTT assay as parameters of the cell number (Fig. 1*D*).

On the other hand, MCF7 clones with a silenced luciferase gene could not respond to 5-aza-dC, but expressed luciferase after treatment by TSA (Fig. 1*C*). Among these clones, 5′-LTR was not methylated, indicating that silencing of the reporter gene promoter in MCF7 cells was not associated with DNA methylation (data not shown).

Halogenated thymidine analogues exhibit antisilencing activity. With this system, we screened >100 ribonucleoside analogues provided by Yamasa Corporation (Choshi, Japan) or purchased from Sigma, and identified a group of halogenated thymidines that could reactivate the transcription of luciferase gene in K5 cells (Fig. 2*A* and *B*). The identified thymidines were similar in their chemical structures (i.e., halogenated at position 5 of the pyrimidine ring; Fig. 2*A*). Among identified compounds, the effect of BrdUrd and CldU was prominent (Fig. 2*B*). In addition to thymidine analogues, CldC also showed antisilencing activity. Because CldC showed a weak antisilencing activity, it is possible that CldC is deaminated, resulting in incorporation into DNA as CldU. On the other hand, it was shown that this system successfully evaluated the antisilencing activities of the two control compounds, 5-aza-dC and 5-aza-C (Fig. 2*B*), indicating that our system was applicable for screening of antisilencing compounds.

By performing single-tube luciferase assay, we confirmed that these compounds reactivated the silenced retroviral promoter in K5 cells in a dose-dependent manner (Fig. 2*C*). Halogenated deoxyuridines substitute thymidine during incorporating into DNA, whereas 5-aza-dC is incorporated instead of deoxycytidine. Therefore, we concurrently exposed K5 cells to 5-aza-dC and either BrdUrd or CldU in various ratios and found that these compounds significantly enhanced the effect of each other (Fig. 2*C*). Luciferase activity peaked when a suboptimal dose of 5-aza-dC was paired with a high dose of BrdUrd or CldU (data not shown). Dose effect analysis was done using the method developed by Chou and Talalay (17), showing that combinations of 5-aza-dC and either BrdUrd or CldU exhibited strong synergism with the CI value decreasing dose dependently (Fig. 2*D*). These findings suggested that halogenated thymidines and 5-aza-dC exerted their antisilencing effects by different mechanisms.

Effect of 5-bromo-2′-deoxyuridine on the expression of silenced endogenous genes. Because halogenated thymidines showed antisilencing activity on hypermethylated reporter gene, we analyzed the antisilencing activity of BrdUrd on endogenous genes that are silenced through promoter DNA methylation. It has been reported that endogenous *GSTP1* gene is silenced in breast

cancer cells by DNA methylation (26). As shown in Fig. 3, BrdUrd restored *GSTP1* gene expression in a dose-dependent manner. The combination of BrdUrd and 5-aza-dC significantly increased the expression level of this gene. These results showed that BrdUrd could reactivate the silenced transcription of not only retroviral promoter in K5 cells, but also endogenous *GSTP1* gene in MCF7 cells that has been silenced by DNA methylation.

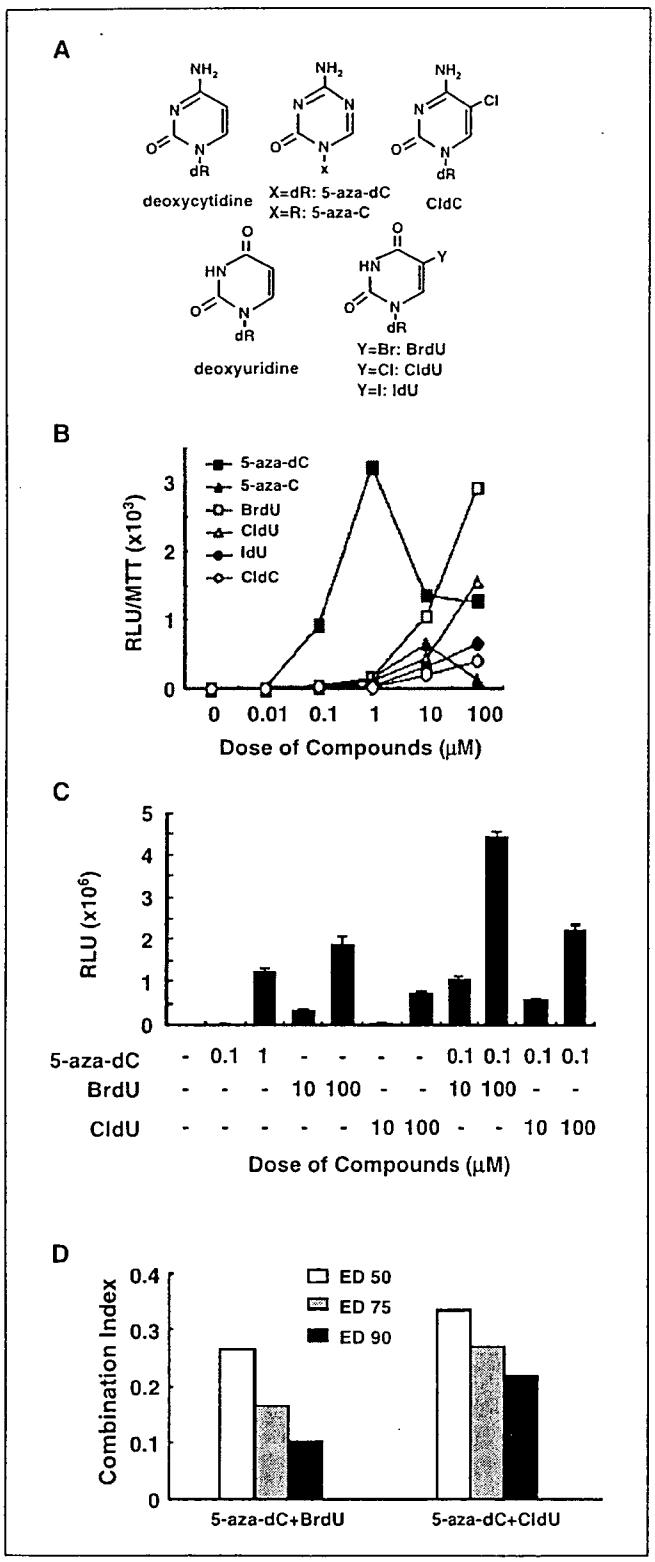

Figure 2. Identification of a group of thymidine analogues as silencing inhibitors. *A*, chemical structures of deoxycytidine, deoxyuridine, and their analogues. *dR*, deoxyribose. *B*, antisilencing activity of the identified compounds unraveled by the method described in Fig. 1*D*. *C*, combination effect of 5-aza-dC and halogenated deoxyuridines. K5 cells were cultured in the presence of a single dose or a combination of compounds for 3 days followed by single-tube luciferase assay. *Columns*, mean from triplicate wells; bars, SD. *D*, dose effect analysis. K5 cells were concomitantly exposed to 5-aza-dC and BrdUrd or CldU for 3 days. Luciferase activity was measured by 96-well plate luciferase assay. The CI was determined by the method based on the computer software Calcusyn. Data plotted are CI values at 50%, 75%, and 90% fractions of luciferase induction (ED). CI < 1, CI = 1, and CI > 1 indicate synergism, additive effect, and antagonism, respectively.

Antisilencing activity of halogenated thymidines is not associated with DNA demethylation. To clarify the mechanisms of antisilencing activities observed in halogenated thymidines, DNA methylation in the promoter region of reporter gene and *GSTP1* gene was examined by bisulfite genomic sequencing. The retroviral 5′-LTR promoter region in K5 cells cultured without G418 (K5/−) was hypermethylated (77%), whereas almost no methylation was detected in K5 cells cultured with G418 (K5/+; Fig. 4*A*). 5-Aza-dC significantly decreased the numbers of methyl-CpG to 34%, which was directly associated with the up-regulated luciferase activity. In contrast, BrdUrd and CldC did not demethylate hypermethylated retroviral promoter although they restored the silenced luciferase gene transcription (Fig. 4*A*). Similarly, BrdUrd did not influence the methylation status of the endogenous *GSTP1* promoter (Fig. 4*B*), whereas 5-aza-dC decreased DNA methylation. Our results showed that halogenated thymidines could activate a hypermethylated promoter by targeting processes other than DNA methylation.

Effect of halogenated thymidines on binding of methyl-CpG binding domain to methylated DNA. It is generally thought that promoter DNA methylation is translated to transcriptional silencing by a family of methyl-CpG binding domain proteins, which bind to methylated DNA through the evolutionarily conserved methyl-CpG binding domain (1). It is possible that incorporated halogenated thymidines disturb the binding of MBD to methyl-CpG sites, resulting in the antisilencing effect. The effect of halogenated thymidines on binding of MBD with methylated DNA was analyzed by gel shift assay using an artificially designed 20 bp double-stranded oligonucleotide probe containing two symmetrically methylated CpG sites with all thymidines replaced by BrdUrd (Fig. 5*A*). BrdUrd incorporation did not affect its binding to a recombinant MBD (Fig. 5*B*). Similar results were obtained when all thymidines were substituted with IdU in gel shift assay (data not shown). It is unlikely that BrdUrd disturbs MBD binding before incorporation into DNA because addition of the monophosphate or triphosphate form of BrdUrd into the protein-DNA binding reaction mixture did not influence the results in our gel shift experiment (data not shown). Taken together, BrdUrd did not disturb the binding of the methyl-CpG binding domain of MBD1 protein with methylated DNA *in vitro*.

Effect of 5-bromo-2′-deoxyuridine on histone modification. DNA methylation is usually associated with changes in the modifications of histone tails, which establish and maintain an inactive chromatin structure (27, 28). To clarify the antisilencing mechanism of BrdUrd, we did quantitative ChIP assay to detect changes in histone modification after BrdUrd treatment. Because acetylation of histone lysine tail is generally associated with

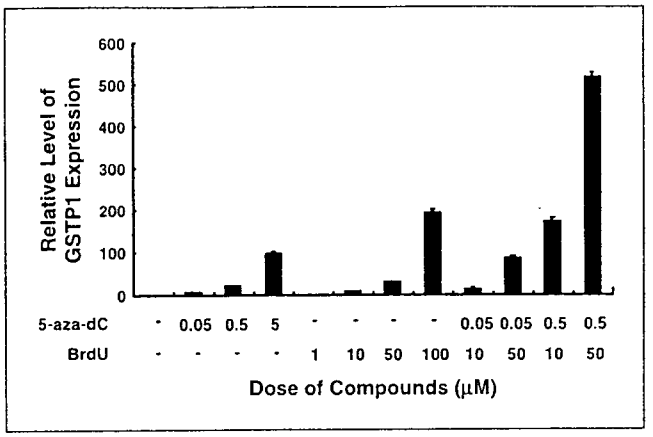

Figure 3. Reactivation of endogenous *GSTP1* gene transcription in MCF7 cells by BrdUrd. MCF7 cells were cultured in medium containing increasing amount of 5-aza-dC or BrdUrd or a combination of both drugs for 3 days. Expression of *GSTP1* gene was analyzed by quantitative real-time PCR assay.

transcriptional active chromatin (29), histone H3 acetylation of the retroviral promoter was studied. K5 cells maintained in G418 selection medium (K5/+) were used as a control, in which retroviral promoter sustained an active transcription state during long-term culture. As shown in Fig. 6*A*, K5/− cells exhibited decreased level of histone H3 acetylation in the retroviral promoter region compared with K5/+ cells, suggesting histone deacetylation in the proviral silencing. By exposing K5/− cells to BrdUrd for 3 days, histone H3 acetylation of the retroviral promoter recovered to a level comparative to that in K5/+ cells (Fig. 6*A*). On the other hand, the close linkage between methylation of histone H3 Lys^9 (H3K9) and transcriptional silencing has been reported (30, 31). In silenced K5/− cells, H3K9 in the retroviral promoter has been methylated (Fig. 6*B*). Treatment with 5-aza-dC or BrdUrd diminished this methylation. Thus, BrdUrd increased acetylation of histone H3 and decreased methylation of H3K9 along with intact DNA methylation.

Discussion

In this study, an MLV-based retroviral vector was used to establish a screening system for antisilencing compounds. This system successfully identified compounds with antisilencing activity. Although it has been reported that silenced retroviral vector becomes resistant to antisilencing agents during passage (32), the responsiveness of K5 cells to 5-aza-dC persisted for >6 months (data not shown). It is noteworthy that clones derived from MCF7 cells respond to TSA, but not to 5-aza-dC, which indicates that silencing of the retroviral promoter in these clones is not associated with DNA methylation, but is correlated with histone deacetylation. Indeed, the promoter region of the reporter gene in a MCF7 clone was not methylated at all (data not shown). This clone will be useful to screen compounds with antisilencing activity that is not associated with DNA methylation.

With this system, a group of halogenated thymidine analogues was identified as silencing inhibitors, which could activate the transcription of silenced genes without DNA demethylation. This is in agreement with the previous observation that BrdUrd-containing genome, even at very high substitution level, had no change in the content of 5-methylcytosine (33). In addition, BrdUrd has been shown to induce the expression from silenced endogenous

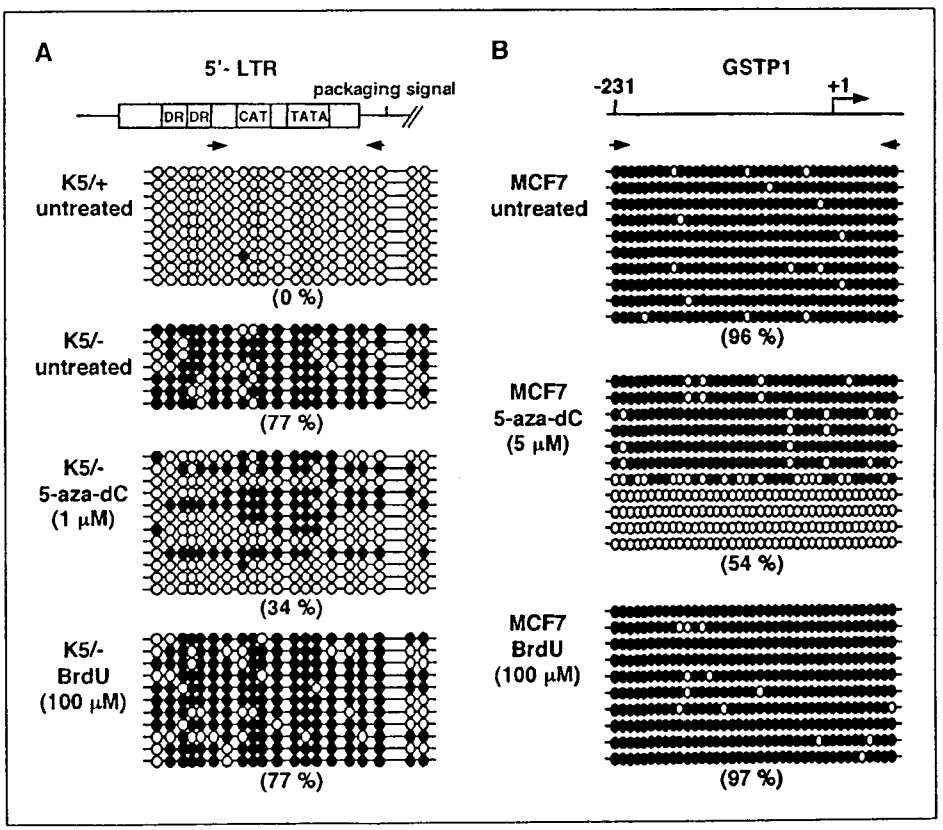

Figure 4. Bisulfite genomic sequencing for the effect of BrdUrd treatment on DNA methylation. *A*, CpG methylation of the retroviral promoter in K5 cells. *B*, methylation of the endogenous *GSTP1* promoter in MCF7 cells. K5 cells (*A*) or MCF7 cells (*B*) were treated with 5-aza-dC, BrdUrd, or CldU for 3 days. Genomic DNA was extracted and subjected to bisulfite genomic sequencing assay. *K5/+*, K5 cells maintained in G418 selection medium; *K5/−*, K5 cells cultured without G418. The analyzed region is shown at the top of each panel. *DR*, direct repeats of enhancer; *CAT*, CAT box; *TATA*, TATA box (39). *Arrows*, location of PCR primers. Open circles indicate unmethylated CpG whereas closed circles indicate methylated CpG. Percentages of methylated CpG sites among total CpG sites are shown in the parenthesis.

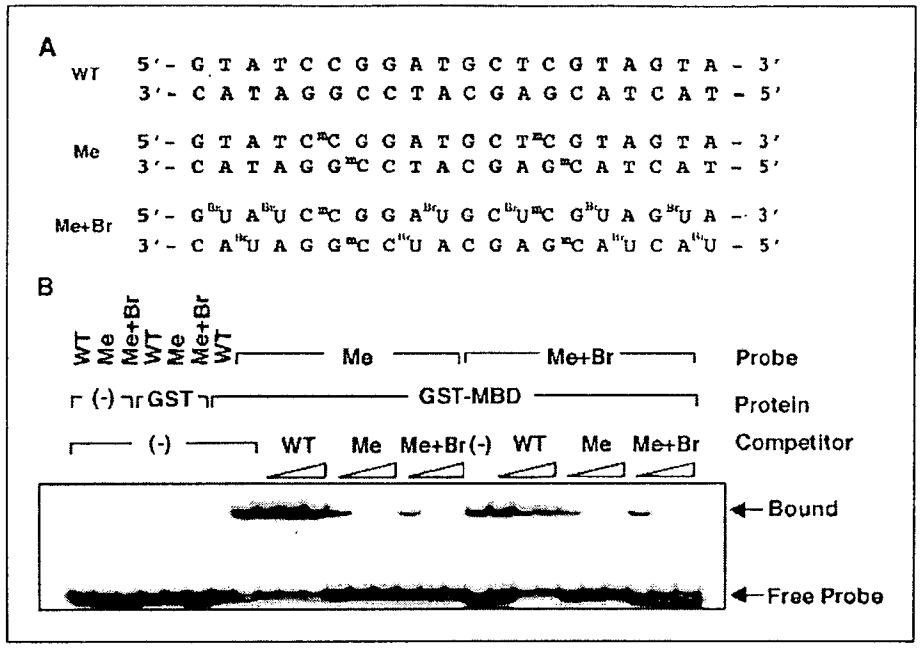

Figure 5. Binding of BrdUrd-incorporated DNA with methyl-CpG binding domain. *A*, oligonucleotides used in gel mobility shift assay. ^{m}C represents 5-methylcytosine. ^{Br}U represents bromouracil. *B*, gel mobility shift assay with GST-tagged recombinant MBD of MBD1 protein (GST-MBD). Triangles indicate an increasing amount of competitors (10-, 100-, and 1,000-fold relative to the probes).

retrovirus without effect on DNA methylation (34), which was also consistent with our findings. However, DNA methylation has not been studied regarding endogenous genes after treatment with BrdUrd. This study first showed that these halogenated thymidines reactivated the transcription of not only retrovirus, but also a heavily methylated endogenous gene. In addition, halogenated thymidines could not reactivate the transcription from the viral promoter in MCF7 clones (data not shown), which could respond to TSA but not to 5-aza-dC, suggesting that the antisilencing effects of halogenated thymidines are closely linked with DNA methylation. This finding coincides with the observation that BrdUrd could reactivate the transcription of endogenous *GSTP1* gene silenced by DNA hypermethylation in MCF7 cells, implicating that halogenated thymidines interfere with cellular protein(s) that function between methylated CpG and HDACs.

Several lines of evidence have indicated that DNA demethylation is not a prerequisite for the reactivation of heavily methylated loci. In helper T cells, GATA3 seems to activate interleukin-4 expression from a hypermethylated promoter by interfering with MBD2 binding to its target CpG sites without altering DNA methylation (35). Similarly, Lembo et al. (36) recently reported that MBDin, a regulatory factor of MBD2, reversed MBD2-mediated transcriptional repression from a methylated promoter by binding with the COOH-terminal region of MBD2. Another example of antisilencing without DNA demethylation has been shown in plants (37). *MOM* is an *Arabidopsis* gene that has been found to be important in maintaining gene silencing in a methylation-independent fashion. Suppression of its expression by siRNA reactivated the transcription of the genes, which existed in several densely methylated loci. It has been reported that BrdUrd-substituted DNA has changed its interaction with some chromatin and nonchromatin proteins (38), showing the possibility that incorporated halogenated thymidines activate transcription by interfering with the function of cellular proteins that are associated with DNA methylation–mediated gene silencing.

The synergistic effects of halogenated thymidines with 5-aza-dC also indicate that the mechanism of halogenated thymidines differs from that of 5-aza-dC. In spite of retained DNA methylation, the level of histone H3 acetylation was increased when treated by

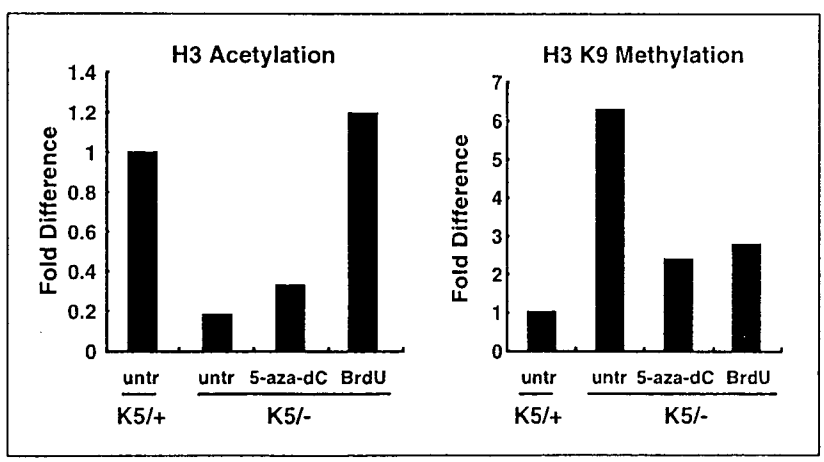

Figure 6. Quantitative ChIP assay for histone acetylation and methylation status of the retroviral promoter in K5 cells. K5 cells cultured in either G418 selection medium (K5/+) or nonselection medium (K5/−) were subjected to ChIP assay after exposure to 5-aza-dC or BrdUrd for 3 days using antiacetylated histone H3 antiserum (*A*) or antidimethyl histone H3 Lys^{9} (*B*). The presented diagram shows the fold difference of acetylated or methylated histone H3, where the data from K5/+ cells was arbitrarily set as 1.0. Three independent ChIP experiments have been done and a representative data is shown.