Development of Cytodifferentiating Agents for Cancer Chemotherapy

by **Ronald Breslow***, **Sandro Belvedere**, and **Leland Gershell**

Department of Chemistry, Columbia University, New York, New York 10027, USA

This paper is dedicated to *Albert Eschenmoser* in honor of his 75th birthday. He has followed the work described almost since its inception.

Starting from the accidental observation that dimethylsulfoxide induces the differentiation of murine erythroleukemia cells, compounds have been designed with increasing potency in transforming this and other cancer-cell types. The target for the most effective of the new compounds has been identified as histone deacetylase, whose natural substrate maps well onto the structures of the particularly effective compounds. Preclinical and early clinical studies suggest that the best of the compounds are promising anticancer agents without excessive toxicity.

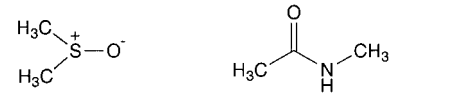
There are many approaches to cancer chemotherapy, but one of the most interesting was discovered some years ago. Mature normal biological cells are produced from stem cells, which can be thought of as juvenile forms that have two functions. The stem cells must proliferate, forming additional stem cells, and they must differentiate to form mature adult cell types. For example, erythrocytes are formed by the differentiation of pre-erythrocytes, which are quite different in several respects. Pre-erythrocytes are spherical, while erythrocytes are discoid, pre-erythrocytes have cell nuclei that are lost when they differentiate into erythrocytes, and pre-erythocytes have no hemoglobin, in contrast with erythrocytes. Mature erythrocytes, lacking nuclei, do not proliferate.

One description of some cancers is that they are cases of arrested development before differentiation, in which cancer cells behave in part like stem cells: they multiply, but they do not differentiate. Other cancers involve adult cells that have de-differentiated, reverting to a proliferative stem-cell-like behavior. Thus, agents that can induce such undifferentiated or de-differentiated cells to transform to an adult non-invasive form have interesting anti-cancer potential. Such agents need not in principal kill the cancer cells, simply 'reform' them.

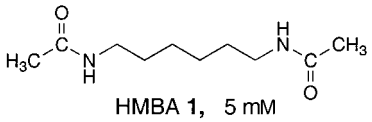
Dr. *Charlotte Friend* had been studying murine erythroleukemia cells – which are now generally called *Friend* cells or MEL cells – and, in order to transfect them, she soaked them in 280 mM dimethyl sulfoxide (DMSO) in H_2O. These MEL cells are stem cells that have been infected with a virus, and, as a result, they proliferate but do not differentiate to adult erythrocytes. That is, they retain the spherical morphology of pre-erythrocyte stem cells rather than the disc shape of mature erythrocytes, unlike mature erythrocytes they have nuclei, and, most strikingly, they have no hemoglobin. When Dr. *Friend* examined the MEL cells that had been sitting in aqueous DMSO she found that they had turned red, indicating the presence of hemoglobin, and that two-thirds of the cells now had adopted the flat shape of mature erythrocytes.

She called this result to the attention of Dr. *Paul Marks* and Dr. *Richard Rifkind* of the Columbia University Medical School (now at the *Sloan Kettering Institute for Cancer Research*, *SKI*), and they came to see me about the chemistry. This started our long-term collaboration. We wanted to develop effective compounds that differentiate cancer cells without causing undesired side effects, as weapons in the war against cancer.

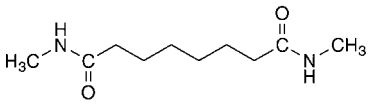
We quickly established that other polar solvents such as dimethylformamide (DMF) and *N*-methylacetamide had the same effect, and indeed they transformed the MEL cells at concentrations of only 50 mM or so in H_2O, lower than for DMSO [1]. However, it seemed clear that such concentrations would still be completely impractical for treatment of human cancer patients, so we set out to invent more effective compounds. (*Note:* Throughout this discussion concentrations will be indicated for *optimal* induction of differentiation. These are not the ED_{50} values for the compounds; the optimal concentrations lead to differentiations that are frequently of the order of 90%. In these cases, the ED_{50} value is lower.)



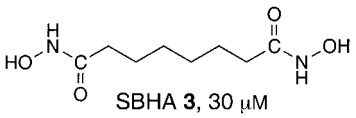
DMSO, 280 mM *N*-Methylacetamide, 50 mM



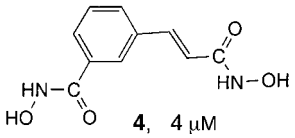
HMBA **1**, 5 mM



Suberoyl-bis[*N*-methylamide], **2**, *ca.* 5 mM



SBHA **3**, 30 μM



4, 4 μM

Assuming that the molecule was binding to a biological receptor, we proposed that there could well be two binding sites, possibly near each other, and that we thus might get more effective compounds if we took advantage of the chelate effect by using a bis-amide with a linker chain. We examined such molecules and saw that the optimum activity was seen with a molecule, hexamethylenebis[acetamide] (HMBA, **1**), that had

two acetamide groups linked at their N-atoms by a flexible $-(CH_2)_6-$ chain [2]. Analogs with $-(CH_2)_5-$ or $-(CH_2)_7-$ chains also had activity, but the optimum over the range of 2 to 9 methylenes came at 6.

We also showed that the amide groups could be reversed. Suberoyl-bis[*N*-methylamide] (= *N*,*N*′-dimethyloctanediamide; **2**) had similar activity to HMBA (**1**), again with an optimum at a six-methylene spacer [3][4]. This six-methylene preference also appeared in a later series of compounds that have a completely different mode of action from that of the bis-amides.

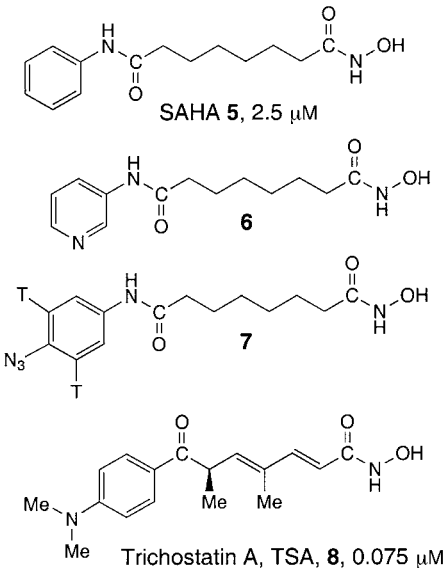
HMBA (**1**) was extensively studied. In tissue culture it induced the differentiation of a great range of cancer cells besides MEL cells, and indeed it even had clinical trials, leading to some modest success. It was effective with MEL at *ca.* 5 mM, an improvement over the 50 mM of *N*-methylacetamide, for instance, but still not really effective enough. The doses required to achieve blood levels of *ca.* 5 mM in human patients led to some undesirable side effects, chiefly major loss of white cells [5–8]. Many bis-amides were examined, and also tris- and tetra-amides [9], but without a large increase in effectiveness. Thus, we tried a different binding group.

An amide can bind either by making hydrogen bonds or by coordinating to a metal ion. In either case it seemed possible that a hydroxamic acid would be more effective. Thus we made suberyl bis[hydroxamic acid] (SBHA; *N*,*N*′-dihydroxyoctanediamide, **3**), an analog of some of our bis-amides with an additional *N*-OH group, with a $-(CH_2)_6-$ chain separating the hydroxamic-acid groups [10]. This compound was a more effective cytodifferentiating agent, requiring only 30 μM for optimum cytodifferentiation of MEL cells. In this series, we also saw that 6 for methylenes was the magic number for optimal effectiveness, with a curve from 2 to 9 methylenes, in which the 5- and 7-methylene compounds were reasonably effective but 6 was the best.

At the time this was not surprising, but now it is astonishing. As I will describe below, we have identified the biological target for the action of cytodifferentiating agents with hydroxamic-acid groups, and the bis-amides have *no effect* on that target. The two series have a different mode of action, but the same spacer preference. We have not yet identified the target for the original bis-amide series, but since those compounds were less effective than the hydroxamic-acid series, we have not devoted much effort to the question.

We made a number of bis[hydroxamic acids] with various spacers, including spacers with C=C bonds and benzene rings, and some such as compound **4** were even more effective than SBHA (**3**) [10]. However, we had no evidence that *two* hydroxamic-acid groups were required. Thus, we made a compound with a hydroxamic-acid group at one end of suberic acid (= octanedioic acid) and an anilide group at the other end, to pick up a possible hydrophobic pocket in the receptor [11]. This compound, with the trivial name suberylanilide hydroxamic acid (SAHA; *N*-hydroxy-*N*′-phenyloctanediamide; **5**), was even more effective; it required only 2.5 μM to give optimal cytodifferentiation of MEL cells.

SAHA (**5**) was effective in preventing the growth of a variety of human tumor cell lines, including those 75 or so in the standard screen performed at the *U.S. National Cancer Institute* (*NCI*). SAHA (**5**) has thus had significant examination. In unpublished work at *SKI*, it has been found that intra-peritoneal administration of



SAHA (**5**) to 'nude' immunocompromised mice with implanted human neuroblastomas and human prostate cancers blocked the further growth of these tumors.

In feeding experiments, SAHA (**5**) was included in the normal diet of a group of F-344 female rats at concentrations likely to achieve the desired blood levels [12]. There were no health problems observed with these rats relative to the control group whose feed did not include SAHA (**5**), but both groups were then injected in the tail vein with the potent carcinogen *N*-methyl-*N*-nitrosourea. The control group developed massive and progressing mammary tumors, but in the group with SAHA (**5**) in their diets there was '*marked inhibition of mammary tumor number and volume*'. In another study, SAHA (**5**) was put in the diet after mammary tumors had already started. '*These studies suggest that SAHA acts not only as a chemopreventive agent but also inhibits the continued development of palpable mammary tumors*'.

In a second study [13], female A/J mice had SAHA (**5**) included in their diets, and then they were injected intraperitoneally with a carcinogen found in cigarette smoke. The control group got lung cancer, but there was '*significant inhibition*' of tumor development in the group with SAHA in their diet, and no observable toxic effects of the SAHA.

Studies of the toxicity of SAHA (**5**) were performed by scientists at *Sterling-Winthrop Pharmaceutical Company*, who found minimal problems in mice and in dogs. Studies at *SKI* confirm that there are no serious toxic symptoms in animals. Human trials of SAHA (**5**) have been started recently, and the preliminary results are promising.

One of the problems with SAHA (**5**) is that it has somewhat limited water solubility. To address this we created the analog **6** of SAHA in which the Ph ring is replaced by a pyridine ring. Compound **6** is more soluble than SAHA (**5**), and it has undergone extensive testing at the *NCI* with respect to efficacy and toxicity. Clinical trials will start shortly for **6**.

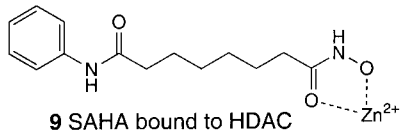
When the effectiveness and low toxicity of SAHA (**5**) became apparent, we set out to identify its target. We synthesized *N*-(4-azido[3,5-$^3H_2$]phenyl)-*N'*-hydroxyoctanediamide (**7**), a radioactive photoaffinity agent that we saw to be an effective drug [14][15]. That is, the N_3 group did not interfere with its biological activity relative to that of SAHA (**5**). We then went fishing in cells and cell fractions to identify a protein that was labelled by **7** on irradiation, but whose labelling could be competitively suppressed by the binding of SAHA (**5**). The first target was isolated and partially sequenced, and revealed as the protein S3, a nuclease with DNA repair functions [14][15].

However, at that point we became aware of work by *Yoshida et al.*, and others [16], indicating that trichostatin A (TSA; 7-[4-(dimethylamino)phenyl]-*N*-hydroxy-4,6-dimethyl-7-oxohepta-2,4-dienamide; **8**) is an inhibitor of histone deacetylase (HDAC). Since TSA (**8**) has some resemblance to SAHA (**5**), we examined the possibility that HDAC might be the target of SAHA. Indeed SAHA was an effective inhibitor of HDAC, as were the other hydroxamic acids we had made [17]. We showed that HDAC was photoaffinity labelled by **7**, and also that the biological effectiveness of our hydroxamic acid cytodifferentiating agents ran parallel with their effectiveness as inhibitors of HDAC. In the cells that were transformed by SAHA (**5**), we saw high levels of acetylated histone. At the present time it seems clear that this is the mode of action of the hydroxamic acids, but remarkably we found that bis-amides such as HMBA (**1**) had *no effect* on the enzymic activity of HDAC. We still do not know what the biological target is for the bis-amides.

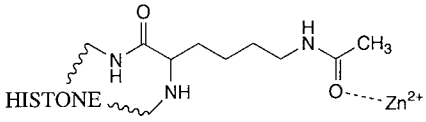
DNA wraps around histones, largely because of electrostatic interaction of histone-surface lysine cations with DNA phosphate anions. When histone is acetylated on those lysines by acetyl coenzyme A, catalyzed by histone transacetylase [18][19], the positive charge is lost, and the DNA is unwrapped, at least in part, making the DNA available for transcription. HDAC hydrolytically removes the Ac groups from the acetylated lysines, restoring the positive charge and suppressing the expression of the DNA. Thus, inhibition of HDAC by SAHA (**5**) and other inhibitors promotes the unwrapping and transcription of DNA. We believe that this leads to the transformation of cancer cells in the cytodifferentiation process [20].

TSA (**8**) is actually *ca.* 30 times more active than is SAHA (**5**) in some tests such as MEL differentiation, even though no better in others, so we set out to make more effective compounds. In unpublished work, we have expanded the hydrophobic region of SAHA (**5**) beyond a simple Ph ring, and found very effective inhibitors of the enzyme HDAC, with ID_{50} values in the single digit nM region.

It has not yet been possible to prepare crystals of HDAC, with or without drugs such as SAHA (**5**) or TSA (**8**) bound to it. However, *Pavletich* and co-workers at *SKI* were able to obtain crystals of both TSA (**8**) and SAHA (**5**) bound to a homolog of HDAC, and to solve their structures by X-ray crystallography [21]. The structure explains why our newest compounds are so effective. The protein histone sits on an open area of the enzyme HDAC, while the acetyllysine side chain passes down a narrow hole to reach a bound Zn^{II} in the enzyme interior (see **9**). In the X-ray structure, the inhibitors SAHA (**5**) and TSA (**8**) pass their chains carrying a terminal hydroxamic acid down the same hole to permit the hydroxamic-acid group to coordinate with the Zn^{II} (see **10**). The hydrophobic group on the other end of TSA (**8**) or of SAHA (**5**) sits in an open



9 SAHA bound to HDAC

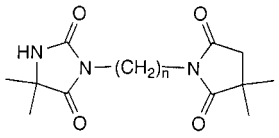


10 Acetylhistone bound to HDAC

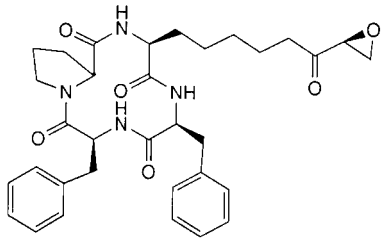
hydrophobic area where the histone core normally sits. The increased hydrophobicity of our new compounds allows them to bind even better to this open hydrophobic surface.

Thus, at the present time we have excellent indications that some of our compounds are promising leads for the treatment of various cancers, although only after human trials will this become clearer. Also, we have identified the target of our compounds, and the X-ray structural work is now an excellent guide for further development of even more effective pharmaceutical agents.

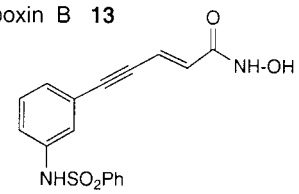
After we had described the cytodifferentiating activity of HMBA (**1**), chemists at *NIH* [22] prepared compounds **11** and **12**, and reported some cytodifferentiating



11. $n = 5$
12. $n = 6$



Trapoxin B **13**



Oxamflatin **14**

activity. We examined these compounds and found that they did not differentiate murine erythroleukemia (MEL) cells but were effective against human leukemia (HL-60), in contrast to our bis-amides such as HMBA (**1**), which were effective with both [10].

There has been a lot of work on inhibitors of HDAC. As mentioned earlier, it was found by *Yoshida et al.* [16] that TSA (**8**) is an effective inhibitor, and we have confirmed both this report and the effectiveness of **8** as a cytodifferentiating agent. Trapoxin B (**13**) is also effective, but as an irreversible inhibitor. Normally it is preferred not to use irreversible reactive drugs because of the likelihood that they can alkylate proteins and produce antigenic materials.

Interestingly, butyric acid is a weak inhibitor of HDAC. One could imagine that the carboxylate group might bind to the Zn^{II} in HDAC, but we have found that carboxy groups cannot substitute for the hydroxamic-acid groups in our compounds; the corresponding carboxylic acids are inactive. Also, some studies, apparently inspired by our publication on the properties of **4**, on a compound called oxamflatin (**14**) have been reported recently [23]. This is reported to be quite effective as an HDAC inhibitor and cytodifferentiating agent.

We should mention important studies on HDAC by *Schreiber* and co-workers [24]. *Allfrey* had detected the enzymatic activity in nuclear extracts in 1964 [25], and showed that there was a correlation between the acetylation state of chromatin and the rate of transcription of its DNA. *Schreiber* and co-workers [26] succeeded in isolating and characterizing one of the HDAC enzymes, and is apparently also pursuing HDAC inhibitors as potential antitumor agents. Also, some recent work from Germany and Austria [27][28] reports studies related to ours on compounds similar to SAHA (**5**).

We thank *Paul Marks*, *Richard Rifkind*, and *Victoria Richon* at *Sloan Kettering Institute for Cancer Research* for the biological studies on our compounds. Our work has been supported by the *U.S. National Institutes of Health*, including support of *Leland Gershell* by the *NIH Medical Scientist Training Program*.

REFERENCES

[1] M. Tanaka, J. Levy, M. Terada, R. Breslow, R. A. Rifkind, P. A. Marks, *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 1003.
[2] R. C. Reuben, R. L. Wife, R. Breslow, R. A. Rifkind, P. A. Marks, *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 862.
[3] P. A. Marks, R. Reuben, E. Epner, R. Breslow, W. Cobb, A. E. Bogden, R. A. Rifkind, *Antibiot. Chemother.* **1978**, *23*, 33.
[4] R. C. Reuben, P. L. Khanna, Y. Gazitt, R. Breslow, R. A. Rifkind, P. A. Marks, *J. Biol. Chem.* **1978**, *253*, 4214.
[5] R. C. Reuben, P. A. Marks, R. A. Rifkind, M. Terada, E. Fibach, U. Nudel, Y. Gazitt, R. Breslow, *Proc. Oji Int. Semin. Genet. Aspects Friend Virus Friend Cells* **1979**, 5th, 303.
[6] P. A. Marks, R. Breslow, R. A. Rifkind, in 'Anticancer Drugs', Eds. H. Tapiero, J. Robert, T. J. Lampidis, John Libbey Eurotext, Montrouge, 1989, pp. 153–165.
[7] P. A. Marks, R. A. Rifkind, R. Breslow, in 'Molecular Basis of Haematopoiesis', Eds. L. Sachs, N. G. Abraham, C. Weidemann, A. S. Levine, G. Konwalinka, Intercept Scientific, Andover, Great Britain, 1990, pp. 579–586.
[8] P. A. Marks, R. A. Rifkind, V. Richon, T. Powell, X. Busquets, L. Leng, H. Kiyokawa, J. Michaeli, B. Jursic, R. Breslow, in 'Concise Reviews in Experimental and Clinical Hematology', Ed. M. J. Murphy, AlphaMed Press, Dayton, Ohio, 1992, pp. 91–99.
[9] P. A. Marks, R. Breslow, R. A. Rifkind, L. Ngo, R. Singh, *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 6358.
[10] R. Breslow, B. Jursic, Z. F. Yan, E. Friedman, L. Leng, L. Ngo, R. A. Rifkind, P. A. Marks, *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 5542.

[11.] V. M. Richon, Y. Webb, R. Merger, T. Sheppard, B. Jursic, L. Ngo, F. Civoli, R. Breslow, R. A. Rifkind, P. A. Marks, *Proc. Natl. Acad. Sci. U.S.A*. **1996**, *93*, 5705.
[12] L. A. Cohen, V. M. Richon, P. Marks, D. Desai, S. Amin, *Proc. Amer. Assoc. Cancer Res.* **1998**, *39*, 108.
[13] D. Desai, K. El-Bayoumy, S. Amin, *Proc. Am. Assoc. Cancer Res.* **1999**, *40*, 2396.
[14] Y. Webb, L. Ngo, V. M. Richon, R. Breslow, R. Rifkind, P. A. Marks, *Proc. Am. Assoc. Cancer Res.* **1998**, *39*, 108.
[15] Y. Webb, X. Zhou, L. Ngo, V. Cornish, J. Stahl, H. Erdjument-Bromage, P. Tempst, R. A. Rifkind, P. A. Marks, R. Breslow, V. M. Richon, *J. Biol. Chem*. **1999**, *274*, 14280.
[16] M. Yoshida, S. Horinouchi, T. Beppu, *BioEssays* **1995**, *17*, 423, and refs. cit. therein.
[17] V. M. Richon, S. Emiliani, E. Verdin, Y. Webb, R. Breslow, R. A. Rifkind, P. A. Marks, *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 3003.
[18] J. E. Brownell, J. X. Zhou, T. Ranalli, R. Kobayashi, D. G. Edmondson, S. Y. Roth, C. D. Allis, *Cell* **1996**, *84*, 843.
[19] Y. Lin, C. M. Fletcher, J. Zhou, C. D. Allis, G. Wagner, *Nature* **1999**, *400*, 86.
[20] P. A. Marks, V. M. Richon, R. Breslow, R. A. Rifkind, *C. R. Acad. Sci. Paris/Life Sciences* **1999**, *322*, 161.
[21] M. S. Finnin, J. R. Donigian, A. Cohen, V. M. Richon, R. A. Rifkind, P. A. Marks, R. Breslow, N. A. Pavletich, *Nature* **1999**, *401*, 188.
[22] A. Haces, T. R. Breitman, J. S. Driscoll, *J. Med. Chem.* **1987**, *30*, 405.
[23] Y. B. Kim, K.-H. Lee, K. Sugita, M. Yoshida, S. Horinouchi, *Oncogene* **1999**, *18*, 2461.
[24] C. M. Grozinger, C. A. Hassig, S. L. Schreiber, *Proc. Natl. Acad. Sci. U.S.A*. **1999**, *96*, 4868, and refs. cit. therein.
[25] V. G. Allfrey, R. Faulkner, A. E. Mirsky, *Proc. Natl. Acad. Sci. U.S.A*. **1964**, *51*, 786.
[26] J. Taunton, C. A. Hassig, S. L. Schreiber, *Science* **1996**, *272*, 408.
[27] M. Jung, K. Hoffmann, G. Brosch, P. Loidl, *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1655.
[28] M. Jung, G. Brosch, D. Kölle, H. Scherf, C. Gerhäuser, P. Loidl, *J. Med. Chem.* **1999**, *42*, 4669.

Received March 27, 2000