Differential Sensitivity of Various Pediatric Cancers and Squamous Cell Carcinomas to Lovastatin-induced Apoptosis: Therapeutic Implications[1]

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ABSTRACT

3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase is the rate-limiting enzyme of the mevalonate pathway, the diverse array of end products of which are vital for a variety of cellular functions, including cholesterol synthesis and cell cycle progression. We showed previously that this enzyme holds a critical role in regulating tumor cell fate, including cell death, as its expression is down-regulated in response to retinoic acid, a potent anticancer therapeutic. Indeed, direct inhibition of HMG-CoA reductase with lovastatin, a competitive inhibitor of this enzyme, induced a pronounced apoptotic response in neuroblastoma and acute myeloid leukemic cells. We have now extended this work and evaluated a wide variety and large number of tumor-derived cell lines for their sensitivity to lovastatin-induced apoptosis. These cell lines were exposed to a wide range (0–100 μM) of lovastatin for 2 days and assayed for cell viability using the 3,4,5-dimethyl thiazlyl-2,2,5-diphenyltetrazolium bromide assay and the induction of apoptosis by flow cytometric and ultrastructural analyses. Lovastatin induced a pronounced apoptotic response in cells derived from juvenile monomyelocytic leukemia, pediatric solid malignancies (rhabdomyosarcoma and medulloblastoma), and squamous cell carcinoma of the cervix and of the head and neck. Interestingly, the subset of malignancies that are particularly sensitive to lovastatin-induced apoptosis correspond to those tumor subtypes that are sensitive to the biological and antiproliferative effects of retinoids *in vitro*. The nature of the biologically active form of lovastatin has been challenged recently as the growth-inhibitory effects of this drug were attributed to its prodrug lactone form that does not inhibit HMG-CoA reductase function. In this report, we demonstrate that the apoptotic properties of lovastatin are triggered by the open ring acid form that is a potent inhibitor of HMG-CoA reductase activity. Thus, we have identified a subset of tumors that are sensitive to lovastatin-induced apoptosis and show HMG-CoA reductase as a potential therapeutic target of these cancers.

INTRODUCTION

Apoptosis or programmed cell death is an essential process for tissue development and homeostasis as well as the elimination of damaged cells (1–3). Apoptosis is a highly regulated cellular process that can be activated as a result of aberrant proliferation or differentiation, abrogation of cell survival signals, or in response to cellular damage (1–4). Failure to adequately control any of these cell fates, including proliferation, differentiation, cell survival, and apoptosis, contributes to neoplastic transformation (2, 5, 6). Most chemotherapeutic agents target tumor cell proliferation, leading to the induction of an apoptotic response (2, 5, 6). The effectiveness of these agents can be limited by collateral damage to replicating normal cells as well as intrinsic and acquired resistance by tumor cells (7–9). More recently, agents that regulate cell survival signaling are being and have been developed as a direct result of progress in elucidating the cellular components of these pathways (10, 11). The clinical utility of modulators of the survival signaling cascade to date, however, has been limited because of specificity and toxicity difficulties (11, 12). Refinements in target identification and validation may uncover agents with greater therapeutic potential.

Tumor cells generally display stalled or arrested differentiation of their normal cellular counterparts (13–15). Several biological and clinical features suggest that this differentiation arrest may be reversible because tumor cells can often display a spectrum of maturation along their respective lineages of tissue origin (3, 14, 15). Indeed, differentiation-based therapeutics can restore the regulation of differentiation and enhance the predetermined differentiation programs in tumor cells, often leading to an apoptotic end point (14, 15). A hallmark or characteristic of differentiation-based therapeutics is that these agents typically display tumor specificity, targeting distinct subsets of

malignancies based on cell lineage (13–15). Despite intensive laboratory and clinical research in identifying potential differentiation-based cancer therapeutics, retinoids remain the most active and promising group of agents of this class (16–18). The utilization of retinoids, particularly retinoic acid, in therapeutic protocols has been limited because of significant associated toxicity and the acquisition of both systemic and cellular resistance to these agents (13, 19, 20). Because the biological effects of retinoids are mediated through transcriptional regulation by their nuclear receptors (13, 21), identification of their gene targets may potentially uncover novel therapeutic approaches circumventing the toxicity and resistance limitations of retinoids (13, 21).

To this end, we recently identified the enzyme HMG-CoA[3] reductase, the rate-limiting enzyme of the mevalonate pathway, as a retinoic acid-repressed gene and a mediator of the biological effects of retinoic acid (22, 23). Mevalonate is a critical intermediate in a complex biochemical pathway, the products of which, including *de novo* cholesterol, are vital for a variety of key cellular functions affecting membrane integrity, cell signaling, protein synthesis, and cell cycle progression (24). Lovastatin is a specific, competitive inhibitor of HMG-CoA reductase (25, 26), the active open ring form of which can effectively block this critical metabolic pathway, has been used extensively for the treatment of hypercholesterolemia (25, 26). Although the biological effects of lovastatin has been attributed to its ability to block HMG-CoA reductase function, a recent report has suggested that the prodrug closed ring form that does not target HMG-CoA reductase is also functional (27).

The ability of lovastatin to induce growth arrest in a number of tumor-derived cell lines (28) led to its evaluation as a potential anticancer therapeutic agent. In a Phase I clinical trial of 88 patients with solid tumors, where prostate, breast, and central nervous system cancers were evaluated, high doses of lovastatin were well tolerated but did not display any significant anticancer activity (29). However, a number of recent studies (30–32), including our own (23, 33), have shown that lovastatin can induce a pronounced apoptotic response in certain tumor types. We have also noted that in the two sensitive tumor types identified in our laboratory, neuroblastoma and acute myeloid leukemia (23, 33), lovastatin induces differentiation of these cells prior to apoptosis (34). This is consistent with our identification of HMG-CoA reductase as a biological mediator of the effects of retinoids (23). Taken together, these studies suggest that lovastatin, a potential differentiation-based therapeutic that can induce apoptosis, may target a specific and wider subset of tumors and, therefore, should be reevaluated as a therapeutic approach. To test this hypothesis, we determined the sensitivity of a large and varied panel of tumor-derived cell lines to lovastatin-induced apoptosis. We have now extended these studies and have demonstrated that a number of specific cancers are

particularly sensitive to the apoptotic effects of lovastatin and include various pediatric malignancies (juvenile monomyelocytic leukemia, rhabdomyosarcoma, and medulloblastoma), squamous cell carcinoma of the cervix, and HNSCC. Moreover, we have shown that the open ring form, and not the prodrug form, of lovastatin is the effective agonist of apoptosis. Thus, targeting HMG-CoA reductase may represent a novel therapeutic approach in the treatment of these cancers.

MATERIALS AND METHODS

Cell Culture. The acute lymphocytic leukemia cell lines B1, C1, W1, G2, KK, and NGR, as well as the juvenile monomyelocytic leukemia patient cell cultures, were derived from primary patient samples as described previously (35, 36). The acute myeloid leukemia cell lines OCI-AML-1, OCI-AML-2, OCI-AML-3, OCI-AML-4, OCI-AML-5, NB-4, and HL60 were kindly provided by Dr. E. A. McCulloch (Ontario Cancer Institute, Toronto, Ontario, Canada). Normal bone marrow cells were obtained with informed consent and kindly provided by Dr. H. Messner (Ontario Cancer Institute, Toronto, Ontario, Canada). All of the pediatric (medulloblastoma: UW228, DOAY, D341, UW402, D283, and MB-1; rhabdomyosarcoma: SJRH, RD, A204, A673, and HS729T; neuroblastoma: SK-N-SH, NUB-7, SMS-MSN, NBL-S, LAN-5, IMR-32, and GOTO; embryonal carcinoma: P19, TERA-2; hepatoblastoma: HEPG2; and neuroepithelioma: EW-2), choriocarcinoma (JAR, BeWo, and JEG-3), and renal cell carcinoma (CAKI-1 and ACHN) cell lines used in this study were provided by Dr. H. Yeger (Hospital for Sick Children, Toronto, Ontario, Canada). The prostate carcinoma (PC3, LNCAP, and DU145), melanoma (SK-MEL2, 1232, WM9, 457, WM35, WM793, and WM983), breast carcinoma (BT20, MDA 468, MDA 231, SK BR3, ZR-75-1, and MCF-7), cervical carcinoma (SIHA, CASKI, HT-1, and ME180), and HNSCC (SCC4, SCC25, SCC15, 2650 PI, SCC9, FADU, and CAL27) derived cell lines were provided by Drs. J. Squire (Ontario Cancer Institute, Toronto, Ontario, Canada), N. Lassam (University of Toronto, Toronto, Ontario, Canada), the laboratory of R. Buick (Ontario Cancer Institute, Toronto, Ontario, Canada), D. Hill (Ontario Cancer Institute, Toronto, Ontario, Canada), and S. Kamel-Reid (Ontario Cancer Institute, Toronto, Ontario, Canada), respectively. The cell lines used in this study were maintained in α-MEM (Princess Margaret Hospital Media Services, Toronto, Ontario, Canada) supplemented with 10% fetal bovine serum (Sigma Chemical Co., St. Louis). Cells were exposed to solvent control or to 1–100 μM lovastatin [generously provided by Merck Research Laboratories, Rahway, NJ; diluted from a 10 mM stock in ethanol prepared as described previously (28, 33)] and processed for MTT, flow cytometric, and electron microscopic analyses.

MTT Assay. In a 96-well, flat-bottomed plate (Nunc, Naperville, IL), ~10,000 cells/150 μl of cell suspension were used to seed each well. After 2 days of lovastatin treatment (0–100 μM), 50 μl of a 5-mg/ml solution in PBS of the MTT tetrazolium substrate (ICN, Toronto, Ontario, Canada) were added and incubated for 6 h at 37°C. The resulting violet formazan precipitate formed was solubilized by the addition of 100 μl of a 0.01 M HCl/10% SDS (Sigma) solution, shaking overnight at 37°C. The plates were then analyzed on an SLT

Labinstruments 340 ATTC ELISA plate reader at 450 nm to determine the absorbance of the samples.

Flow Cytometry and Electron Microscopy. Cell cycle parameters were determined by flow cytometry using propidium iodide labeling of single cells as described previously (33). Single-cell suspensions were labeled with 50 μg/ml propidium iodide (Sigma), and ~10^6 cells in 100 μl analyzed by flow cytometry. Ten thousand cells were evaluated, and the percentage of cells in pre-G_1 phase was determined using the Modfit LT program (Verity Software House, Topsham, ME). For electron microscopy, ultrathin sections of cultured cell pellets were cut and prepared as described previously (33). Cultured cell pellets were fixed in phosphate-buffered 2% glutaraldehyde and 1% osmium tetroxide, dehydrated through acetone, and embedded in epon araldite.

Mass Spectroscopy and HPLC. Mass spectral analyses were performed at the Mass Spectrometry Facility of the Biotechnology Service Center at The Hospital for Sick Children (Toronto, Ontario, Canada). Briefly, electrospray ionization mass spectrometry was used for the analysis of lovastatin prodrug and the activated form. Analyses were performed on a model Q-T Mass Spectrometer using MassLynx Software version 3.2 (Micromass, Montreal, Ontario, Canada). Samples (10 μl) were introduced into the ionization source via flow injection (10 μl/min) with 1:1 acetonitrile:water plus 0.2% (vol/vol) acetic acid. Spectra were collected in the mass range 50–1500 amu, over a period of about 4 min, at a sampling rate of 1.90/spectrum, with a 0.10-min time lag between two consecutive spectra. Lovastatin lactone and the open ring acid form were measured using reversed phase HPLC with solid phase extraction.[4] The internal standard used was cerivastatin, a member of the vastatin family of HMG-CoA reductase inhibitors. The lower limit of quantitation of lovastatin was 100 ng/ml.

RESULTS

Sensitivity of Tumor-derived Cell Lines to Lovastatin-induced Cytotoxicity. In this study, we evaluated a number of tumor-derived cell lines for their lovastatin sensitivity to identify tumor cells that may be as sensitive to lovastatin-induced apoptosis as we had shown previously for neuroblastoma (23) and acute myeloid leukemia (33). Fifty-nine cell lines have been evaluated for lovastatin sensitivity and compared with the sensitive (acute myeloid leukemia and neuroblastoma) and insensitive (acute lymphocytic leukemia and normal bone marrow) cells evaluated in our previous studies (23, 33). All of the cells were exposed to a wide range (0–100 μM) of lovastatin for 2 days and assayed for cell viability using the MTT assay (37). The MTT assay is a measure of mitochondrial dehydrogenase activity in viable cells and is used to evaluate cytotoxicity *in vitro* (37, 38). The use of the MTT assay at the 2-day time point is consistent with the NCI anticancer cell line screen that is currently used to evaluate the cytotoxicity and specificity of a wide array of agents (39, 40).

Using a similar approach to the NCI, we compared the

sensitivity of the cell lines used in our survey to lovastatin. Experimental evidence from our previous work with leukemia cell lines and primary cell cultures showed that significant apoptosis after 2 days of exposure to lovastatin was characteristic of MTT values falling to <30% (33). Evaluating the MTT50 and the MTT30 values (MTT values at 50 and 30% of control) distinguished the sensitive acute myeloid leukemias from the insensitive acute lymphocytic leukemias (33). On the basis of these data, a S.I. of each cell line was calculated by adding the differences between the calculated means of the MTT50 and MTT30 of all 80 cell lines with the cell line of interest. Each MTT50 and MTT30 value was determined by averaging two independent experiments with six replicates in each experiment. The S.I. for each cell line was then calculated using the following formula S.I. = (MTT50$_{n=1}$ − meanMTT50$_{n=80}$) + (MTT30$_{n=1}$ − meanMTT30$_{n=80}$). On the basis of our identification of HMG-CoA reductase as a potential mediator of the biological effects of retinoids and the sensitivity of neuroblastomas and acute myeloid leukemias to lovastatin-induced apoptosis (23, 33), we included a wide range of retinoid-responsive and nonresponsive cancers in this survey (20, 41, 42). Cell lines derived from juvenile monomyelocytic leukemias, medulloblastomas, rhabdomyosarcomas, prostate carcinomas, melanomas, breast carcinomas, choriocarcinomas, cervical carcinomas, HNSCCs, and renal cell carcinomas were evaluated. Of these, only prostate carcinoma, melanoma, breast carcinoma, and renal cell carcinoma are represented within the NCI screen (39). Furthermore, we also included four nontransformed fibroblast cell lines.

The sensitivity index of all 80 cell lines, including the leukemic and neuroblastoma cell lines analyzed in our previous studies (23, 33) for comparison purposes, are shown in Table 1 along with their MTT50 and MTT30 values. The S.I. data are also presented in columnar form to facilitate comparison of lovastatin sensitivity between the cell lines (Fig. 1). The range of the calculated S.I. was from 96 to −97, representing the most resistant lines to the most sensitive cell lines examined, respectively. On the basis of our MTT results, the cell lines segregated into three groups: a nonresponsive group, the MTT50 and MTT30 values of which were both >100 μM; an intermediate group, the MTT50 values but not their MTT30 values of which were <100 μM; and a more sensitive group, where both the MTT50 and MTT30 values fell below 100 μM. Representative MTT profiles of a number of the cell lines used in this study are shown in Fig. 2, *A–C*, highlighting the differences of the three sensitivity groups. The difference in responsiveness to lovastatin of acute myeloid and acute lymphocytic leukemias was well established in our previous study and validated this analytical approach as a measure of responsiveness to lovastatin in this cell line series (Fig. 1A). In the pediatric tumor types evaluated (juvenile monomyelocytic leukemias, medulloblastomas, and rhabdomyosarcomas,), sensitive, intermediate, and nonresponsive cell lines were evident within each subtype (Fig. 1*B*). The adult solid tumor-derived cell lines response to lovastatin was linked to tumor type. Although the majority of prostate and breast carcinomas and melanomas did not display significant cytotoxicity in response to this agent (Fig. 1*C*), the majority of choriocarcinomas, cervical carcinomas, and HNSCCs were sensitive to lovastatin-induced cytotoxicity (Fig. 1*D*). The adult

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[4] L. Y. Ye and M. J. Moore, manuscript in preparation.

Table 1 Sensitivity of various tumor-derived cell lines to lovastatin-induced cytotoxicity: MTT analysis

Each MTT50 and MTT30 value was determined by averaging two independent experiments with six replicates in each experiment. The sensitivity index for each cell line was then calculated using the following formula S.I. = (MTT50$_{n=1}$ − meanMTT50$_{n=80}$) + (MTT30$_{n=1}$ − meanMTT30$_{n=80}$).

Leukemia-derived cell lines[a]

Cell lines	MTT50	MTT30	S.I.
AML			
AML-4	22	>100	18
HL-60	5	27	−72
AML-1	4	19	−86
AML-5	3	13	−88
AML-2	4	7	−93
NB-4	2	8	−94
AML-3	1	6	−97
ALL			
G2	>100	>100	96
KK	>100	>100	96
B1	>100	>100	96
C1	>100	>100	96
W1	86	>100	82
NGR	72	>100	68
Bone marrow			
BM1	>100	>100	96
BM2	>100	>100	96
BM3	>100	>100	96

Pediatric tumor-derived cell lines

Cell lines	MTT50	MTT30	S.I.
JMML			
28	>100	>100	96
24	92	>100	88
29	52	>100	48
26	17	85	−2
25	18	55	−31
30	12	17	−75
Neuroblastoma[a]			
GOTO	>100	>100	96
IMR-32	>100	>100	96
LAN-5	>100	>100	96
NBL-S	60	85	41
SMS-MSN	20	74	−10
NUB-7	25	35	−44
SK-N-SH	10	34	−58
Rhabdomyosarcoma			
HS729T	>100	>100	96
A673	8	>100	4
A204	14	50	−40
FD	9	23	−72
SJRH	8	20	−76
Medulloblastoma			
MB-1	>100	>100	96
D283	18	>100	14
UW402	8	>100	4
D341	36	61	−7
DOAY	15	55	−34
UW228	6	11	−87
Other			
EW-2	51	>100	47
HEPG2	11	28	−69
TERA-2	7	14	−83
P19	4	12	−88

Adult tumor-derived cell lines

Cell lines	MTT50	MTT30	S.I.	Cell lines	MTT50	MTT30	S.I.
Fibroblast				Renal cell carcinoma			
417902	>100	>100	96	ACHN	1	>100	−3
MRC5	>100	>100	96	CAKI-1	0.8	6	−97
385810	>100	>100	96	HNSCC			
HEL	9	>100	5	CAL27	50	59	5
Breast carcinoma				FADU	12	25	−67
MCF-7	>100	>100	96	SCC9	8	18	−78
ZR-75-1	>100	>100	96	2650 PI	7	14	−83
SK BR3	17	>100	14	SSC15	6	13	−85
MDA 231	10	>100	6	SCC25	0.8	1.6	−87
MDA 468	8	>100	4	SCC4	0.3	12	−89
BT20	8	40	−56	Cervical carcinoma			
Melanoma				ME180	>100	>100	96
WM983	>100	>100	96	HT-1	8	19	−77
WM793	>100	>100	96	CASKI	2	9	−93
WM35	70	>100	66	SIHA	3	8	−93
457	18	>100	14	Choriocarcinoma			
WM9	15	>100	11	JEG-3	>100	>100	96
1232	13	>100	9	BeWo	22	56	−31
SK-MEL2	9	>100	5	JAR	3	7	−94
Prostate carcinoma							
DU145	>100	>100	96				
LNCAP	8	>100	4				
PC3	1	>100	−3				

[a] Extrapolated from data in Refs. 23 and 33 and used only as a comparator to the other cell lines based in this study.
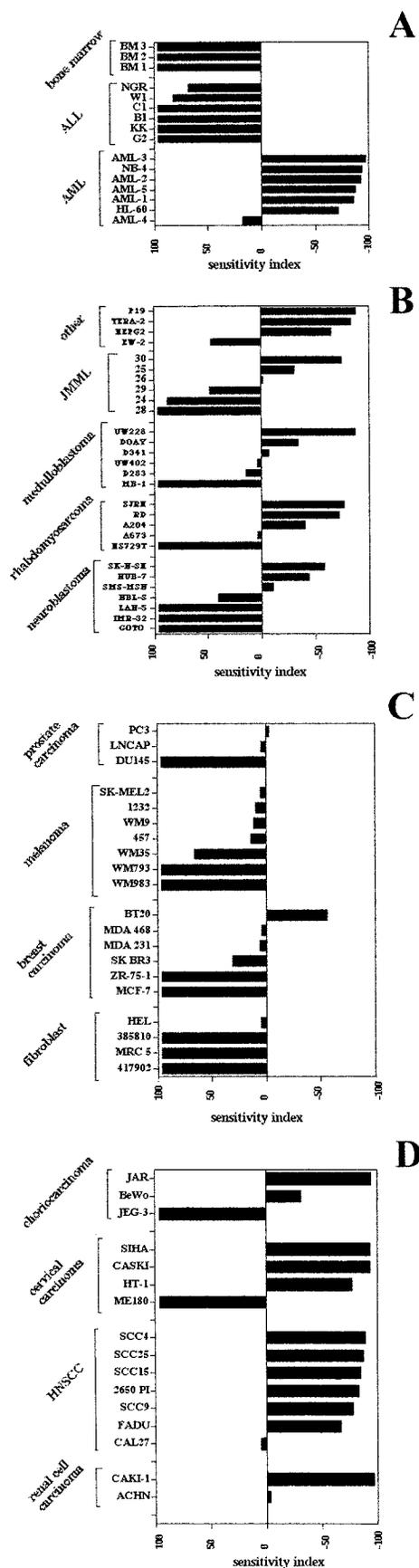
solid tumor subtypes were segregated, based on their reported responsiveness to retinoic acid. Tumor types that typically do not display dramatic growth inhibition or differentiation within therapeutically relevant concentrations of retinoic acid are grouped in Fig. 1*C,* whereas retinoid responsive cancers are grouped in Fig. 1*D* (20, 41, 42).

Lovastatin Induces Apoptosis of Sensitive Cell Lines. To determine whether the drop in MTT activity after exposure to lovastatin was attributable to induction of apoptosis, flow cytometric and ultrastructural characteristics of the cells were evaluated. By flow cytometric analysis, cells undergoing apoptosis typically show a pre-G_1 peak because of nuclear and cellular fragmentation (43, 44). Flow cytometric analysis of the majority of the cell lines used in this survey also distinguished the differences in the nonresponsive, intermediate, and sensitive cell lines highlighted by the MTT analysis. For example, no significant apoptosis was observed in the nonresponsive breast carcinoma cell line MCF-7 after exposure to 20 μM lovastatin for up to 2 days (Fig. 2*D*). The intermediate rhabdomyosarcoma cell line A673 exhibited a weak apoptotic response, as highlighted by the presence of a pre-G_1 peak containing 9.1% of the cells after a 2-day treatment of 20 μM lovastatin (Fig. 2*D*). The sensitive pediatric teratocarcinoma cell line TERA-2, under identical experimental conditions, showed a significant apoptotic response highlighted by the presence of a prominent pre-G_1 peak of 46.8% (Fig. 2*D*).

Ultrastructural features of apoptotic cell death include chromatin and cytoplasmic condensation, followed by nuclear and cellular fragmentation (2, 5). Electron microscopic analysis of MCF-7 and TERA-2 cell lines either untreated or exposed to 20 μM lovastatin for 2 days clearly highlighted their distinct responses to lovastatin treatment. Only lovastatin-treated TERA-2 cells displayed ultrastructural features of apoptosis including nuclear and cytoplasmic condensation (Fig. 3). Therefore, the cytotoxic effects of lovastatin are the result of the induction of an pronounced apoptotic response that is evident in a number of tumor types and is absent or muted in the nonresponsive and the intermediate responsive cell lines analyzed in this study.

Apoptotic Effects of Lovastatin Are Mediated by the Open Ring Activated Form. The nature of the biologically active form of lovastatin has been challenged recently (27). Lovastatin is a closed ring lactone prodrug that is enzymatically or chemically altered to its open ring acid form (25, 26). The open ring structure acts as a molecular mimic of the intermediate formed in the conversion of HMG to mevalonate by HMG-CoA reductase (Fig. 4*A*). The open ring form binds to the active site at a 10× greater affinity than the natural product, making lovastatin a potent inhibitor of HMG-CoA reductase (25, 26). A

Fig. 1 Lovastatin S.I. of the 80 cell lines assayed by MTT. *A,* leukemia and bone marrow samples (33) shown as a comparator for the other cell lines tested. *B,* pediatric tumor-derived cell lines tested. *C,* nonretinoid responsive adult solid tumor derived cell lines. *D,* retinoid-responsive, adult solid tumor-derived cell lines. The mean MTT50 (dose at 50% MTT activity) and MTT30 (dose at 30% MTT activity) are calculated within all of the cell lines, and a S.I. is determined. Each MTT50 and MTT30 value was determined by averaging two independent experiments with six replicates in each experiment.
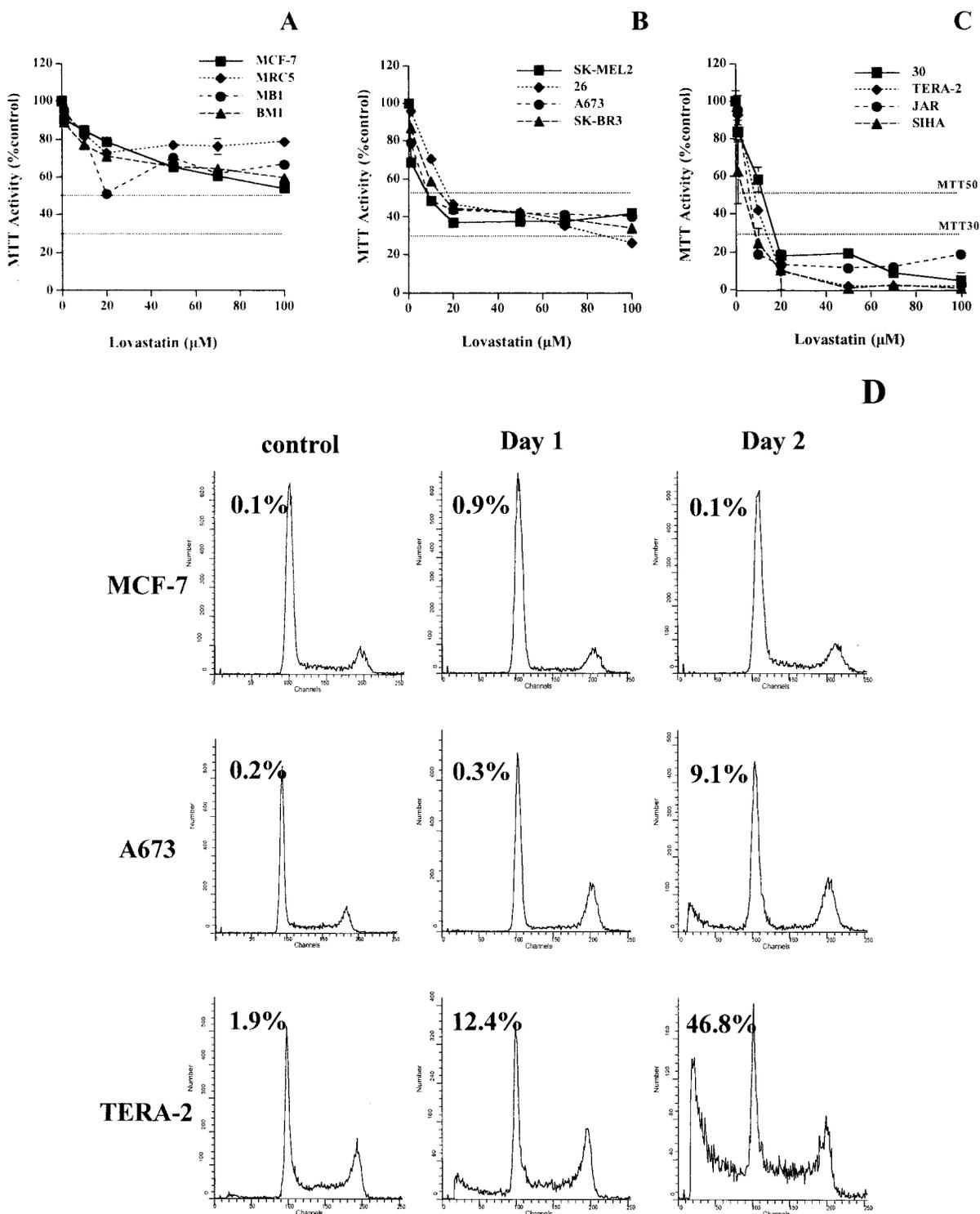
Fig. 2 Evaluating the effects of lovastatin on the viability of tumor-derived cell lines using the MTT assay. MTT enzyme activity, after exposure to 0–100 μM lovastatin for 2 days, distinguished three responses to this agent. *A,* nonresponsive; *B,* intermediate; and *C,* sensitive to the cytotoxic effects of lovastatin. *Dashed lines* highlight the MTT50 and MTT30 values. *D,* representative flow cytometric analysis of tumor-derived cell lines after exposure to solvent control or 20 μM lovastatin for 1 and 2 days. The percentage of cells in the pre-G_1 (apoptotic) fraction is shown in the *upper left region* of the individual histograms. The cell lines segregated into three groups based on their apoptotic response to lovastatin exposure: nonresponsive (MCF-7), intermediate (A673), and sensitive (TERA-2).
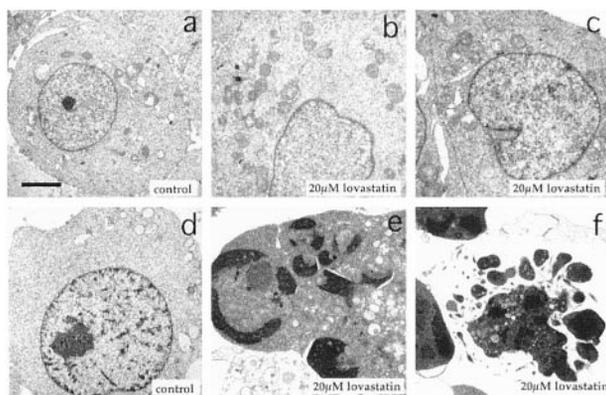
Fig. 3 Ultrastructural changes in the MCF-7 (*a*–*c*) and the TERA-2 (*d*–*f*) cell lines after 2 days of exposure to 20 μM lovastatin. *a,* control MCF-7 cells; *b* and *c,* lovastatin treatment produced no significant changes; *d,* control TERA-2 cells; *e* and *f,* lovastatin treatment induced morphological features typical of apoptosis in the majority of cells examined.

recent report has implicated the lactone form as a potential mediator of the growth-inhibitory properties of this drug (27). We convert lovastatin to its activated form under basic conditions (NaOH), and the solution neutralized with the addition of equimolar acid (HCl) as described previously (28, 33). To determine whether this treatment had indeed resulted in the formation of the open ring structure, we evaluated the molecular mass of our lovastatin preparation before and after chemical activation. Using electrospray ionization mass spectrometry, the molecular mass of the prodrug was 405.4 Da, as predicted by its chemical structure (Fig. 4, *A* and *B*). After chemical treatment, the predominant molecular mass in our preparation was 445.4 Da (Fig. 4, *A* and *B*). The observed mass coincides with the addition of a water molecule and a sodium ion as a consequence of opening the ring structure and the activation protocol, respectively. To quantitate the extent of activation, HPLC analysis of the prodrug and the activated forms was performed. As documented previously, the prodrug and activated forms of lovastatin are distinguishable by HPLC (27). The conversion of the prodrug to the activated form of lovastatin was shown to be >98% under our activation procedure (Fig. 4*C*).

Mevalonate Reversal of Lovastatin-induced Apoptosis. To determine the role of the open ring acid form of lovastatin as a mediator of its apoptotic effects, we exposed the nonresponsive MCF-7 (breast carcinoma) and the sensitive TERA-2 (embryonal carcinoma) cell lines to both the prodrug and the activated forms of lovastatin. Both the prodrug and the activated preparations had minor effects on MCF-7 MTT activity with no morphological evidence of toxicity. Addition of 200 μM mevalonate, the end product of the reaction catalyzed by HMG-CoA reductase, did not affect the MTT activity of the MCF-7 cell line treated with the prodrug and activated forms of lovastatin (Fig. 5*A*). By contrast, exposure of the TERA-2 cell line to either preparation induced a prominent cytotoxic response evident by the MTT assay (Fig. 5*B*) as well as morphologically (data not shown). The prodrug version was slightly less effective than its activated counterpart. With both the prodrug and activated prep-

arations, addition of 200 μM mevalonate abrogated the cytotoxic responses observed (Fig. 5*B*). To test whether the open ring form may be produced spontaneously as a consequence of hydrating the prodrug form of lovastatin in the media used, we analyzed the HPLC profile of lovastatin at various time points after the addition of the prodrug to media. In culture medium, 80% of the prodrug converts to the activated form within 9 h, with complete conversion occurring within 24 h (data not shown). Taken together, these results indicate that the cytotoxic effects induced by lovastatin in this cell line survey were mediated by the activated form of this drug that inhibits HMG-CoA reductase activity and function.

DISCUSSION

In this study, we evaluated the sensitivity of a variety of tumor-derived cell lines to lovastatin-induced apoptosis using a similar approach to the NCI 60 cell line anticancer agent screen (39). Because of the potential mechanistic link between HMG-CoA reductase and the anticancer properties of retinoids (23), we evaluated the lovastatin sensitivity of a number of retinoid responsive and nonresponsive cancer-derived cell lines, including many that have not been analyzed previously in the NCI anticancer screen (39). In our cell line screen, we demonstrated that a number of retinoid-responsive tumor types (20, 41, 42) that included a variety of pediatric solid malignancies, JMML, HNSCC, and cervical carcinomas are particularly sensitive to lovastatin-induced apoptosis. This work suggests that lovastatin has potential as an immediate, novel therapeutic approach in the treatment of these responsive tumors: (*a*) lovastatin induced a specific apoptotic response in these tumor cells within the achievable therapeutic range; and (*b*) it has a proven record in the clinic as a safe and effective drug (25, 26).

The therapeutic potential of lovastatin on the apoptosis-sensitive tumor types identified in our studies were not evaluated in the previous Phase I trial (29). Indeed, we show here that cell lines derived from the tumor subtypes evaluated in the above Phase I trial did not undergo significant apoptosis in response to lovastatin exposure *in vitro*. This is consistent with previous reports showing that lovastatin can trigger a cytostatic response in these types of cancer cells (28, 29). Furthermore, in a 5-year safety and efficacy study of lovastatin in the treatment of 745 patients with severe hypercholesterolemia, a lower incidence of cancer (14 cases *versus* 21 expected) was observed (45). Although this patient population was relatively small, the incidence of breast and prostate carcinomas was as expected; however, no cancers identified as lovastatin sensitive in this study were documented (45). Using a larger cohort of patients, the effect of prolonged exposure of lovastatin to cancer incidence may determine whether this agent has anticancer and/or chemopreventative capabilities. Therefore, the tumor-specific apoptosis induction as well as the biological properties of the HMG-CoA reductase inhibitors suggests that they are potentially ideal therapeutic agents in the treatment of a specific subset of cancers.

The mechanism of action and the tumor specificity of lovastatin-induced apoptosis remain unclear. The sensitivity to lovastatin-induced apoptosis appears to be cell type dependent and may result from the depletion of critical mevalonate metab-
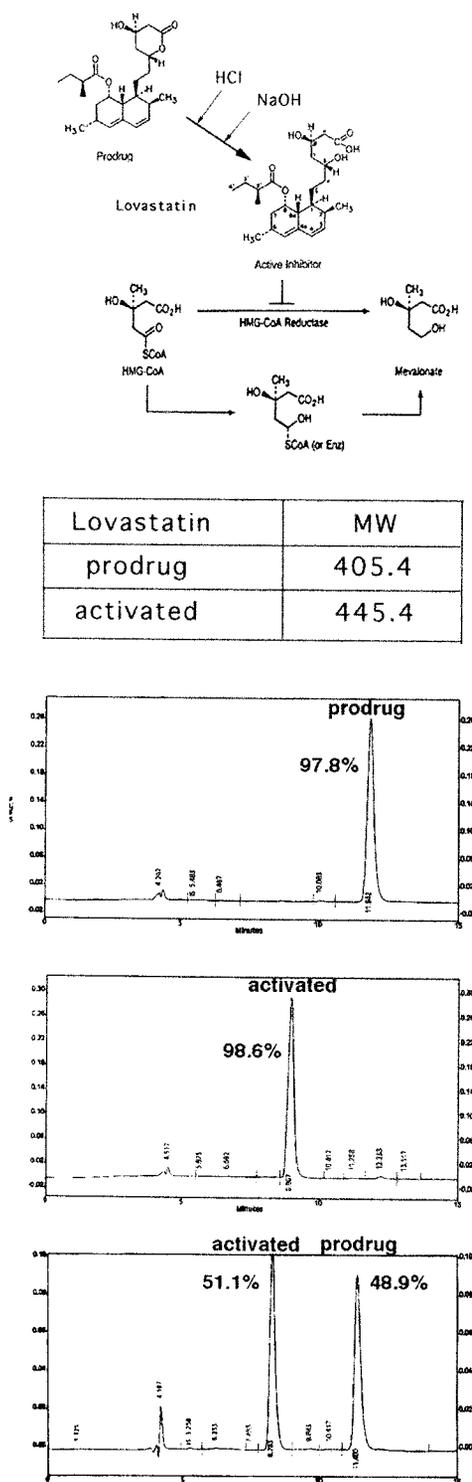
Fig. 4 Analyses of lovastatin in its prodrug and activated forms. *A,* the chemical structures of lovastatin, HMG-CoA, and mevalonate (25, 26). The active inhibitor of HMG-CoA reductase is the open ring conformation of this drug that is a molecular mimic of the intermediate formed within the active site of this enzyme. *B,* the molecular mass of the prodrug and the chemically activated form corresponds to the expected mass as predicted from their chemical structures. *C,* HPLC analysis of the prodrug form (*top panel*), after activation (*middle panel*) and a 1:1 mixture of the two preparations (*bottom panel*) are shown. Greater than 98% of the lovastatin used in this study was in the activated form.
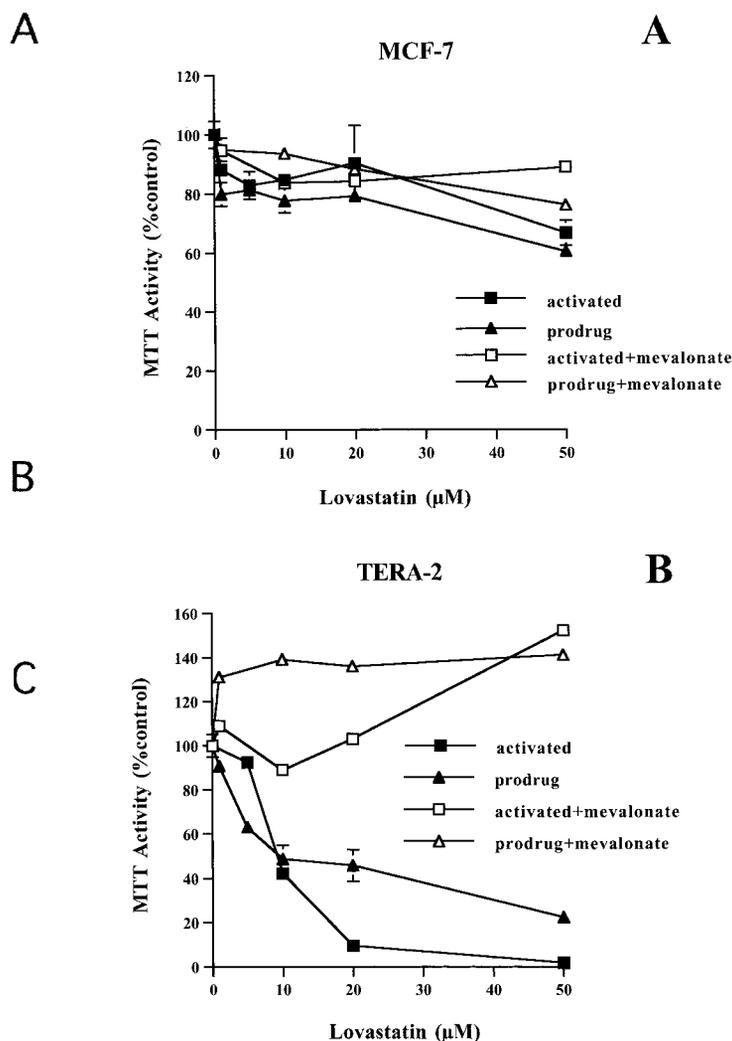

Fig. 5 The open ring activated form of lovastatin triggers apoptosis. *A,* MTT analysis of MCF-7 cells treated with the activated and prodrug forms of lovastatin with or without the addition of mevalonate after 2 days exposure. No significant effects on MTT activity were observed under any of these conditions. *B,* MTT analysis of TERA-2 cells treated with the activated and prodrug forms of lovastatin with or without the addition of mevalonate after 2 days of treatment. Both the activated and the prodrug forms of lovastatin produced significant toxicity in TERA-2 that was abrogated by the addition of mevalonate. *Bars,* SE.

olites required for cellular survival in this subset of tumors. Alternatively, nontransformed cells and some tumor-derived cell lines have been shown to respond to the acute depletion of HMG-CoA reductase after lovastatin exposure with growth arrest in the G_1 phase of the cell cycle (28). This suggests that lovastatin triggers a cell cycle checkpoint that may be abolished in the transformation process and lead to increased sensitivity to lovastatin-induced apoptosis in tumors that lack this checkpoint. On the other hand, the level of HMG-CoA reductase activity may determine sensitivity to lovastatin-induced apoptosis; however, our previous work suggests that lovastatin-triggered cytotoxicity is independent of expression levels of this enzyme (23). Finally, lovastatin may inhibit cell growth by a mechanism

independent of this rate-limiting enzyme of the mevalonate pathway (27). Clearly, understanding the sensitivity and specificity of tumor responsiveness to lovastatin as well as the molecular mechanism of lovastatin-induced apoptosis requires further investigation.

Similar to the effects of other differentiation-based therapeutics, lovastatin induces a pronounced differentiation prior to evidence of overt apoptosis (23, 33, 34) and shows tumor specificity. These findings indicate that lovastatin may represent a novel differentiation-based therapeutic approach. Retinoids, derived from mevalonate metabolites, are potent differentiating and growth-inhibitory agents during embryogenesis that have demonstrated efficacy in the prevention and treatment of specific cancers (13, 41, 42). The tumor types responsive to retinoids are generally derived from tissues of mesenchymal, neuroectodermal, hematopoietic, and epithelial origins and include a variety of pediatric tumors, myeloid leukemias, as well as HNSCC and cervical carcinomas (13, 41, 42). This is not surprising because agents that modulate differentiation are generally lineage specific (13). This spectrum of retinoid-responsive cancers parallels the tumor-derived cell lines that are responsive to lovastatin-induced apoptosis, suggesting a potential mechanistic link.

In this study, we have also demonstrated that apoptosis induced by lovastatin is mediated by the open ring activated form of this drug that targets HMG-CoA reductase. As we propose a new application for HMG-CoA reductase inhibitors, it will be interesting to determine whether the new generation of vastatins will show increased efficacy as anticancer drugs compared with lovastatin. These synthetic compounds have modified structures and/or are in the active open ring conformation and do not require activation (25, 26, 46). Moreover, these synthetic compounds have increased specificity of binding to the intermediate, suggesting elevated activity with relatively low doses *in vivo* (25, 26). Taken together, these vastatins may possess increased bioavailability *in vivo* and may also represent a therapeutic approach.

Therefore, the tumor-specific apoptosis induction as well as the biological properties of the HMG-CoA reductase inhibitors suggests that they are potentially valuable therapeutic agents in the treatment of these retinoid-responsive cancers. Targeting HMG-CoA reductase may represent a therapeutic alternative in these refractory cancers. An acute myeloid leukemia patient that was treated at our institute using lower lovastatin doses (2 mg/kg/day) for 52 consecutive days demonstrated control of leukemic blast counts that lasted >6 months after cessation of treatment (47). We are currently conducting Phase I trials for toxicity and efficacy of prolonged low-dose lovastatin treatment in recurrent acute myeloid leukemias, HNSCC, and cervical carcinoma patients as a result of this work.

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Differential Sensitivity of Various Pediatric Cancers and Squamous Cell Carcinomas to Lovastatin-induced Apoptosis: Therapeutic Implications

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