z-Leucinyl-Leucinyl-Norleucinal Induces Apoptosis of Human Glioblastoma Tumor–Initiating Cells by Proteasome Inhibition and Mitotic Arrest Response

Massimiliano Monticone,1 Emanuela Biollo,2 Andrea Fabiano,4 Marina Fabbri,3 Antonio Daga,3 Francesco Romeo,3 Massimo Maffei,3 Alice Melotti,3,4 Walter Giaretti,3 Giorgio Corte,3,4 and Patrizio Castagnola3

1Centro Biotecnologie Avanzate; 2Dip. Chimica e Tecnologie Farmaceutiche ed Alimentare, Università di Genova; 3Istituto Nazionale per la Ricerca sul Cancro; and 4Dip. Oncologia Biologica e Genetica, Università di Genova, Italy

Abstract
γ-secretase inhibitors have been proposed as drugs able to kill cancer cells by targeting the NOTCH pathway. Here, we investigated two of such inhibitors, the Benzyloxicarbonyl-Leu-Leu-Nle-CHO (LLNle) and the N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), to assess whether they were effective in killing human glioblastoma tumor–initiating cells (GBM TIC) in vitro. We found that only LLNle was able at the micromolar range to induce the death of GBM TICs by apoptosis. To determine the cellular processes that were activated in GBM TICs by treatment with LLNle, we analyzed the amount of the NOTCH intracellular domain and the gene expression profiles following treatment with LLNle, DAPT, and DMSO (vehicle). We found that LLNle, beside inhibiting the generation of the NOTCH intracellular domain, also induces proteasome inhibition, proteolytic stress, and mitotic arrest in these cells by repressing genes required for DNA synthesis and mitotic progression and by activating genes acting as mitotic inhibitors. DNA content flow cytometry clearly showed that cells treated with LLNle undergo arrest in the G2-M phases of the cell cycle. We also found that DAPT and L-685,458, another selective Notch inhibitor, were unable to kill GBM TICs, whereas lactacystin, a pure proteasome inhibitor, was effective although at a much less extent than LLNle. These data show that LLNle kills GBM TIC cells by inhibiting the proteasome activity. We suggest that LLNle, being able to target two relevant pathways for GBM TIC survival, may have a potential therapeutic value that deserves further investigation in animal models. (Mol Cancer Res 2009;7(11):1822–34)

Introduction
Glioblastomas (GBM) are poorly differentiated astrocytic tumors arising in the central nervous system, which despite recent improved treatment modalities are still characterized by very poor prognosis. Several studies have shown the existence of a subpopulation of cells within glioma tumors displaying cancer stem cells properties (1-3). The term “tumor initiating cells” (TIC) is frequently used to describe such cells (4), and we shall use it throughout this article to indicate cells with cancer stem cell capacity. Due to the fact that TICs promote the tumor chemoresistance (5, 6), radioresistance (3, 7), and angiogenesis (5, 8), it is conceivable that finding a manner to kill them would improve GBM therapy (9, 10).

The NOTCH pathway plays important roles during the central nervous system development, contributing to the maintenance of neural stem/progenitor cell pool (11), to promote the neural lineage entry of embryonic stem cells (12) and in the differentiation of astrogia from the adult hippocampus-derived multipotent progenitors in rat (13). NOTCH signaling activation requires the proteolytic processing of this type I integral membrane protein by a two-step cleavage process catalyzed first by a metalloprotease and then by the γ-secretase complex composed of the integral membrane proteins presenilin, nicastrin, Aph1, and Pen-2 (14-17). Increased activation of the NOTCH signaling has been reported in several tumor types (18). Recent studies showed that this pathway induces the survival and/or proliferation in GBM and glioma cells (19-22), and the expression of stem cell markers in glioma cells (21, 22). According to these findings, the inhibition of this pathway leads to depletion of stem-like cells and to the block of the engraftment in embryonal brain tumors (23). Furthermore, enhanced NOTCH signaling may lead to the tumor radioreistance mechanisms deployed by GBM (24). Targeting the NOTCH pathway specifically in GBM TICs seems therefore a rational approach for exploring novel and hopefully more effective therapeutic strategies for the management of this malignancy.

Several molecular tools are available for targeting the Notch pathway such as specific siRNAs, shRNAs, or drugs
such as γ-secretase inhibitors. The latter are small cell permeant molecules able to inhibit the γ-secretase by distinct mechanisms (25). Many researchers are carefully studying these molecules because they can be potentially effective in cancer and in the Alzheimer disease (18, 26). Recently, the results of a phase I trial and of phase II trials (in Alzheimer disease) with the γ-secretase inhibitor LY450139 have been published (27-29).

We presently investigated, by evaluating by flow cytometry and gene expression profiling, two drugs, N-[N-(3,5-difluorophenacetyl)-t-alanyl]-S-phenylglycine t-buty1 ester (DAPT) and Benzylxilcarbonyl-Leu-Leu-Nle-CHO (LLNle), known to be active as γ-secretase inhibitors, for their ability to interfere in vitro specifically with survival properties of GBM TICs previously obtained in our laboratory (30). Here, we clearly show that LLNle is effective in killing these cells by inhibiting, in addition to the NOTCH, also the ubiquitin-proteasome pathway. These data suggest that preclinical studies should definitely be carried on to evaluate whether LLNle is able to significantly improve the survival of immunodeficient mouse xenotransplanted with human GBM TICs.

**Results**

**The γ-Secretase Inhibitor LLNleu Kills GBM TICs in Culture**

To determine whether the γ-secretase inhibitors LLNleu and DAPT have any effect on GBM TICs in vitro, cells obtained from patient (PT)1 (PT1) were cultured with medium alone or supplemented with different amount of DMSO (vehicle), LLNleu, and DAPT. After 48 hours, the cell viability was determined by using the MTT compound. PT1 GBM TICs treated with LLNleu displayed a dose-dependent reduction of cell viability compared with untreated cells (Fig. 1). Cells treated with DMSO or with DAPT displayed a reduction of viability ≥50% of the untreated controls only at the higher end of the concentrations tested, corresponding to 3.6% and 270 μmol/L, respectively (Fig. 1). These experiments also showed that the viability obtained with DMSO and DAPT followed a similar trend (Fig. 1). Consistent results were also obtained from PT2 and PT3 (data not shown). We used the 7.5 μmol/L concentration in subsequent experiments with LLNleu because it was the concentration yielding ~50% killing activity in the GBM TICs. We arbitrarily decided to use the 7.5 μmol/L concentration also for DAPT for the following reasons: to use an equimolar concentration yielding a higher killing activity than the 7.5 μmol/L concentration (Fig. 1).

**LLNle Inhibits the Generation of the NOTCH Intracellular Domain**

To verify whether LLNleu and DAPT actually decrease the extent of the Notch protein processing by γ-secretase in PT1 GBM TICs and therefore the generation of the transcriptionally active intracellular domain (Notch1 intracellular domain, NICD), we subjected both floating and adherent cells to cell lysis and Western blot analysis using an antibody able to recognize uniquely this portion of the Notch1 protein (Fig. 2). After 24 hours of treatment with DAPT or LLNleu, TICs did not show major changes in the amount of NICD in the samples when compared with the control sample. At 48 hours, instead, a dramatic decrease of the signal corresponding to the NICD in the LLNleu-treated TICs with respect to the DMSO and DAPT-treated cells was observed (Fig. 2). No detectable protein degradation was observed by Ponceau Red staining performed before challenging with the primary antibody (Fig. 2). Similar results were obtained with TICs from PT2 and PT3 (see Supplementary Data S1 and S2, respectively).

**LLNle Induces Apoptosis in GBM TICs**

To investigate the killing mechanism underlying the activity of LLNleu in GBM TICs, we took advantage of a multicolor labeling system able to detect apoptosis and necrosis at the same time. This system utilizes the use of allophycocyanin-conjugated Annexin V, C12-resazurine, and Sytox green to discriminate metabolically active cells, early apoptotic, late apoptotic, and necrotic cells. The nonfluorescent C12-resazurine is reduced to orange-fluorescent resorufin by live cells; the far-red fluorescent allopheocyanin-Annexin V binds to exposed phosphatidylserine on the membrane of apoptotic cells; and Sytox green, being cell impermeant, is able to bind to the DNA of cells with compromised membranes such as late apoptotic and necrotic cells. These cell subpopulations can be discriminated by flow cytometry. After 48 hours of treatment with LLNleu, PT1 GBM TICs showed an increase in the far-red and green-labeled cell subpopulation (late apoptotic) and in the far-red and orange-labeled cell subpopulations (early apoptotic) compared with the DMSO treatment (R3 and R7, respectively, in Fig. 3). It should be pointed that most cells in the region R1, after 48 hours of treatment with LLNleu, are still resorufin positive and hence metabolically active (Fig. 3). The flow cytometric results obtained after 48 hours of exposure of TICs to DAPT were very similar to those showed by the TICs exposed to DMSO (data not shown). Similar results were obtained also for PT2 and PT3 GBM TICs (see Supplementary Data S3).

**LLNle Affects the Expression of Genes That Have a Role in Proteasome Activation and Cell Cycle Arrest**

To investigate the molecular basis underlying the LLNle-induced apoptosis in GBM TICs, we determined the gene expression profile of these cells after exposure to LLNleu, DAPT, and DMSO by using GeneChip microarrays. Analysis of GBM TIC gene expression profile by Principal Component Analysis (PCA) revealed a distinct partition between those treated with LLNleu and those treated with DAPT and DMSO (Fig. 4). Each dot in the two-dimensional plot represents one sample. The distance between any pair of dots was related to the similarity between the two observations in high-dimensional space. Samples that were near each other in the plot were similar in a large number of variables, i.e., expression level of individual genes. Conversely, samples that were far apart in the plot were different in a large number of variables. PCA showed that for each patient, GBM TIC samples were clustered closer to each other at 24 hours than at 48 hours (Fig. 4). Furthermore, the 24-hour time point samples treated with DMSO and DAPT were clustered closer to each other than to those treated with LLNleu. This distribution in the two-dimensional space was less evident at
48 hours (Fig. 4). To gain a more mechanistic understanding of the processes affected by LLNle, the Expression Analysis Systemic Explore score (31) was used to identify Gene Ontology (GO) functional categories, which were significantly over-represented. After filtering the results to avoid redundant and/or generic categories, statistically significant GO terms associated with LLNle-regulated genes were found (Table 1).

The results clearly show that the genes whose expression changed by a factor 2, after treatment with LLNle in the cells from the three patients examined, are associated to catabolic processes and proteasome activity at 24 hours (Table 1) and to cell cycle, cell proliferation, chromosome segregation, response to stress, and p53 signaling pathway at the 48-hours time point (Table 1). In particular, after 24 hours, LLNle induced the upregulation of transcript coding for several proteasome subunits, whereas after 48 hours, it downregulated several genes required for cell cycle progression, cell proliferation, chromosome segregation, and upregulated several genes having a role as inhibitors of these processes (Table 1). Differential gene expression by the GBM TICs of the three patients subjected to the three different treatments (LLNle, DAPT, and DMSO) was visualized by a heat map visualization obtained by hierarchical clustering (HCL), which generates a tree (dendrogram) to group similar objects together (Fig. 5). Again, the DAPT gene expression profile resulted very similar to the one determined in the DMSO-treated cells (Table 1; Fig. 5), whereas the one determined for LLNle was clearly distinct from those determined in the DAPT and DMSO-treated cells.

Expression changes were confirmed by Real-time PCR analysis of arbitrarily selected genes resulting as deregulated from the gene array experiments (Fig. 6). A complete list of significantly regulated genes in the comparison between the LLNle-treated samples versus DAPT-treated samples or control samples (DMSO) is provided (see Supplementary Data S4).

**LLNle and DAPT Effects on NOTCH Target Genes**

To assess the effects of LLNle and DAPT on the NOTCH pathway in GBM TICs, we examined the expression of NICD transcriptional target genes JAG1, HES1, HEY1, and HEY2 (32-34) as evaluated by the GeneChip microarray studies. The values of mRNA expression for these genes showed that the effects of the two drugs were not homogeneous across the three patients (see Supplementary Data S5). In particular, we observed, in some cases, only negligible changes in gene expression due to the treatment with DAPT and LLNle, and downregulation of gene expression for some genes in treated cells of some patients at 24 hours but upregulation for the same genes at 48 hours in treated cells of the same patients (see Supplementary Data S5). The only consistent change was observed for the HES1 transcript, which was downregulated by both DAPT and LLNle in the cells of all three patients examined at the 24-hours time point (see Supplementary Data S5). However, even for HES1 at 24 hours, the changes in the expression levels were <2-fold in the DAPT and LLNle TIC samples with respect to the DMSO-treated cells (see Supplementary Data S5).

**LLNle Induces Hsp70 Expression and the Accumulation of Poly-Ubiquitinated Proteins in a Dose-Dependent Fashion**

To investigate in more detail the effects of LLNle on the proteasome pathway in GBM TICs, we studied the expression of the Hsp70, a known marker of proteasome inhibition, by Western blot analysis (35, 36). Our results show that Hsp70 expression is higher in PT1 cells treated with LLNle for 24 hours than...
in PT1 cells treated with DAPT or with DMSO (Fig. 2). This increase was maintained after 48 hours of exposure to LLNle (Fig. 2). Similar results were obtained with TICs from PT2 and PT3 (see Supplementary Data S1 and S3, respectively). To investigate whether proteasome inhibition was dose dependent, we subjected PT1 GBM TIC cells to a concentration series of LLNle and analyzed by immunoblot analysis the expression of Hsp70 along with that of poly-ubiquitinylated proteins and that of the NICD (Fig. 7). This experiment clearly shows that a dose-dependent increase in Hsp70 exists and that it can be detected after 48 hours of exposure even at the 1.25 μmol/L LLNle concentration (Fig. 7). This concentration of LLNle is the lowest at which a decrease of the NICD is detectable (Fig. 6). A clear-cut increasing of poly-ubiquitinylated protein accumulation was also observed at 1.25 μmol/L LLNle with respect to lower doses. This accumulation peaked at 7.5 μmol/L (Fig. 7).

**LLNle Induces Cell Cycle Arrest and Mitotic Catastrophe in GBM TICs**

On the basis of the results obtained by the DAVID analysis, we hypothesized that GBM TICs would undergo cell cycle arrest and mitotic catastrophe following LLNle treatment. To test this hypothesis, we analyzed the nuclear DNA content by 4',6-diamidino-2-phenylindole (DAPI) staining of TICs after treatment with LLNle, DAPT, and DMSO. Since this dye binds with a stoichiometric ratio to DNA, and increases its fluorescent emission dramatically with respect to the unbound state, DNA content can be derived by the intensity of the fluorescent signal examined by flow cytometry. In Fig. 8, the DNA content of untreated nuclei in the G1 phase peaks at ~200 units of fluorescent light emission, whereas G2+M nuclei have a DNA content corresponding to the double value of 400 units and S phase nuclei have fluorescence values comprised between 200 and 400 units. After 24 hours of treatment with LLNle, PT1 GBM TICs showed an increase of the peak corresponding to G2+M nuclei with respect to the control samples and an increase of nuclei in the S phase with respect to controls (Fig. 8). In addition, one could observe the clear appearance of particles related to fragmented (apoptotic) nuclear bodies having lower fluorescent emission, hence lower DNA content, than untreated G1 nuclei (Fig. 8). After 48 hours of exposure to LLNle, a dramatic increase of fragmented nuclei having less than G1 DNA content was observed along with a still higher peak of nuclei corresponding to the G2+M phase DNA content, and higher fraction of nuclei in S phase (Fig. 8).

**Proteasome but not Notch-Specific Inhibitors Kill GBM TICs**

Although DAPT is one of the most selective Notch inhibitors (37), to establish whether another selective Notch inhibitor with high solubility and reported specificity (nmol/L range) would be more effective in killing GBM TICs, the compound L-685,458 was used. The experiments performed,
however, showed that the viability obtained with DMSO and L-685,458 followed a similar trend and no inhibition of PT1 GBM TIC viability was observed up to the 78.1 μmol/L concentration (see Supplementary Data S7). The effects obtained at the highest concentration of L-685,458 used, 390.5 μmol/L, were due to the solvent toxicity (see Supplementary Data S7).

To assess whether L-685,458, although ineffective in killing GBM TICs, was able to inhibit the generation of NICD, we analyzed by Western blot PT1 GBM TICs after 48 hours of treatment with this compound. This analysis showed no detectable changes in the amount of NICD at concentrations up to 15.63 μmol/L of L-685,458 (see Supplementary Data S8).

As we proved that LLNle inhibits the poly-ubiquitin proteasome pathway and kills GBM TICs, we expected that a pure proteasome inhibitor would yield similar results. Therefore, we treated PT1 GBM TICs with lactacystin for 48 hours and found that this compound was indeed active with a IC₅₀ of 145.8 μmol/L (Fig. 9). Western blot analysis performed with GBM TICs treated for 48 hours with lactacystin showed a steady increase in the amount of protein poly-ubiquitination up to the 145.8 μmol/L concentration (see Supplementary Data S10). However, on molar basis, the effects of lactacystin on both inhibition of cell viability and protein poly-ubiquitination were achieved at least at one order of magnitude higher than LLNle (compare Fig. 1 with Supplementary Fig. S9 and Fig. 7 with Supplementary Data S10).

**Discussion**

In this study, we investigated the potential killing activity on GBM TICs of two well-known γ-secretase inhibitors with distinct chemical structure, LLNle and DAPT. The hypothesis that γ-secretase inhibitors would have caused GBM TIC cell death was mainly based on recently published literature, which

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**FIGURE 4.** Microarray analysis performed with The Institute for Genomic Research multiple experimental viewer program: PCA. Microarray analysis of GBM TICs treated with 0.1% (v/v) DMSO (vehicle) or with 7.5 μmol/L DAPT or LLNle for the indicated time period. Probe sets associated to dysregulation of gene expression levels among the nine samples for each time point were identified using SAM (see Materials and Methods). PCA is shown to provide the two-dimensional projections onto the plane spanned by the two principal components for the three different data sets for each patient for each time point.
reported that γ-secretase inhibitors kill Kaposi’s tumor cell lines (38), enhance chemotherapy-induced apoptosis in colon cancer cells (39) and in myeloma cells (40), and reduce the proliferation of lung cancer cells (41).

Our results indicate that LLNle is indeed able to induce death by apoptosis in human GBM TICs. We also show that after challenge with LLNle, these cells undergo a G2-M cell cycle arrest that is already detectable after 24 hours of treatment and more evident after 48 hours. Such an arrest is concomitant to the appearance of sub-G0-G1 nuclei, or nuclear fragments, and Annexin V binding, both indicative of apoptosis.

On the other hand, treatment with DAPT, one of the most selective Notch inhibitors, in our experimental conditions yields little or no effective GBM stem cell killing and inhibition of the Notch1 processing. Furthermore, here, we show that another selective Notch inhibitor, L-685,458, is equally ineffective in impairing both GBM TIC viability and Notch1 processing, even when used at concentrations higher than the ones previously reported as effective (42-45). Interestingly, the potency in growth suppression of DAPT was reported as mild compared with LLNle also in a different cellular system (46). Some of the possible explanations for these results could be the requirement for a much higher concentration, an extremely short half-life of the molecules, or the presence of a molecular sink in the cells used for the study.

Genome-wide expression analysis of the effects of LLNle on GBM TICs was a further aim of the study. The results indicate that the apoptotic process induced by LLNle in the low micromolar range is most likely related to the inhibition of proteasomal degradation of cellular proteins followed by a reduced expression of a number of genes required for cell cycle, cell proliferation, and chromosome segregation, together with an increased expression of genes able to actively inhibit these processes and those belonging to the p53 signaling pathway.

More in detail, after 24 hours of exposure to LLNle, we found upregulation of genes coding for several proteasomal subunits, for proteins involved in ubiquitinylated protein degradation (i.e., UFD1L, CYLD, UBR1, VCP), and in cell death signaling (DEDD2). After 48 hours, we found upregulation of a number of genes implicated in the negative regulation of cell cycle and cellular proliferation (i.e., DUSP1, RGS2, PPP1R15A, IL11, SGK1, DNAJB1, DDT3, CHEK1, ADAMS1, HBEGF, LIF, CLU, TPX2, TIMELESS, CENPF, BUB1B, BUB1, KIF2C, PLK1, CDC7, MKI67, DLG7, GADD45B, GADD45A, CHEK2, CCNE2, CHEK1, ESPL1, CDC20, MCM6, SKP2, BUB1B, BUB1, CCNB2, CCNB1, PLK1, CCNA2, SERPINE1, GADD45B, GADD45A, SESN2, CHEK2, CCNE2, CHEK1, CCNB2, CCNB1).

<table>
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<tr>
<th>Table 1. GO (at level 2) and KEGG Pathway Analyses of the Comparison between the LLNle-Treated and the Control (DMSO) GBM TICs from PT1-3</th>
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<td>System Gene Category-Term</td>
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<tr>
<td>GO biological process</td>
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<td>Catabolic process</td>
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NOTE: Nonredundant functional categories, number of genes contained within each category and percentages ranked by the degree of over-representation in the category as determined by Expression Analysis Systemic Explore (P) are shown. Redundant categories with similar gene members were removed to yield a single representative category. Up to 15 of the most regulated gene members, ordered from the most upregulated one (bold) to the most downregulated one, are shown in the last column. Only categories associated with a P < 5.0E-4 are shown. Gene name symbols used are those approved by the Human Genome Organisation Gene Nomenclature Committee (http://www.genenames.org/).
the induction of genes related to the unfolded protein response and endoplasmic reticulum stress (including HSPA1B). Similar results have been previously described in different cell types with proteasome inhibitors (35, 36, 52-54). Furthermore, the close similarity of the chemical structure of LLNle with those of GM132 and N-acetyl-leucyl-leucyl-norleucinal, well-known proteasome inhibitors, should be underlined (55). In particular, it should be pointed that the latter was reported as a cystein-proteinase inhibitor (56). The subsequent replacement of the small and hydrophilic acetyl group with a bulkier and hydrophobic benzyloxycarbonyl group (z group) at the amino end of the tripeptide led to the z-LLNle molecule, used in the present study. To the best of our knowledge, there are no reports specifically addressing the equivalence or difference in proteinases specificity of the parent and daughter molecules. However, as a matter of fact, z-LLNle has been

\[ z-LLNle \]

Figure 5. Microarray analysis performed with The Institute for Genomic Research multiple experimental viewer program: HCL. Microarray analysis of GBM TICs treated with 0.1% (v/v) DMSO (vehicle) or with 7.5 μmol/L DAPT or LLNle for the indicated time period. Heat map visualization obtained by HCL. Ratios for each probe relative to the mean value were used to rearrange the gene list on the basis of their expression pattern. Probes corresponding to genes with similar regulation trend were placed close to each other. The color-ratio bar indicates intensity of gene upregulation (red), downregulation (green), and no change (black).
widely used as γ-secretase and hence as NOTCH inhibitor in an increasing number of articles (38, 57-66). Here, we also show that lactacystin, a pure proteasome inhibitor, inhibits GBM TIC viability as expected from the results obtained with LLNle. However, we show that in our experimental context, its potency is at least one order of magnitude lower than that of LLNle.

Consistently with our data, two studies published during the revision of this article showed that lactacystin, a pure proteasome inhibitor, inhibits GBM TIC viability as expected from the results obtained with LLNle. However, we show that in our experimental context, its potency is at least one order of magnitude lower than that of LLNle.

With respect to the LLNle effect upon the NOTCH pathway, the following issues should be stressed: first, we showed that LLNle downregulates in GBM TICs the generation of the NICD, which is responsible for the activation of the nuclear signal cascade of the NOTCH pathway. This effect was slightly more evident after 48 hours of treatment. On the contrary, second, we did not observe a consistent change in the expression levels of several known NOTCH direct target gene transcripts in TICs of the three patients examined.

Notwithstanding the lack of clear-cut downregulation of NOTCH target genes after treatment of GBM TICs with LLNle, we cannot rule out that the inhibition of the generation of the
NICD observed contributes to some extent to the killing of these cells. However, our gene expression results along with those obtained with lactacystin lead us to favor the hypothesis that proteasome inhibition is the key process unleashed by LLNle, which drives GBM TICs to programmed cell death.

The observation that the inhibitory effects in GBM TICs on both γ-secretase and proteasome activity by LLNle are dose dependent and occur concomitantly at the same concentration range raises a caveat on the use of this molecule as a tout court γ-secretase inhibitor. However, we believe that this dual specificity may be exploited to target at the same time two relevant pathways for cancer onset and progression (69, 70). Interestingly, the association of γ-secretase inhibitors with chemotherapy has been proposed very recently as a novel approach for the treatment of metastatic colon cancer (71), whereas the use of proteasome inhibitors into the clinic dates back of a decade and now is part of the standard treatment of some hematologic malignancies (72). Although a molecule having dual specificity may be associated to a higher toxicity with respect to more specific inhibitors, it may have the advantage of a later onset of drug resistance due to the possibility of targeting efficiently two targets (72, 73). Furthermore, our results showing that on a molar basis LLNle is more effective than lactacystin in GBM TIC in vitro killing suggest the possibility that similar results could be achieved also in vivo at lower doses with respect to other proteasome specific inhibitors.

This study shows effective in vitro killing activity by LLNle on GBM TICs via apoptotic cell death following γ-secretase and proteasome inhibition. On these bases, future studies in animal models bearing human GBM orthotopic transplant may be foreseen with the goal to assess the efficacy of drugs targeting both the proteasome and γ-secretase, either alone or in combination with other chemotherapeutic or biological response modifiers, in the therapy of this fatal disease.

Materials and Methods

Cell Culture

GBM TICs were obtained from tumor surgical samples provided by the Neurosurgery Department of the San Martino Hospital in Genoa. An informed consent was obtained from all patients before surgery as required by the Ethic Board.

Cell isolation was described in detail elsewhere (30). Proliferation medium was DMEM-F12/Neurobasal added with 1% v/v B27 supplement, (Life Technologies Ltd), 2 mmol/L L-glutamine (LifeTechnologies Ltd), recombinant human fibroblast growth factor (FGF-2; 10 ng/mL; Peprotech), and recombinant human epidermal growth factor (20 ng/mL; Peprotech). The medium was changed twice a week. Under these conditions and were blotted on Immobilon membrane (Millipore) according to the manufacturer’s instructions. After blotting, the membrane was briefly stained with Ponceau S solution (Sigma-Aldrich); washed with deionized water; solved at the 7.5 mmol/L concentration in DMSO, aliquoted, and stored in the dark at −20°C. Cells were treated with 7.5 μmol/L LLNle or DAPT or vehicle alone (DMSO 0.1%), unless otherwise indicated, and kept in a humidified 5% CO2 atmosphere at 37°C for the indicated time period. This concentration is in the low range reported by several previous in vitro studies (38, 74-77). The γ-secretase inhibitor L-685,458 and the proteasome inhibitor lactacystin were obtained from Sigma-Aldrich, dissolved at 10 mmol/L in DMSO and 30 mmol/L in H2O, respectively, aliquoted, and stored in the dark at −20°C.

Western Blot Analysis

Cells were detached from Matrigel-coated flasks by incubation in PBS containing 1 mmol/L EDTA and harvested by centrifugation at 450 g for 5 min. Protein cell extracts were prepared by lysis in 5 mmol/L EDTA, 1% Triton ×100, and 0.1% SDS in PBS buffer on ice for 30 min. Cell lysates were then centrifuged through Qiashredder (Qiagen) to homogenate the sample and fragment the DNA. Protein concentrations were quantified (BCA protein assay kit, Thermo Scientific) and equal amounts of protein were run on NuPage 4-12% Bis-Tris Glycine polyacrylamide gels (Invitrogen) under reducing conditions and were blotted on Immobilon membrane (Millipore) according to the manufacturer’s instructions. After blotting, the membrane was briefly stained with Ponceau S solution (Sigma-Aldrich); washed with deionized water; solved at the 7.5 mmol/L concentration in DMSO, aliquoted, and stored in the dark at −20°C. Cells were treated with 7.5 μmol/L LLNle or DAPT or vehicle alone (DMSO 0.1%), unless otherwise indicated, and kept in a humidified 5% CO2 atmosphere at 37°C for the indicated time period. This concentration is in the low range reported by several previous in vitro studies (38, 74-77). The γ-secretase inhibitor L-685,458 and the proteasome inhibitor lactacystin were obtained from Sigma-Aldrich, dissolved at 10 mmol/L in DMSO and 30 mmol/L in H2O, respectively, aliquoted, and stored in the dark at −20°C.

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saturated with 5% milk in 20 mmol/L Tris-HCl (pH 7.5), 500 mmol/L NaCl, and 0.05% Tween 20; and then challenged with a primary antibody. Horse radish peroxidase–conjugated goat anti-rabbit or goat anti-mouse IgGs and the SuperSignal west Pico Chemiluminescent substrate (Thermo Scientific) were used for immunodetection. Chemiluminescent signals were acquired by a ChemiDoc XRS apparatus (Bio-Rad Laboratories). The rabbit polyclonal antiserum ab8925 specific for NICD and the mouse monoclonal ab2787 specific for Hsp70 were from Abcam. The mouse monoclonal antibody anti–poly-ubiquitinylated-protein (04-262) was purchased from Millipore.

Flow Cytometric Analysis

Cells harvested with their media to ensure collection of floating cells along with adherent cells were centrifuged at 980 g for 5 min.

To measure the DNA content in cell nuclei, each sample was stained with DAPI as described by Otto (78). Briefly, 1 million cells were incubated in 2 mL of detergent solution (0.1 mol/L Citric acid, 0.5% Tween 20 in water) for 20 min at room temperature. Nuclei were passed over a nylon sieve with a pore size of 50 μm (CellTrics yellow filters, Partec GmbH), pelleted, resuspended in 200 μL of detergent solution, and incubated for 10 min at room temperature with gentle shaking. Finally, six volumes of staining solution (0.4 mol/L Na2HPO4, 5 μmol/L DAPI in water) were added. Each sample was then analyzed, after 15 min of incubation, using a CyflowML multiparameter flow cytometer (Partek). Excitation of DAPI was provided by an UV mercury lamp (HBO-100 long life, 100W) and the emitted fluorescence was collected by the Gratz setting (488 blue solid laser shutdown; 435 nm long pass filter).

To determine the extent of apoptosis, the Vybrant apoptosis assay kit (Invitrogen) was used. Cells were labeled according to the manufacturer’s instruction. Samples were subjected to flow cytometric analysis using a CyAn ADP analyzer equipped with

FIGURE 8. LLNle induces cell cycle arrest in G2-M phase. High-resolution flow cytometric analysis of DAPI-stained nuclei of PT1 GBM TICs treated with 7.5 μmol/L LLNle (right panels) reveals a substantial increase of nuclei in the S, G2, and M phase of the cell cycle at 24 h followed by an increase of nuclei/fragment having a sub-G1 DNA content (arrowheads) at 48 h with respect to cells treated with 0.1% DMSO (v/v); left panels.

FIGURE 9. The proteasome inhibitor lactacystin kills GBM stem cells in culture. Survival curves of GBM TIC cells derived from PT1 are based on the results of MTT assays after continuous exposure to drugs for 48 h. Average values from at least three independent experiments are shown; bars, SD. Concentration (v/v) for H2O (vehicle; solid line) was (1) 0.027%, (2) 0.081%, (3) 0.0167%, (4) 0.243%, (5) 0.729%, and (6) 2.187%. Concentration for Lactacystin (dashed line) was (1) 2.7 μmol/L, (2) 8.1 μmol/L, (3) 24.3 μmol/L, (4) 72.9 μmol/L, (5) 218.7 μmol/L, and (6) 656.1 μmol/L.
488 and 635 nm lasers and 530/40, 575/25, and 665/20 nm band pass filters to detect the three fluorochromes (Dako).

RNA Extraction and Quality Analysis

Total RNA was isolated using miRNeasy mini kit (Qiagen) with DNase treatment. RNA concentration and purity were determined from measuring absorbance at 260 and 280 nm; 2 μg total RNA were run on a 1% denaturing gel and 100 ng were loaded on the 2100 Bioanalyzer (Agilent) to verify RNA integrity.

Amplification of RNA and Array Hybridization

According to the recommendations of the manufacturer, 100 ng of total RNA were used in the first-round synthesis of double-stranded cDNA. The RNA was reverse transcribed using a Whole Transcript cDNA synthesis and amplification kit (Affymetrix UK Ltd.). The resulting biotin-labeled cRNA was purified using an IVT clean-up kit (Affymetrix) and quantified using a UV spectrophotometer (A260/280; Beckman). An aliquot (15 μg) of cRNA was fragmented by heat and ion-mediated hydrolysis at 94°C for 35 min. Fragmented cRNA, run on the Bioanalyzer (Agilent Technologies,) to verify the correct electropherogram, was hybridized in a hybridization oven (16 h, 45°C) to a Human Gene 1.0 ST array (Affymetrix) representing whole-transcript coverage. Each one of the 28869 genes is represented on the array by ~26 probes spread across the full length of the gene, providing a more complete and more accurate picture of gene expression than the 3’-based expression array design. The washing and staining procedures of the arrays with phycocerythrin-conjugated streptavidin (Invitrogen) was completed in the Fluidics Station 450 (Affymetrix). The arrays were subsequently scanned using a confocal laser GeneChip Scanner 3000 7G and the GeneChip Command Console (Affymetrix).

GeneChip Microarray Analysis and Data Normalization

Affymetrix raw data files (cell intensity files) were used as input files in expression console environment (Affymetrix). Briefly, cell intensity files were processed using the Robust Multi-Array Analysis procedure (79), an algorithm that is publicly available at the Bioconductor.org website. The Robust Multi-Array Analysis method was used to convert the intensities from the multiple probes of a probe set into a single expression value with greater precision and reduced background noise (relaying on the perfect match probes only and thus ignoring the mismatch probes) and then to normalize by sketch quantile normalization. Quality assessments were also done in the expression console environment. This procedure, based on various metrics, allowed us to identify a chip as an outlier (see for details Quality assessment of exon and gene arrays). Significance Analysis of Microarrays (SAM), PCA of variance and HCL, after mean scaling and log2 transformation were performed with the software tool from The Institute for Genomic Research multiple experimental viewer7 (80).

Individual genes with different expression levels, among the three groups, were identified using SAM (81). The false discovery rate expressed as q-value was used to evaluate statistical significance, and its threshold was set at 0.02 (2%). For comparison purposes, an arbitrary filter was applied excluding all genes that did not exhibit a difference in expression of at least 2-fold. Genes differentially expressed were investigated using (a) a multiclass analysis to test differences among the three groups of cells and (b) a two-class analysis within each pair groups to specify expression changes.

We used PCA to reduce the complexity of high-dimensional data and to simplify the task of identifying patterns and sources of variability in these large data sets.

The results from SAM were visualized using HCL (82). All the microarray information has been submitted to the National Center for Biotechnology Information Gene Expression Omnibus Web site.8

Pathways Identification by Expression Analysis Systemic Explore

Gene lists from Affymetrix results were examined using the Expression Analysis Systemic Explore program, accessible via the DAVID Bioinformatics Resources Web site.9 Expression Analysis Systemic Explore is a customized stand-alone software application with statistical functions for discovering biological themes within gene lists. This software assigns genes of interest into functional categories based on the GO database10 and uses the Fisher’s exact test statistics to determine the probability of observing the number of genes within a list of interest versus the number of genes in each category on the array. A more detailed analysis of the genes’ association with physiologic pathways was done using the Kyoto Encyclopedia of Genes and Genomes (KEGG).11 Each identified process was confirmed through PubMed/Medline.12

Reverse Transcription-PCR Analysis

Starting from ~1 μg of total RNA, cDNA was synthesized by using Oligo(dT)20, random hexamers mix, and a Superscript III first-strand synthesis system supermix for reverse transcription-PCR (RT-PCR; Invitrogen). cDNAs were diluted 5 to 20 times, then subjected to PCR analysis.

Relative quantification was done by real-time quantitative RT-PCR. Briefly, quantitative RT-PCR was done and analyzed using real-time PCR (ABI Prism 7700 Sequence Detector, Applied Biosystems). Primers were designed across a common exon-exon splice junction by the Primer Express 2.0 software (Applied Biosystems) to avoid possible signal production from potential contaminating genomic DNA. Reactions were carried out in triplicates and amplitcons were measured by SYBR Green fluorescence (Applied Biosystems) as per manufacturer’s recommendations. Dissociation curve analysis defined the specificity of the products by the presence of a single dissociation peak on the thermal melting curve.
The gene coding for the low-density lipoprotein receptor–related protein-associated protein 1 (LRPAP1) was used as the endogenous control for normalization because, in the microarray data, it showed in all conditions the steadiest expression in our experimental setting when compared with other housekeeping genes.

To avoid possible signal arising from potential contaminating genomic DNA, specific primers for each gene were designed across a common exon-exon splice junction by the Primer Express software (Applied Biosystems; see Supplementary Data S6).

**Disclosure of Potential Conflicts of Interest**

The authors declare that they have no competing interests in relation to this article.

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