伽马-分泌酶抑制剂下调Notch1可诱导卵巢癌细胞A2780生长抑制及凋亡

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Down-regulation of Notch1 by Gamma-secretase Inhibitor Contributes to Growth Inhibition and Apoptosis of Ovarian Cancer Cells A2780

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Abstract: **Objective**　To investigate the inhibiting effect of gamma-secretase inhibitor, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), on Notch1 and its effect on the growth and apoptosis of ovarian cancer cells. **Methods**　Expression of Notch 1 and hes1 in four human ovarian cancer cell lines, A2780, SKOV3, HO-8910 and HO-8910PM, and one ovarian surface cell line IOSE 144 were detected by Western blot and quantitative real-time RT-PCR. The effects of DAPT on ovarian cancer cells were measured by MTT assay, flow cytometry, ELISA and colony formation assay. **Results**　Expression of Notch1 and hes1 were found in IOSE144 and all the four human ovarian cancer cell lines, and they were the highest in ovarian cancer cells A2780 compared with other four ovarian cells. Down-regulation of Notch1 expression by DAPT was able to substantially inhibit the growth, induce G_1 cell cycle arrest and the apoptosis of A2780 cells in a dose- and time-dependent manner. In addition, hes1 was found to be down-regulated in a dose- and time-dependent manner by DAPT in A2780. **Conclusion**　DAPT could inhibit the growth and induce the apoptosis of A2780 cells in a dose- and time-dependent manner. DAPT inhibiting Notch1 activity represents a potentially attractive strategy of targeted therapy for ovarian cancer.

Key words:Ovarian carcinoma; Notch1; Notch signaling

摘　要:**目的**　探讨γ-分泌酶抑制剂对Notch1的抑制作用及对卵巢癌细胞生长抑制及凋亡的影响。**方法**　采用Western blot及实时定量PCR检测四株卵巢癌细胞（A2780, SKOV3, HO-8910, HO-8910PM）及一株卵巢上皮细胞（IOSE144）Notch1及其下游基因hes1的表达情况；采用MTT、流式细胞术、ELISA及克隆形成实验检测γ-分泌酶抑制剂（DAPT）对卵巢癌细胞的影响。**结果**　Notch1、hes1在IOSE 144及四株卵巢癌细胞系中均有表达，且在A2780细胞系中的表达最高；DAPT下调Notch1可抑制A2780细胞生长、诱导细胞G[1]期阻滞、诱导细胞凋亡并呈现时间和剂量依赖性，同时A2780细胞中DAPT下游hes1基因的表达也呈现时间和剂量依赖性。**结论**　γ-分泌酶抑制剂DAPT可抑制卵巢癌细胞A2780生长、诱导细胞凋亡，γ-分泌酶抑制剂下调Notch1可能是卵巢癌治疗的潜在靶点。

关键词：卵巢癌；Notch1；Notch信号通路

中图分类号：R73-3；R737.31　　**文献标识码**：A

0　引言

　　卵巢癌起病隐匿，死亡率位居妇科恶性肿瘤首位。虽然手术及化疗方法不断改进和创新，卵巢癌仍居妇女死亡原因的首位[1]。这要求我们进一步研究探索，寻找一个可以控制卵巢癌发生发展的方法。

　　Notch信号通路是一条高度保守的信号通路，

Notch信号分子及其目的基因在细胞增殖、分化、凋亡过程中有着重要作用[2]。异常Notch信号通路与多种恶性肿瘤的发生有关[3-4]，Notch1在卵巢癌中有促癌作用[5]，并与卵巢癌组织分化程度相关[6]，而γ-分泌酶抑制剂可通过Notch信号通路抑制细胞生长、诱导细胞凋亡[7-8]。但γ-分泌酶抑制剂在卵巢癌细胞中能否抑制Notch1信号通路，并抑制细胞生长、诱导细胞死亡则有待进一步验证。因此，本研究的目的就是验证γ-分泌酶抑制剂对Notch1的抑制能否引起卵巢癌细胞生长抑制及凋亡。

1　材料与方法

1.1　细胞培养

将卵巢癌细胞系A2780、SKOV3、HO-8910、HO-8910PM及卵巢上皮细胞IOSE144培养于含10%小牛血清（FCS）、青霉素（100 u/ml）、链霉素（100 μg/ml）的RPMI1640培养液中，置于37℃、5%CO_2培养箱内备用。每次实验均选用处于对数生长期的细胞进行指标检测。

1.2　γ-分泌酶抑制剂处理

γ-分泌酶抑制剂（DAPT）用于阻断Notch1信号通路。处于对数生长期、密度为每毫升$1×10^5$个细胞采用不同浓度DAPT（25、50、75 μmol/L）进行处理，对照组用0.1%DMSO处理。在不同时间点检测Notch1及hes1的表达，同时检测细胞生长、细胞周期分布、细胞凋亡及克隆形成情况。

1.3　反转录聚合酶链反应(RT-PCR)

四株卵巢癌细胞及一株卵巢上皮细胞IOSE144细胞用Trizol裂解后按RNA提取方法提取细胞总RNA，并用紫外分光光度计检测其浓度及纯度。Notch1及β-actin引物均由TaKaRa公司设计合成。Notch1上游引物：5'- TCA GCG GGA TCC ACT GTG AG-3'，下游引物：5'- ACA CAG GCA GGT GAA CGA GTT G-3'；hes1上游引物：5'-TGGAAA TGACAGTGAAGCACCTC-3'，下游引物：5'-TCG TTCATGCACTCGCTGAAG-3'；β-actin上游引物：5'-TGG CAC CCA GCA CAA TGA A-3'，下游引物：5'- CTA AGT CAT AGT CCG CCT AGA AGC A-3'。实时RT-PCR过程按照产品说明书操作，反转录条件：37℃水浴15 min, 85℃ 5 s；RT-PCR反应条件：95℃变性10 s，95℃退火5 s，60℃延伸31 s。经ABI PRISM 7000所得的CT值用$2^{-\triangle\triangle Ct}$法分析处理。

1.4　Western blot 分析

所有卵巢细胞组织加入适量裂解液，所得裂解液，于4℃下12 000 r/min离心10 min。取上清液加入1/4体积的5×SDS蛋白加样缓冲液，煮沸10 min。蛋白电泳及转膜：所得蛋白样品，经3%SDS-PAGE分离后，电转至硝酸纤维素膜上，丽春红染色验证蛋白转移成功。抗体杂交及显色：以50 g/L的脱脂奶粉封闭2 h后，依次加入Notch1羊多克隆抗体(室温2 h，TBS/T洗2次，TBS洗1次)、兔抗羊二抗(室温2 h，TBS/T洗2次，TBS洗1次)，最后加入NBT/BCIP底物显色。

1.5　MTT实验

取对数生长期的细胞调整密度为$1×10^5$/ml悬液，接种于96孔培养板内，每孔100 μl（$1×10^4$个）。实验组分别加入终浓度为25、50、75 μmol/L DAPT。分别培养24、48、72 h后离心吸去上清液，每孔加无血清培养液MTT（5 mg/ml）10 μl，37℃、5%CO_2继续培养4 h后，终止培养，吸去上清液，每孔加DMSO150 μl，充分振荡10 min，在酶联免疫检测仪570 nm处测量吸光度(OD)值，与对照组比较，计算各组细胞生长抑制率。实验重复3次取平均值。

1.6　流式细胞术与细胞周期分析

取对数生长期细胞，调整密度为$1×10^5$/ml，接种于50 ml培养瓶，加入浓度分别为25、50、75 μmol/L DAPT，作用24 h后胰酶消化收集细胞，调整待测细胞浓度为（$5×10^5$ ~ $1×10^6$）/ml，缓慢加入PBS，轻轻振荡使细胞悬浮，1 000 r/min，离心5 min，重复3次，将洗涤后的细胞加入1 ml PBS混匀，再加入2 ml无水乙醇固定，固定1 h后用PBS洗涤3次，将等体积的细胞悬液和PI染液混合，4℃放置30 min，放入FCM样本室，以激发波长488 nm测定，并用modfit LTV2.0软件分析细胞周期。

1.7　凋亡实验

按照ELISA凋亡试剂盒说明步骤进行实验。取样品离心后（1 000 r/min，10 min），吸取20 μl上清液，加入链霉亲合素包被的培养板孔中；另加入80 μl免疫反应试剂，室温下孵育2 h（置摇床上，250 r/min）；弃上清液，用300 μl温育缓冲液洗涤3次，小心移去洗涤液；加入100 μl底物缓冲液，室温下孵育至颜色变化至适合；然后作比色分析，用底物缓冲液作空白对照，检测波长405 nm，参考波长492 nm。

1.8　克隆形成实验

A2780细胞及DAPT处理组（$5×10^4$/ml）按照说明接种于6孔板内，置于含5%CO_2培养箱37℃孵育14天，倒置显微镜下计数细胞克隆形成数。

1.9　统计学方法

SPSS14.0软件t检验对结果进行分析，采用均数±标准差表示，$P<0.05$为差异有统计学意义。

2　结果

2.1　Notch1及hes1在卵巢癌细胞A2780中的表达

实时定量PCR及Western blot结果发现五株卵巢癌细胞中均有Notch1的表达，其中Notch1在A2780和HO-8910细胞中的表达高于在IOSE144细胞中的表达，见图1A、1B；Notch1下游基因hes1在A2780细胞中的表达明显高于在IOSE144细胞中的表达，见图1C、1D。以上结果显示，Notch1及其下游hes1基因

在卵巢癌细胞A2780中高表达，因此选用A2780细胞进行随后的实验。

2.2 DAPT对Notch1信号通路的抑制作用

不同浓度DAPT均能明显下调Notch1 mRNA的表达，见图2A；为了探讨Notch1转录水平的改变是否引起翻译水平的变化，采用Western blot方法对Notch1蛋白进行分析，结果证实DAPT可引起Notch1蛋白的下调，见图2B。这表明DAPT能抑制卵巢癌细胞A2780 Notch1信号通路的表达，并呈剂量依赖性。因此选用50 μmol/L浓度进行下面的实验。

随后采用50 μmol/L DAPT处理A2780细胞后发现，Notch1 mRNA明显受到抑制并呈现时间依赖性，见图2C，Notch1蛋白表达也同样降低，见图2D。这些结果表明，DAPT能够抑制A2780细胞Notch1信号通路，并呈时间依赖性。

2.3 DAPT对Notch1下游hes1基因表达的影响

采用实时定量PCR对DAPT处理A2780细胞的mRNA进行检测，DAPT同样引起Notch1下游hes1基因mRNA及蛋白的下调，并呈现剂量依赖性，见图3A、3B。

同样，采用50 μmol/LDAPT处理A2780细胞后，发现Notch1下游hes1基因mRNA及蛋白也随时间的不断延长表达逐渐降低，呈现时间依赖性，见图3C、3D。

2.4 DAPT下调Notch1表达后对A2780细胞生长的作用及对细胞阻滞的诱导作用

采用不同浓度DAPT作用A2780细胞在不同时间点进行分析，发现A2780细胞生长受到抑制并呈时间和剂量依赖性，且在50 μmol/L、75 μmol/L时抑制更显著，见图4A。克隆形成实验也表明DAPT处理组细胞克隆形成数明显低于实验组，见图4B。DAPT处理组细胞G_1期细胞比例增加、G_2期细胞比例减少，细胞周期分析发现，A2780细胞G_1期阻滞并呈剂量依赖性（$P < 0.05$），见图4C~F。这些结果都表明，DAPT下调Notch1可抑制A2780细胞生长并诱导细胞G_1期阻滞。

2.5 DAPT下调Notch1表达后对A2780细胞凋亡的诱导作用

采用50 μmol/L DAPT处理A2780细胞，在24、48、72 h时间点进行检测发现，A2780细胞凋亡呈现时间依赖性，且在48、72 h时凋亡数更多，见图5。
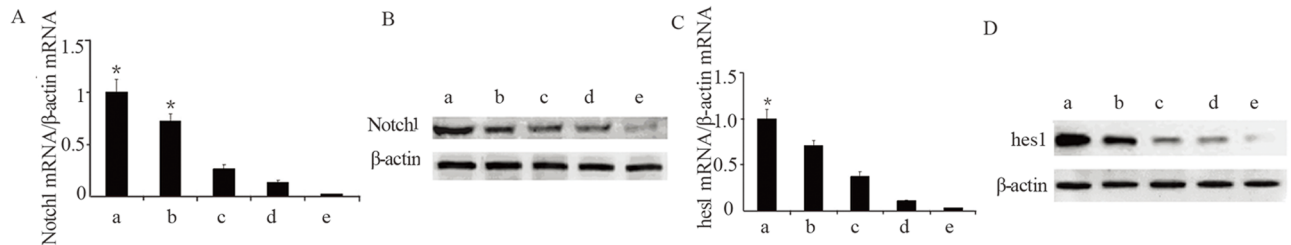
A：mRNA expression of Notch1 in the five ovarian cell lines were measured by real-time RT-PCR；B：protein levels of Notch1 in the five ovarian cell lines were measured by Western blot；C：mRNA expression of hes1 in the five ovarian cell lines were measured by real-time RT-PCR；D：protein levels of hes1 in the five ovarian cell lines were measured by Western blot；*: $P< 0.05$, compared with IOSE144; a:A2780;b:8910;c:IOSE144; d:SKOV3;e:8910PM

**图1 Notch1和hes1在卵巢癌细胞A2780、SKOV3、HO-8910、HO-8910PM及卵巢上皮细胞IOSE144中的表达**

Figure1 Expression of Notch1 and hes1 in human ovarian cancer cell lines A2780, SKOV3, HO-8910 and HO-8910PM, and ovarian surface cell line IOSE144
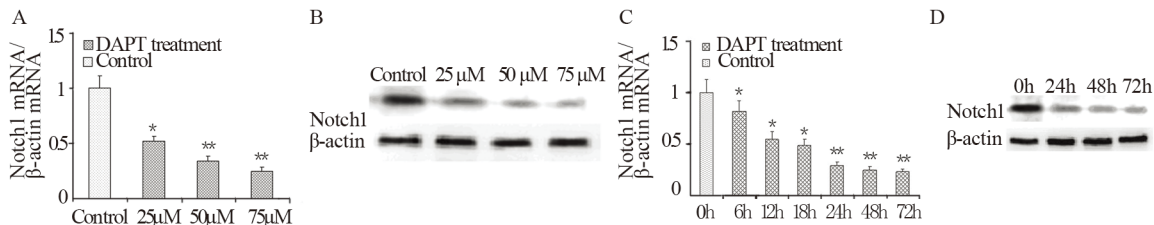
A：Notch1 mRNA expression in ovarian cancer cells A2780 treated with different concentrations of DAPT；B：Western blot analysis showed that the protein levels of Notch1 were down-regulated in DAPT-treated A2780 in a dose-dependent manner；C：Notch1 mRNA expression in ovarian cancer cells A2780 treated with 50 μM/L DAPT for 6, 12, 18, 24, 48, and 72 h；D：After A2780 were treated with 50 μM/L DAPT for 24, 48, and 72 h, Western blot analysis showed that the protein levels of Notch1 were down-regulated in a time-dependent manner. *: $P < 0.05$; **: $P < 0.01$, compared with control group

图2 DAPT对Notch1表达的影响

Figure2 Effect of DAPT on Notch1 expression
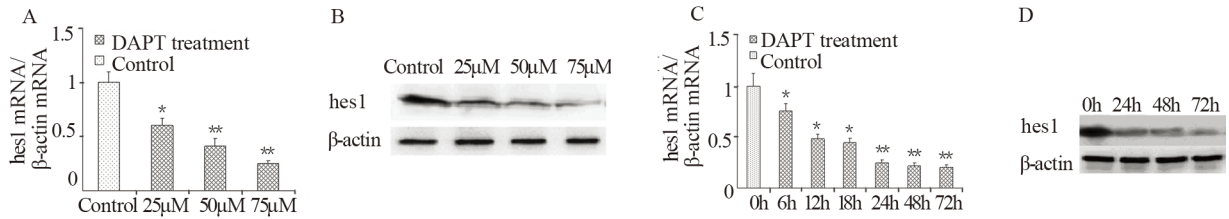
A:hes1 mRNA expression in ovarian cancer cells A2780 treated with different concentrations of DAPT; B:Western blot analysis showed that the protein levels of hes1 were down-regulated in DAPT-treated A2780 in a dose-dependent manner;C:hes1 mRNA expression in ovarian cancer cells A2780 treated with 50 μM/L DAPT for 6, 12, 18, 24, 48, and 72 h;D:After A2780 were treated with 50 μM/L DAPT for 24, 48, and 72 h, Western blot analysis showed that the protein levels of hes1 were down-regulated in a time-dependent manner; *: $P < 0.05$; **:$P < 0.01$, compared with control group

图3　DAPT对hes1表达的影响
Figure3　Effect of DAPT on hes1 expression
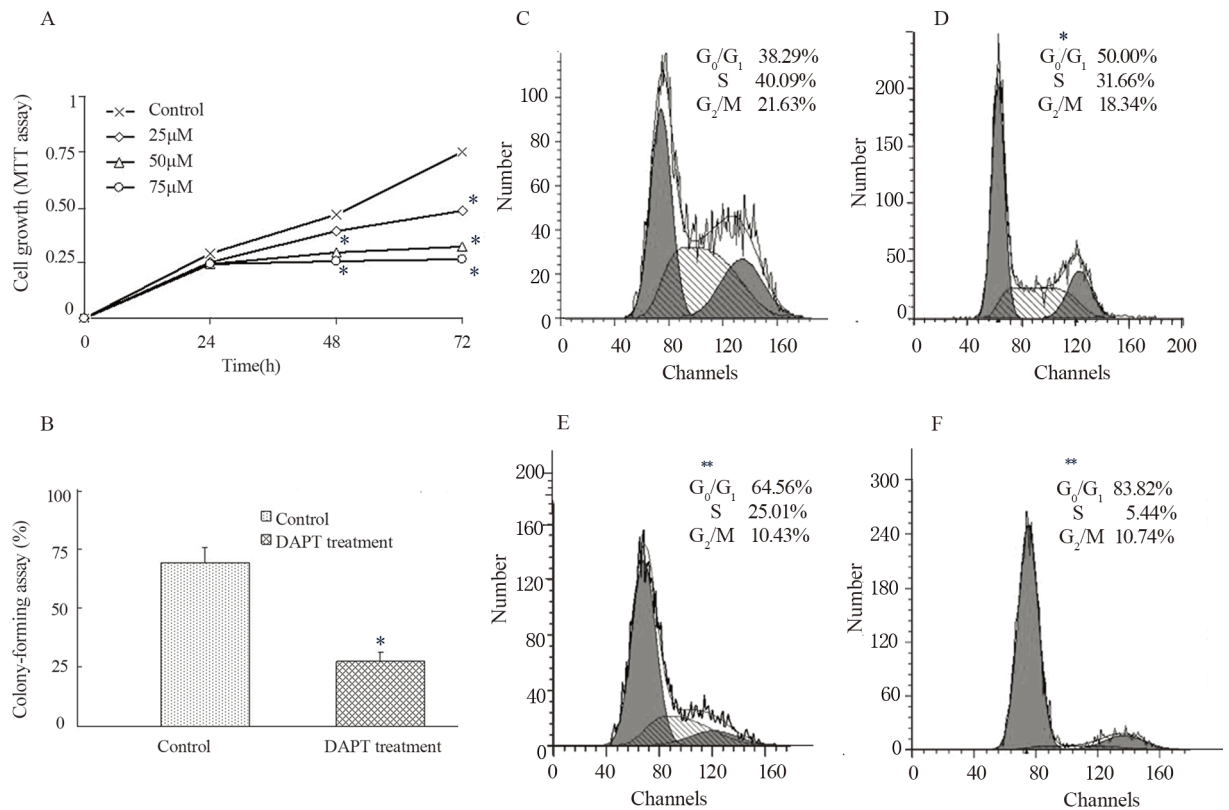
A:After A2780 cells were treated with 25, 50, and 75 μM DAPT for 3 d, MTT assay showed the cell growth was inhibited in a dose- and time-dependent manner;B: After A2780 cells were treated with 50 μM DAPT for 14 d, colony formation assay showed the clonal growth of A2780 cells was significantly inhibited; C-F: effect of down-regulation of Notch1 on cell cycle distribution of A2780 detected by flow cytometry; C: Control group; D: 25 μM DAPT treatment group;E: 50 μM DAPT treatment group;F: 75 μM DAPT treatment group. Down-regulation of Notch1 caused G_1 cell cycle arrest in a dose-dependent manner; *: $P < 0.05$; **:$P < 0.01$, compared with control group

图4　DAPT对卵巢癌细胞周期及细胞生物学的影响
Figure4　Effect of DAPT on cell cycle and biology of ovarian cancer

3　讨论

　　Notch信号通路在细胞生长过程中决定着细胞命运，同时在许多恶性肿瘤发生发展、浸润及血管形成过程中起着重要作用[9-11]。Notch1的功能是癌基因还是抑癌基因取决于细胞类型的不同，它在皮肤癌和非小细胞型肺癌中起抑癌基因作用[12-13]，而在其他许多肿瘤中起癌基因作用，如：肾癌、胰腺癌、乳腺癌和前列腺癌等[14]。Notch1在妇科宫颈癌中也起癌基因作用，其影响细胞生长分化的

下游基因hes1在宫颈癌细胞系中有高表达[5]。我们先前的研究[6]发现Notch1在卵巢癌中的表达与卵巢癌组织分化程度相关。本实验研究了Notch1在卵巢癌细胞A2780增殖及凋亡过程中的作用，发现下调Notch1能抑制A2780生长并诱导凋亡，进一步证明Notch1在卵巢癌中起到癌基因的作用。

　　hes1是Notch信号通路下游、影响细胞增殖及分化的靶基因[15-17]。本研究发现在A2780细胞中DAPT下调Notch1能下调其下游hes1基因的表达，
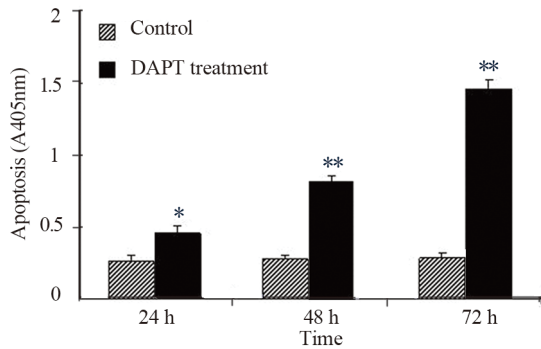
DAPT-induced apoptosis in ovarian cancer cells A2780 was measured by ELISA. After treated with 50 μM/L DAPT for 24, 48, or 72 h, the apoptosis of A2780 was induced in a time-dependent manner; *:$P< 0.05$; **:$P< 0.01$, compared with control group

图5　DAPT对卵巢癌细胞凋亡的影响
Figure5　Effect of DAPT on the apoptosis of ovarian cancer cells

并呈现时间和剂量依赖性；下调Notch1能引起A2780细胞生长抑制、细胞克隆数减少，同时引起细胞G$_1$期阻滞；还能引起A2780细胞凋亡，并呈时间依赖性。然而，Notch信号通路介导的、影响A2780细胞生长分化的下游基因的分子过程还不是十分清楚，也许hes1只是Notch信号通路下游众多功能性靶基因中的一个，其功能的完成需要Notch信号通路下游的其他靶基因的共同完成。在卵巢癌中，Notch信号通路及其下游基因的功能还不是完全清楚，因此，需要更多的实验进一步阐明Notch信号通路与卵巢癌发生发展分子机制之间的关系。

γ-分泌酶是Notch信号胞内段NICD释放的一个关键蛋白[3]，γ-分泌酶抑制剂可阻断Notch信号胞内段的释放并抑制Notch信号通路的激活[18]。研究报道，γ-分泌酶抑制剂已经运用于Notch激活引起的相关疾病，如γ-分泌酶抑制剂可用于Alzheimer's疾病的治疗[19]；γ-分泌酶抑制剂在非白血病性白血病治疗中不良反应小、安全性更高[20]；在急性淋巴细胞性白血病（T-ALL）有治疗作用[21]，且与糖皮质激素联合用药还可以降低其毒性等[22]。许多研究认为，有Notch信号通路开关作用的γ-分泌酶是Notch通路钝化的最佳靶点。最近，γ-分泌酶抑制剂与肿瘤基因治疗的研究已成研究热点，因为它可能是Notch激活相关肿瘤的一个新的治疗靶点[23]。本研究中，γ-分泌酶抑制剂DAPT有效抑制了Notch1及其下游hes1基因，同时抑制了A2780细胞增殖并诱导细胞凋亡。然而，在动物实验或临床试验中是否有这样的结果呢？更多的研究还将继续。随着对Notch信号通路与卵巢癌发生机制、相关癌基因和抑癌基因关系的不断深入研究，γ-分泌酶抑制

剂可能成为卵巢癌治疗的一个新靶点。

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