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• 基础研究 •

CDK7抑制剂THZ1对乳腺癌细胞增殖与凋亡的作用及其机制

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Effect of CDK7 Inhibitor THZ1 on Proliferation and Apoptosis of Breast Cancer Cells and Related Mechanism

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Abstract: Objective To investigate the effect of THZ1, a selective inhibitor of cyclin-dependent protein kinase 7(CDK7), on the proliferation and apoptosis of human breast cancer cell lines MCF7, SkBr3 and Hs578T. **Methods** MTT, flow cytometry and Western blot were used in the experiment to detect cell viability, cell cycle and apoptosis. **Results** After treated with 500 nmol/L THZ1, the number of cells was obviously decreased based on microscope observation. MTT assay showed that the proliferation of breast cancer cells was inhibited in a dose- and time-dependent manner after THZ1 treatment. Cells were synchronized at the G_1/S transition by starved for 24h, and then treated with indicated doses of THZ1 for 24h. FCM results showed that the number of cells in G_2/M phase was increased, indicating the G_2/M phase cell cycle arrest was induced. Furthermore, THZ1 induced cells apoptosis in a dose- and time-dependent manner. The expression of Cleaved-PARP were significantly up-regulated and anti-apoptotic gene Bcl-2 were down-regulated, and the NF-κB and GSK3β pathway were activated via increasing the phosphorylation of p65 and GSK3β. **Conclusion** THZ1 can inhibit the proliferation of MCF7, SkBr3 and Hs578T cells, induce cell cycle arrest and cell apoptosis in a dose- and time-dependent manner. It suggested that THZ1 can be used as a potential candidate drug for breast cancer.

Key words: CDK7; Breast cancer; Apoptosis; PARP

摘　要：目的 探讨细胞周期蛋白依赖性蛋白激酶7（CDK7）特异性抑制剂THZ1对人乳腺癌细胞系MCF7、SkBr3及Hs578T增殖及凋亡的作用及其分子机制。**方法** 采用MTT、流式细胞术、Western blot等方法检测细胞活力、细胞周期和凋亡的变化情况。**结果** 500 nmol/L THZ1处理细胞，显微镜观察细胞数明显减少；MTT实验表明THZ1呈剂量及时间依赖性抑制细胞的增殖；细胞饥饿培养24 h以同步化在G_1/S期，THZ1继续处理24 h, 流式细胞术检测G_2/M期细胞数增多。THZ1引起细胞G_2/M期阻滞；THZ1呈剂量及时间依赖性诱导细胞早期凋亡与晚期凋亡；Western blot结果表明，THZ1可导致Cleaved-PARP上调，Bcl-2的显著下调和p65、GSK3蛋白磷酸化水平的明显上调。**结论** THZ1能抑制MCF7、SkBr3及Hs578T细胞增殖，诱导其细胞周期阻滞及细胞早、晚期凋亡，可作为乳腺癌治疗潜在的候选药物。

关键词：CDK7；乳腺癌；凋亡；PARP

中图分类号：R737.9　　**文献标识码：**A

0 引言

乳腺癌是女性最常见的恶性肿瘤之一，据资料统计，美国在2014年新增乳腺癌患者23万例，占女性肿瘤的28.7%，死亡约4万例，其发病率和死亡率列各恶性肿瘤第一位[1]。我国乳腺癌近年来发病率不断增加，占世界范围内乳腺癌总发病率的12.2%，而死亡率则占9.6%[2]，严重威胁着人类的生存和发展。目前乳腺癌的治疗手段有：手术、内分泌治疗、化疗、放疗、分子靶向治疗及基因治疗等。虽然乳腺癌的治疗已经卓有成效，但其发病率和死亡率依旧是构成人类生存健康的强大威胁[3]。

细胞周期蛋白依赖性激酶（cyclin dependent kinases, CDKs）是细胞周期调节的关键激酶，参与细胞增殖、转录、存活等生理过程[4-5]。CDK7属

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于参与转录调节的CDKs家族，通过调节RNA聚合酶Ⅱ的磷酸化来调节转录[6]。由于CDK活性为细胞分裂所必需，而在肿瘤细胞中又常有CDK活性增强，因此长期以来，CDK一直被认为是抗肿瘤及其他增殖失调疾病药物研发的较好靶点。本实验以细胞株MCF7、SkBr3及Hs578T作为研究对象，探讨CDK7的选择性抑制剂THZ1对乳腺癌可能的抗肿瘤作用及其相关分子机制，以期为乳腺癌的治疗提供潜在的药物靶点。

1　材料与方法

1.1　细胞株

人乳腺癌 SkBr3、MCF7、Hs578T细胞均来源于本实验室保存。

1.2　主要试剂

RPMI 1640培养液、0.25% Trysin-EDTA购自美国Gibco公司；胎牛血清购自法国Biowest公司；碘化丙啶、细胞凋亡检测试剂盒购自美国BD Pharmingen）；青霉素-链霉素双抗购自美国HyClone公司；噻唑蓝MTT购自美国Sigma公司；5X Loading Buffer购自杭州弗德生物科技有限公司）；蛋白裂解液、GAPDH抗体购自上海碧云天生物技术有限公司）；CDK7抗体、Cleaved-caspase3抗体、Cleaved-caspase7抗体、Cleaved-caspase9抗体、PARP抗体、Cleaved-PARP抗体均来自美国CST公司；α-tublin、GSK3β及其磷酸化抗体、YAP及其磷酸化抗体、STAT3及其磷酸化抗体、Fox3a及其磷酸化抗体、p65及其磷酸化抗体均来自美国Bioworld公司。

1.3　细胞培养

MCF7、SkBr3细胞贴壁生长于含10%胎牛血清、100 u/ml青霉素和100 μg/ml链霉素的RPMI 1640培养液中，在37℃、5%CO_2饱和湿度下培养，每隔1至2天换液或传代，取对数生长期细胞进行实验。

1.4　显微镜观察THZ1对细胞的增殖及形态影响

细胞以5×10^5的密度接种于6孔板中，次日加入THZ1处理，连续培养5天后，用PBS洗去漂浮的细胞，显微镜观察，并随机挑选明视野拍照。

1.5　MTT法检测细胞增殖情况

0.25%胰酶消化对数期细胞，终止后离心收集，细胞以每孔4 000的密度接种至96孔板。条件培养至细胞贴壁后加入浓度梯度的THZ1（分别为0、1；1×10^1、$1\times10^{1.5}$、1×10^2、$1\times10^{2.5}$、1×10^3、$1\times10^{3.5}$、1×10^4 nmol/L），培养液继续培养24、48或72 h后，每孔加入MTT溶液（5 mg/ml，PBS配制，pH=7.4）20 μl。继续孵育4 h，终止培养，小心吸去培养液，每孔加150 μl DMSO，振荡5 min使结晶溶解，在490 nm波长处测量吸光度*OD*值，实验重复三次。

1.6　流式细胞术检测细胞周期

细胞以5×10^5的密度接种于6孔板中，待细胞贴壁后，撤去血清24 h同步化处理细胞，更换新鲜完全培养液后加入不同浓度的THZ1（0、0.1和0.5 μmol/L）处理24 h，0.25%胰酶消化细胞，1 000 r/min离心后加入70%乙醇固定过夜。次日用PBS清洗细胞两次，加入500 μl PI避光染色15 min后用400目筛网过滤，转至流式细胞仪检测细胞周期。

1.7　流式细胞术检测细胞凋亡

细胞以5×10^5的密度接种于6孔板中，待细胞贴壁后，加入不同浓度的THZ1（分别为0、0.05、0.1、0.2、0.5、1 μmol/L）处理细胞，继续培养48 h后用0.25%胰酶消化细胞，用预冷的PBS清洗细胞两遍后，按说明书加入PI及FITC避光染色15 min。400目筛网过滤后转至流式细胞仪检测。

1.8　蛋白免疫印迹（Western blot）检测蛋白表达

细胞按要求加药处理后，PBS清洗两次，于冰上加入IP裂解液处理25~30 min，12 000 r/min离心20 min，收集上清液，BCA法测定蛋白浓度后，加入四分之一体积的5×Loading buffer 100℃金属浴变性后取30~50 μg总蛋白进行丙烯酰胺凝胶电泳。恒压80~120V电泳后，恒流200 mA，90~120 min的条件下转印至甲醇预处理的PVDF膜上。室温下用5%的脱脂奶粉（PBST配制）封闭PVDF膜2 h，4℃一抗摇床上孵育过夜，次日用PBST（1×PBS加入1:1000吐温–20）清洗三次，每次10 min，用相应的二抗孵育2 h，再用PBST清洗三次，每次10 min。加入发光液进行曝光。

1.9　统计学方法

所有实验结果均由三次重复实验得出，应用SPSS17.0分析软件，配对*t*检验进行数据分析，$P<0.05$认为差异有统计学意义.

2　结果

2.1　THZ1抑制乳腺癌细胞增殖

按实验设计浓度的THZ1处理细胞不同时间后，细胞增殖情况见图1。显微镜直接观察结果表明500 nmol/L THZ1作用5天后，细胞数目就开始减少，见图1A。THZ1显著抑制Hs578T、MCF7细胞的增殖，且随药物浓度增大及时间延长，抑

制效应越显著，其中Hs578T对THZ1最为敏感，10 nmol/L即出现增殖抑制现象，100 nmol/L THZ1对Hs578T的增殖作用最明显，而THZ1对MCF7抑制最明显的浓度在300~400 nmol/L之间，SkBr3相对Hs578T、MCF7细胞对THZ1不敏感，见图1B。不同浓度的THZ1处理细胞组与阴性对照相比，差异均具有统计学意义（$P<0.05$）。THZ1对Hs578T细胞24、48及72 h的IC_{50}分别为1.47、0.45和0.19 μmol/L，而THZ1对MCF7的IC_{50}分别为＞10、6.03和0.91 μmol/L，SkBr3对THZ1相对不敏感，IC_{50}均大于10 μmol/L。

2.2 THZ1对细胞周期的影响

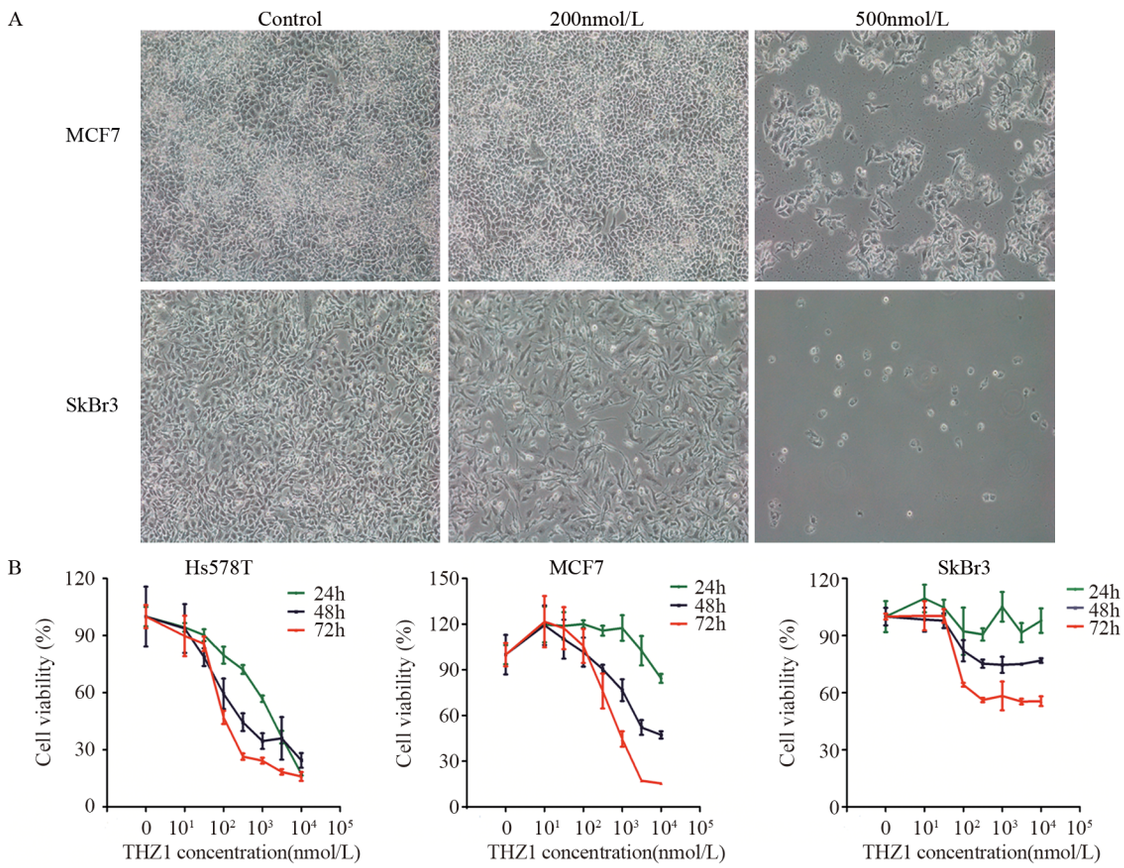
细胞在正常培养条件下所处的细胞周期各不相同，为了消除不同细胞周期对实验的影响，细胞接种贴壁后，撤去血清饥饿培养细胞24 h，使其同步化。Syn1、Syn2检测同步化效果，不同浓度的THZ1处理细胞24 h后，流式细胞术检测细胞周期的变化，结果见图2A。对应细胞各周期分布柱状图见图2B~2C。结果表明经THZ1处理后，细胞在G_2/M期的数量明显增加。

2.3 流式细胞术检测THZ1对细胞凋亡的作用

细胞凋亡是细胞程序化死亡的一种途径，因此我们进一步探讨THZ1对细胞凋亡的作用。设置不同浓度THZ1处理细胞48 h，Annexin V-FITC细胞凋亡检测试剂盒检测细胞凋亡程度。结果显示THZ1同时使细胞早期凋亡与晚期凋亡明显增加，并呈浓度依赖性，见图3。

2.4 凋亡相关蛋白验证THZ1的促凋亡作用

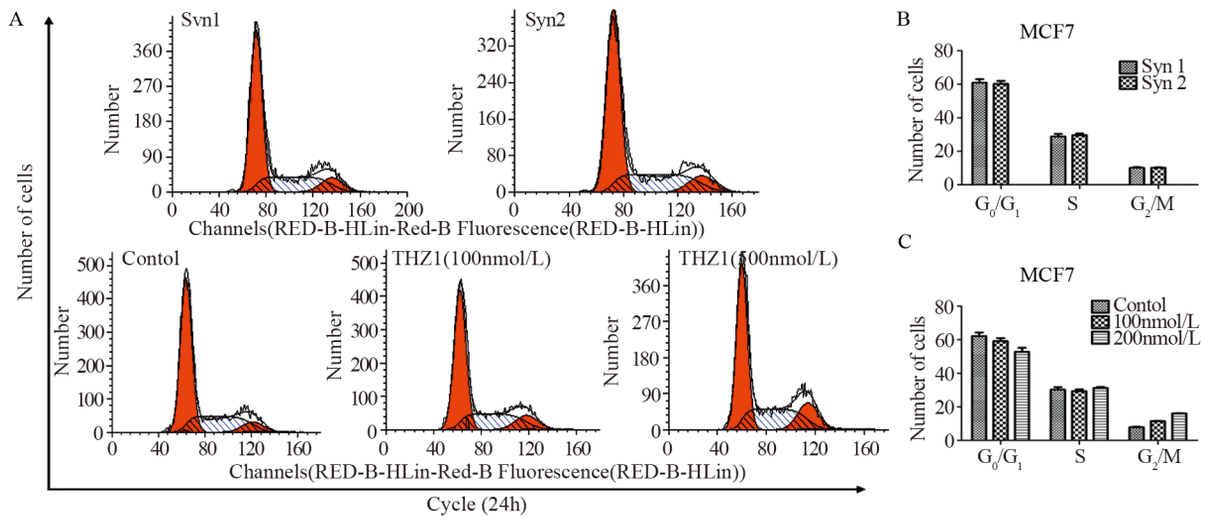
为了进一步验证THZ1促凋亡作用，Western blot检测凋亡相关的蛋白分子的变化。用不同浓度的THZ1处理细胞48 h，Western blot检测结果表明Cleaved-PARP显著上调，而抗凋亡基因Bcl-2明显下调，见图4A，MCF7细胞和SkBr3细胞经THZ1处理不同时间，THZ1的促凋亡作用呈现出时间依赖性，并且在12 h 上调蛋白的作用即达到最大，见图4B~4C。



A: bright-field of MCF7 and SkBr3 cells which were treated with DMSO (left) or 200 nmol/L THZ1 (middle) and 500nmol/L THZ1(right) for 5 days; B: Cells were treated with increasing concentrations of THZ1(0~10 000nmol/L) for 24, 48 and 72h, then the cell viability was assessed by MTT method. With the increasing dose of THZ1, the proliferation of cells was obviously inhibited. The data represented the average of three independent experiments results

图1 MTT法检测不同浓度THZ1对乳腺癌细胞增殖的影响

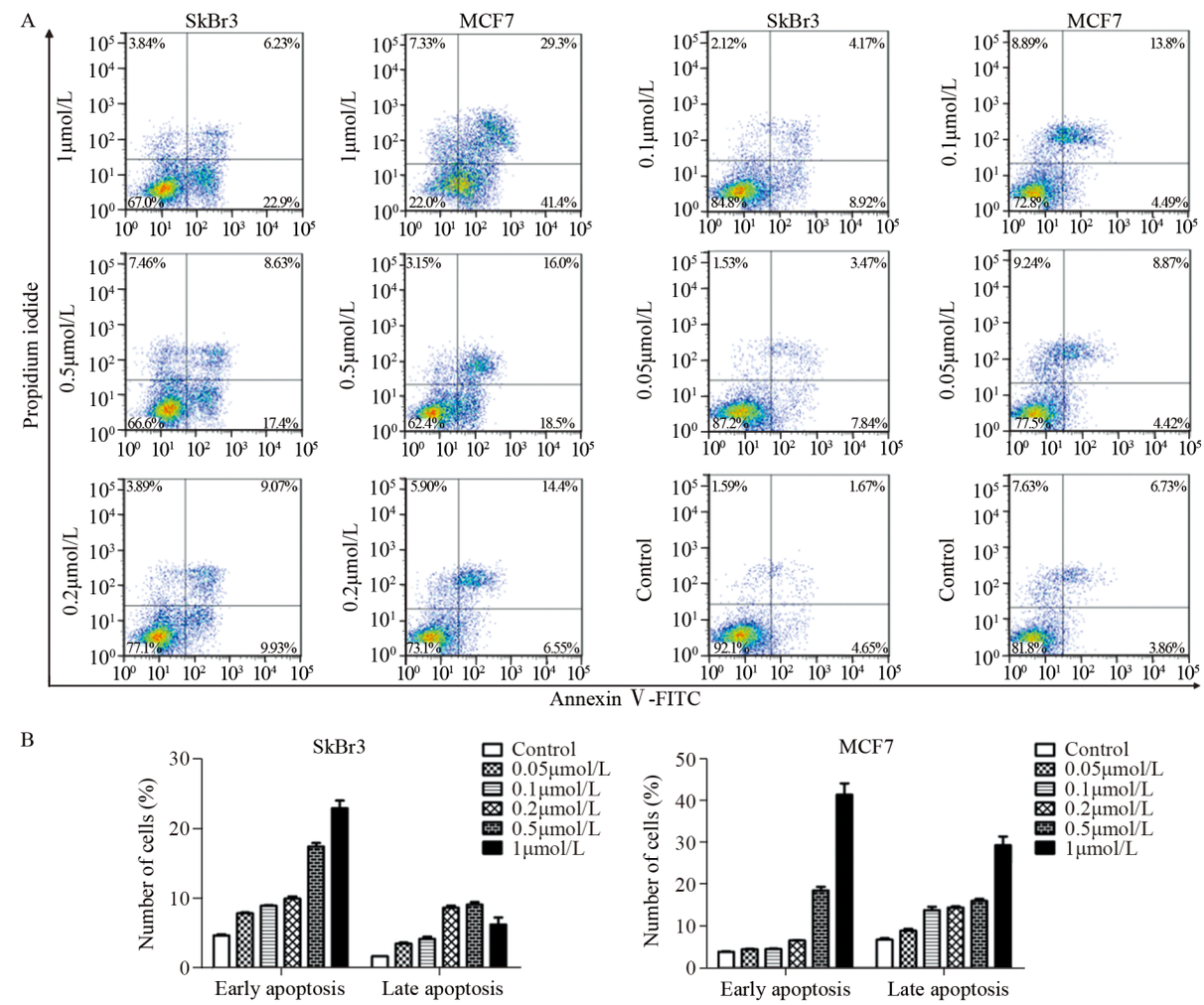
Figure1 Cell proliferation assessed by MTT after various concentrations of THZ1 treatment



A: MCF7 cells were synchronized at the G_1/S transition by starved 24h, and then treated with indicated THZ1 for 24h. The cell cycles were analyzed by FCM; data were presented as means±SD of three independent experiments; B, C: the histogram of corresponding cell cycle distribution

图2　流式细胞术检测不同浓度THZ1处理后对细胞周期的影响

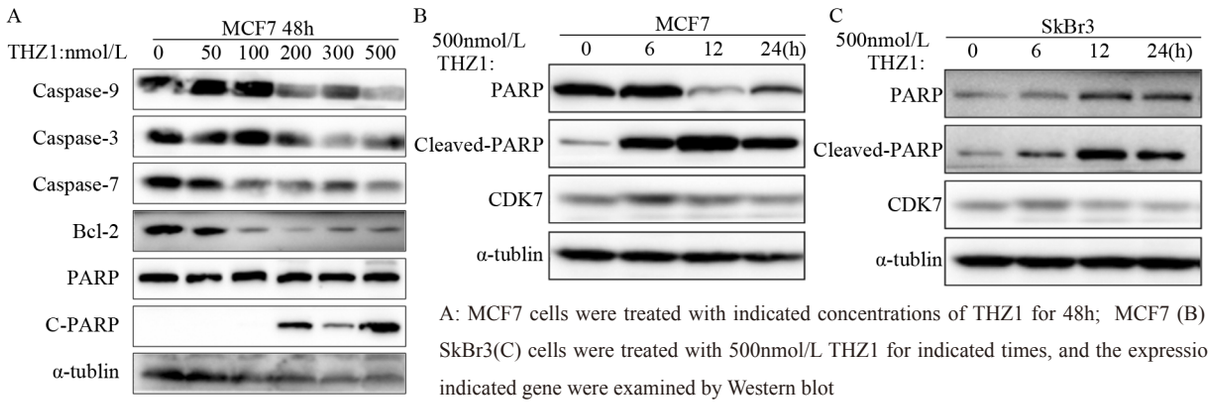
Figurea2　Cell cycle assessed by FCM after various concentrations of THZ1 treatment



A: SkBr3 and MCF7 cells were treated with increasing concentrations of THZ1 for 48h, stained with annexin V-FITC and PI, and then analyzed by flow cytometry for cell apoptosis; B: the histogram of corresponding cell distribution

图3　流式细胞术检测不同浓度THZ1处理后对细胞凋亡的影响

Figure3　Cell apoptosis assessed by FCM after various concentrations of THZ1 treatment



A: MCF7 cells were treated with indicated concentrations of THZ1 for 48h; MCF7 (B) and SkBr3(C) cells were treated with 500nmol/L THZ1 for indicated times, and the expression of indicated gene were examined by Western blot

图4 Western blot检测不同浓度THZ1处理后对乳腺癌细胞凋亡相关蛋白的影响

Figure4 Cell apoptosis related protein assessed by Western blot after various concentrations of THZ1 treatment

2.5 THZ1作用后细胞信号通路的变化

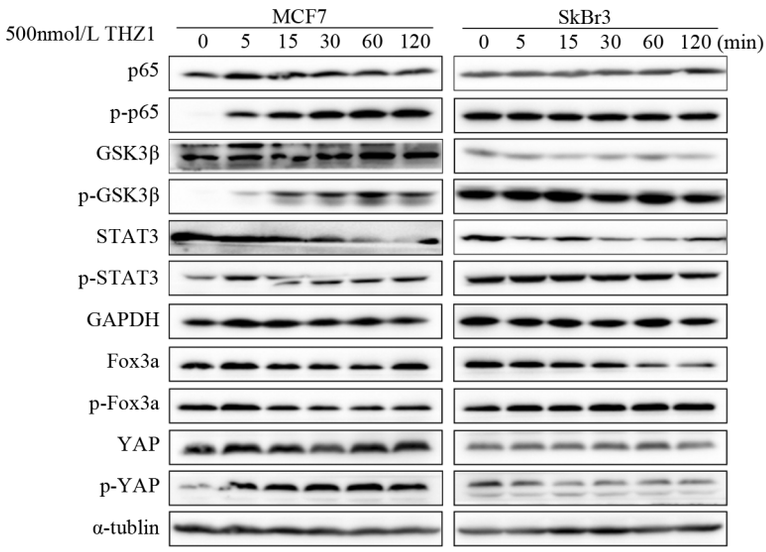
利用Western blot检测调控细胞增殖及凋亡的重要介导信号分子，p65、GSK3β、STAT3、Fox3a、YAP等蛋白及其磷酸化在THZ处理后乳腺癌细胞株中的变化情况，结果显示，THZ1可显著上调p65及GSK3调的磷酸化水平。THZ1的促凋亡作用有可能与p65和GSK3相关的信号通路有关，见图5。

3 讨论

乳腺癌治疗的进展涉及多个方向，随着生物工程及基因技术的发展，分子靶向治疗将为乳腺癌的治疗提供进一步的选择和前景[7]。有研究指出[8]，癌症的发生是细胞增殖和细胞程序性死亡平衡失调引起的，因此，不可遏制的细胞增殖和凋亡程序的失控是癌症发生发展的重要过程。

过度活化、持续的细胞增殖是肿瘤的一个基本特征，而CDK属于丝/苏氨酸蛋白激酶家族，是参与细胞周期调节的关键激酶[9]。CDK活性失调会直接或间接引起细胞增殖失控、基因组不稳定（DNA突变增加，染色体缺失等）和染色体不稳定（染色体数目变化）等，来参与肿瘤的发生发展[10]。根据CDK功能的不同，可将其主要分为两大类：一类CDK参与细胞周期调控，主要包括CDK1、CDK2、CDK4、CDK6等，另一类CDK参与转录调节，主要包括CDK7、CDK8、CDK9、CDK10、CDK11等目前已有1个CDK抑制剂在临床用于抗肿瘤治疗，另有数十个CDK抑制剂正进行临床或临床前研究[11-16]。CDK7属于参与转录调节的CDKs家族，通过调节RNA聚合酶Ⅱ的磷酸化来调节转录。长久以来，靶向基因转录过程进行的癌症治疗一直被认为是困难的，这是因为转录的普遍作用。抑制转录过程会因为缺乏对癌细胞的择性而导致药物毒性。然而，最近的研究已经质疑这一观点，发现某些基因的转录十分敏感于转录抑制剂[17-19]。我们的实验结果表明CDK7选择性抑制剂THZ1可以显著抑制乳腺癌细胞的增殖。这为靶向乳腺癌的治疗提供了一个方向。

有文献指出，THZ1可以在nmol/L级别显著抑制三阴性乳腺癌细胞的增殖[20]，这种抑制肿瘤增殖作用是通过抑制RNA聚合酶Ⅱ的磷酸化实



MCF7 and SkBr3 cells were treated with 500 nmol/L for 0 to 60 min, then the expression of total protein and phosphorylation of p65, GSK3β were examined by Western blot

图5 THZ1对乳腺癌细胞信号通路的影响

Figure5 Effect of THZ1 on signaling pathway of breast cancer cells

现的。而本实验发现，除了三阴性乳腺癌细胞Hs578T对THZ1敏感，雌激素/孕激素阳性的乳腺癌细胞MCF7和SkBr3在THZ1作用后细胞增殖也同样变得缓慢，MCF7经THZ1作用72 h后，IC_{50}只有0.91 μmol/L。这为包括三阴性乳腺癌在内的乳腺癌提供了一个新的潜在治疗方法。MCF7在经THZ1作用后，G_2/M期细胞明显增多，提示THZ1可以诱导细胞发生G_2/M期周期阻滞从而影响细胞的周期进程，这有可能是CDK7的抑制影响了另一类参与细胞周期调控的CDKs的功能，这种猜想为THZ1抑制肿瘤增殖作用的机制提供了一个补充。本实验证实了THZ1可以诱导MCF7及SkBr3细胞凋亡，流式细胞术检测THZ1浓度在0~1 μmol/L范围内，细胞的早期凋亡和晚期凋亡呈剂量依赖性增加，并使Bcl-2及PARP的剪切体显著上调。Bcl-2家族蛋白在调节内在凋亡机制中扮演重要角色，Bcl-2具有抗凋亡活性，能够抑制Bax进入线粒体[21-24]，这些蛋白的变化被认为可介导细胞通过线粒体相关途径诱导细胞凋亡，但THZ1是否通过ROS线粒体途径使细胞凋亡还有待进一步验证。p65、GSK3β、STAT3、Fox3a、YAP等[25-29]都被认为可能参与了细胞增殖与凋亡的过程，所以本实验检测了其在THZ1作用后的变化情况，结果显示p65和GSK3β蛋白的的磷酸化水平显著上调，在短时间（5~10 min）即发挥了作用，这提示促凋亡作用可能通过NF-κB和GSK3β信号通路实现，具体THZ1的促凋亡作用如何通过NF-κB和GSK3β信号通路实验，我们将进行进一步的探究。

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