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· 基础研究(专题) ·

积雪草酸通过抑制耐药相关蛋白表达增强 U87MG 胶质瘤细胞对紫杉醇的敏感性

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[摘要] **目的:** 探索积雪草酸(asiatic acid, AA)对紫杉醇(paclitaxel, PTX)耐药性胶质瘤细胞的抑制作用及其可能的作用机制。**方法:** CCK-8 实验、实时荧光定量 PCR、Western blotting 检测 AA 对成胶质细胞瘤 U87MG 细胞的增殖、凋亡的影响。浓度递增法构建 PTX 耐药性细胞株 PR-U87MG, 以 U87MG 细胞为对照, CCK-8 实验验证 PR-U87MG 细胞对 PTX 的耐药性, 实时荧光定量 PCR、Western blotting 检测 PR-U87MG 细胞中 *MDR1*、*LRP* mRNA 及蛋白的表达水平。AA 和 PTX 单独或联合处理 PR-U87MG 细胞, CCK-8 实验、实时荧光定量 PCR、Western blotting 检测各组细胞增殖活力及凋亡的变化。**结果:** 成功构建 PTX 耐药性细胞株 PR-U87MG。AA 可以剂量依赖方式抑制 U87MG 细胞和 PR-U87MG 细胞的增殖活力($P < 0.01$), 并明显促进其凋亡($P < 0.01$)。与 AA 或 PTX 单独处理组相比, 联合处理组中 PARP1 的蛋白水平显著减少($P < 0.01$), caspase 3 的裂解量显著增加($P < 0.01$), 耐药相关蛋白 P-糖蛋白 1(P-glycoprotein 1, Pgp-1)和 LRP 表达水平显著减少($P < 0.01$)。**结论:** AA 可有效增强 U87MG 胶质瘤细胞株对 PTX 的敏感性, 其机制可能与 AA 抑制具有药物排出功能的耐药蛋白 Pgp-1 和 LRP 表达有关。

[关键词] 积雪草酸; 胶质瘤; 耐药性; 化疗敏感性; 紫杉醇; U87MG

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Asiatic acid enhances the chemosensitivity of U87MG glioma cells to paclitaxel through inhibiting the expression of drug resistance related proteins

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[Abstract] **Objective:** To explore the inhibitive effect of asiatic acid (AA) on paclitaxel (PTX)-resistant glioma cells and its possible mechanism. **Methods:** The effects of AA on the proliferation and apoptosis of glioblastoma U87MG cells were detected by CCK-8 assay, Real-time quantitative polymerase chain reaction (qPCR) and Western blotting. The drug-resistant glioma cell line PR-U87MG was established by culturing the cells in concentration-increasing PTX. With U87MG cells as control, the PTX-resistance of PR-U87MG cells was confirmed using CCK-8 assay, and the mRNA and protein levels of *MDR1* and *LRP* were measured with qPCR and western blotting. PR-U87MG cells were treated with AA, PTX or AA+PTX, and then the cell viability and apoptosis of each group were measured with CCK-8 assay, qPCR and Western blotting. **Results:** PTX-resistant PR-U87MG cell line was successfully established. AA inhibited the viability of U87MG and PR-U87MG cells in a dose-dependent manner ($P < 0.01$) and significantly promoted their apoptosis ($P < 0.01$). Compared with the group treated with AA or PTX alone, the group treated with the combination of AA and PTX had significantly decreased protein levels of PARP1 ($P < 0.01$), drug-resistant related proteins (Pgp-1 and LRP [lung resistance protein]), all $P < 0.01$, and markedly increased caspase 3 ($P < 0.01$). **Conclusion:** AA could effectively enhance the sensitivity of U87MG cells to PTX, and the mechanism may be related to the suppressed expression of drug efflux-associated proteins Pgp-1 and LRP.

[Key words] asiatic acid; glioma; drug resistance; chemosensitivity; paclitaxel; U87MG

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积雪草酸(asiatic acid, AA)又称为亚细亚酸,是积雪草中的主要活性成分,既往相关研究发现 AA 具有抗肿瘤、抗糖尿病、抑菌抗炎、促进皮肤伤口愈合及改善认知功能等生物学活性^[1]。神经胶质瘤又名胶质瘤,也称为胶质细胞瘤,是临床上最常见的脑肿瘤。目前相关文献^[2]报道,紫杉醇(PTX)对胶质瘤细胞具有抗肿瘤的作用。然而,关于 AA 能否增强胶质

瘤细胞对紫杉醇的敏感性的研究在国内还尚无报道。本研究通过 AA 作用于 PTX 诱导构建的耐药性 U87MG 细胞株,旨在探讨 AA 是否能够增强胶质瘤

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细胞对PTX的敏感性。

1 材料与方法

1.1 主要材料与试剂

成胶质细胞瘤U87MG细胞株购自中国科学院上海细胞库,培养于含双抗的高糖DMEM培养液中,放于37℃恒温、5% CO₂饱和湿度培养箱中培养,选取处于对数生长期的U87MG细胞用于实验研究。

高糖DMEM培养基及胎牛血清(fetal bovine serum, FBS)购自HyClone公司,青霉素和链霉素购自Gibco公司,AA(纯度≥98%)购自成都植化纯生物技术有限公司,二甲基亚砜(DMSO)、十二烷基硫酸钠(SDS)购自Sigma Aldrich公司,ECL化学发光试剂盒和蛋白裂解液(RIPA裂解液)均购于碧云天公司,Western blotting实验中一抗PARP1(#5625)、caspase 3(#9662)、Pgp-1(#3578)、LRP(#64099)及GAPDH(#5174)抗体,二抗抗体(#7074)均购于CST公司。QuantiFast® SYBR® Green PCR试剂盒购自Qiagen公司,LightCycler® 480 II型荧光定量PCR仪购自Roche公司。由上海生工生物工程公司合成实验所需引物。

1.2 浓度递增法构建PTX耐药性PR-U87MG细胞株

选取处于对数生长期的U87MG细胞与PTX作用24 h,药物从0.001 μmol/L开始逐渐加至0.3 μmol/L,不同浓度PTX作用于细胞后,药物敏感的细胞渐渐死亡,能耐药的细胞在不含PTX培养液中继续培养,待细胞生长至对数期时,再加PTX诱导,反复多次诱导、换液、传代,直至该细胞株能在0.3 μmol/L PTX浓度下生长,即构建PTX耐药株(PR-U87MG细胞)成功。

1.3 CCK-8测定AA对PTX作用下PR-U87MG细胞增殖活性的影响

当培养皿内的U87MG细胞汇合度达到80%左右,用胰酶将U87MG细胞消化,按一定数量细胞均匀种于96孔板种,培养24 h后进行CCK-8实验。分别处理如下:加入不同浓度(0、1、5、10、40、60、80 μmol/L)的AA分别处理细胞12 h、24 h;分别用0、0.1、1、5、10、20、30 μmol/L PTX处理正常U87MG细胞和耐药性U87MG细胞24 h;以DMSO处理组为对照,用PTX(1 μmol/L)、AA(5 μmol/L)以及两者联合作用于PR-U87MG细胞24 h。最终用酶标仪测定在450 nm处的光密度(D)值,从而计算细胞增殖率。

1.4 流式细胞仪检测AA对PTX作用下PR-U87MG细胞凋亡的影响

将U87MG细胞分为若干组,对照组加DMSO处理,实验组用5 μmol/L AA与1 μmol/L PTX单独或联

合作用于PR-U87MG细胞。处理24 h后,0.25%胰酶将其消化,PBS洗涤、悬浮并计数,约吸取 1×10^5 个细胞,用膜联蛋白结合液将其慢慢重新悬浮,每管加入5 μl的膜联蛋白结合液V-fluor488及PI染色液各1 μl,室温避光孵育后流式细胞仪检测。

1.5 实时荧光定量PCR检测正常和耐药U87MG细胞中MDR1、LRP mRNA的表达水平

引物(5'→3')序列为:对照组为正常U87MG细胞,实验组为PR-U87MG细胞,提取细胞总RNA,以M-MLV反转录酶体系将总RNA(1 μg)逆转录合成cDNA,再进行实时荧光定量PCR检测2组细胞中MDR1、LRP mRNA的表达水平。MDR1上游引物:GGCTACATGAGAGCGGAGGA,下游引物:AGGAATGTTCTGGCTTCCGTT;LRP上游引物:AGGGTGAGAGTTCCCCATCT,下游引物:GGGGAGACGACACAGGATGA;GAPDH上游引物:TTAGGAAAGCCTGCCGGTGA,下游引物:GGCGCCCAATACGACCAAA。实时定量PCR反应体系为SYBRGreen Mix 12.5 μl,上游引物(F)0.5 μl,下游引物(R)0.5 μl,ddH₂O 9.5 μl,cDNA模板2 μl。记录Ct值,mRNA的相对水平用 $2^{-\Delta\Delta Ct}$ 法计算。

1.6 Western blotting检测正常和耐药U87MG细胞中PARP1、caspase 3的表达水平

将正常和耐药U87MG细胞收集起来,用预冷好的PBS清洗细胞,加入细胞裂解液进行裂解约30 min,在4℃离心机中以12 500 r/min离心15 min。用BCA法进行细胞蛋白定量,后水浴变性,将30 μg细胞蛋白进行SDS-PAGE,转膜,封闭,孵育PARP1(稀释度1:2 000)、caspase 3(1:1 500)、Pgp-1(1:1 000)、LRP(1:2 000)及GAPDH(1:2 000)一抗,TBST洗3次,孵育二抗(1:2 000),显影曝光,分析图像。使用ImageJ软件(版本V1.8.0)对蛋白免疫印迹各条带进行灰度分析。

1.7 统计学处理

采用SPSS 17.0软件进行分析,所有数据以 $\bar{x} \pm s$ 表示,两组间均数比较用t检验,多组间比较采用单向方差分析,以 $P < 0.05$ 或 $P < 0.01$ 表示差异有统计学意义。

2 结果

2.1 AA对胶质瘤细胞株U87MG增殖和凋亡的影响

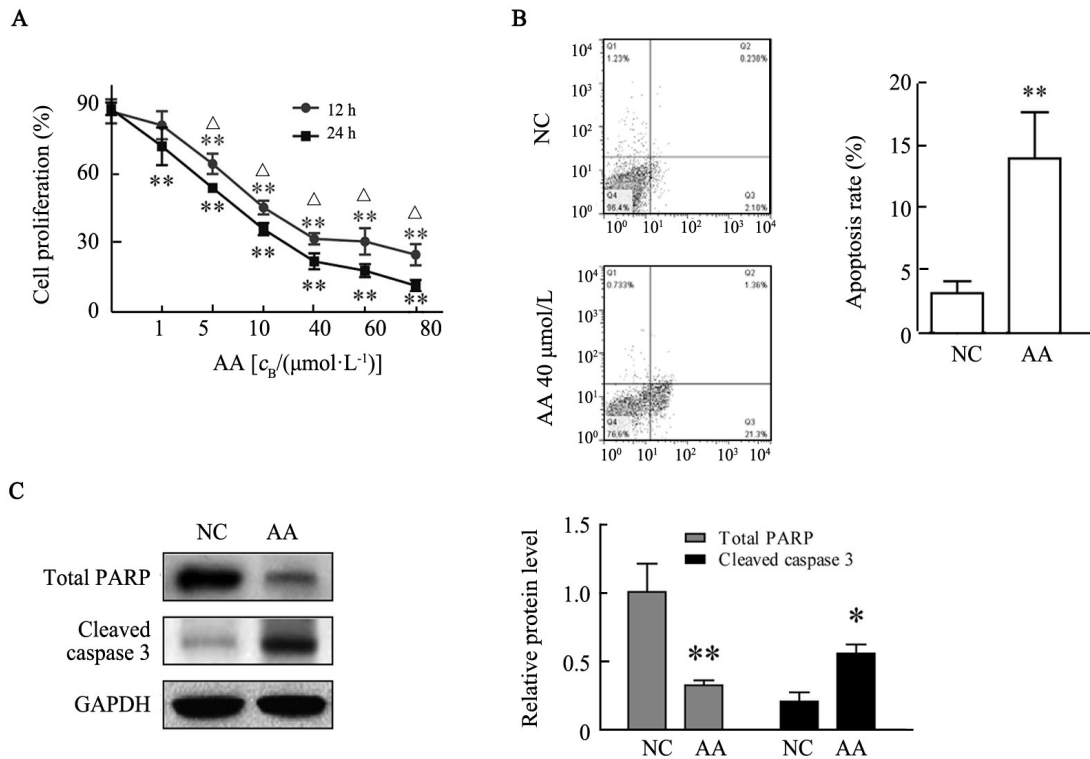
分别用不同浓度的AA处理U87MG细胞,细胞增殖活性被显著抑制,并且抑制作用随着药物浓度和作用时间而增强(图1A, $P < 0.05$)。流式细胞术检测发现,40 μmol/L的AA处理U87MG细胞后,胶质瘤细胞凋亡率显著增加(图1B, $P < 0.01$)。Western

blotting 检测结果显示, 40 $\mu\text{mol/L}$ 的 AA 作用 12 h 后的 U87MG 细胞中, 总 PARP1 的水平显著降低, caspase 3 的裂解显著增强(图 1C, $P < 0.05$)。

2.2 成功构建 PTX 耐药性细胞株 PR-U87MG

CCK-8 检测结果(图 2A)显示, 随着 PTX 作用浓度的增加, PR-U87MG 细胞增殖活力显著高于正常 U87MG 细胞 ($P < 0.05$)。实时荧光定量 PCR 检测结

果(图 2B)显示, 耐药相关分子 *MDR1*、*LRP* 的 mRNA 水平在 PR-U87MG 细胞中表达量较正常 U87MG 细胞显著升高(均 $P < .05$) ; Western blotting 检测结果(图 2C)显示, PR-U87MG 细胞中耐药相关蛋白 Pgp-1 和 LRP 的表达水平较正常 U87MG 细胞增加(均 $P < 0.01$)。



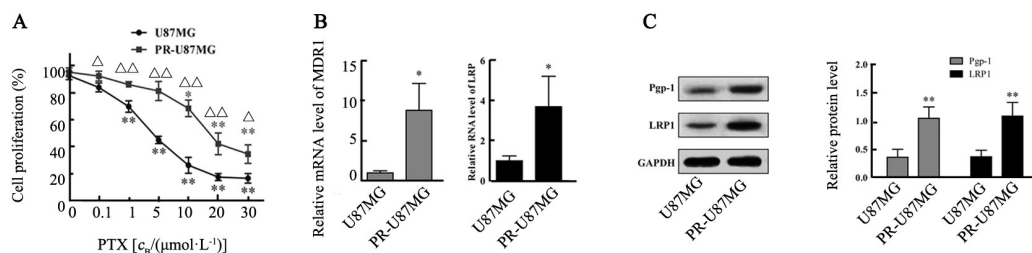
A: CCK-8 was used to determine the effect of AA on the proliferation of U87MG cells at different concentrations, $^*P < 0.05$, $^{**}P < 0.01$ vs AA 0 $\mu\text{mol/L}$ group; $^{\Delta}P < 0.05$ vs 24 h;

B: The effect of 40 $\mu\text{mol/L}$ AA on U87MG cell apoptosis was determined by flow cytometry, $^{**}P < 0.01$ vs NC group;

C: The cleavage levels of PARP1 and caspase 3 were determined by Western blotting analysis, $^*P < 0.05$, $^{**}P < 0.01$ vs NC group

图 1 AA 对胶质瘤细胞株 U87MG 的增殖和凋亡的影响

Fig. 1 Effect of AA on the proliferation and apoptosis of glioma U87MG cells



A: CCK-8 assay was used to determine the effect of PTX on the proliferation of U87MG cells and PR-U87MG cells, $^*P < 0.05$, $^{**}P < 0.01$ vs 0 $\mu\text{mol/L}$ group; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ vs U87MG cells at the same PTX concentration;

B: Real-time quantitative PCR was used to determine the mRNA levels of *MDR1* and *LRP* in U87MG cells and PR-U87MG cells, $^*P < 0.05$ vs U87MG cells;

C: Western blotting analysis of Pgp-1 and LRP protein expression levels, $^*P < 0.05$, $^{**}P < 0.01$ vs U87MG cells

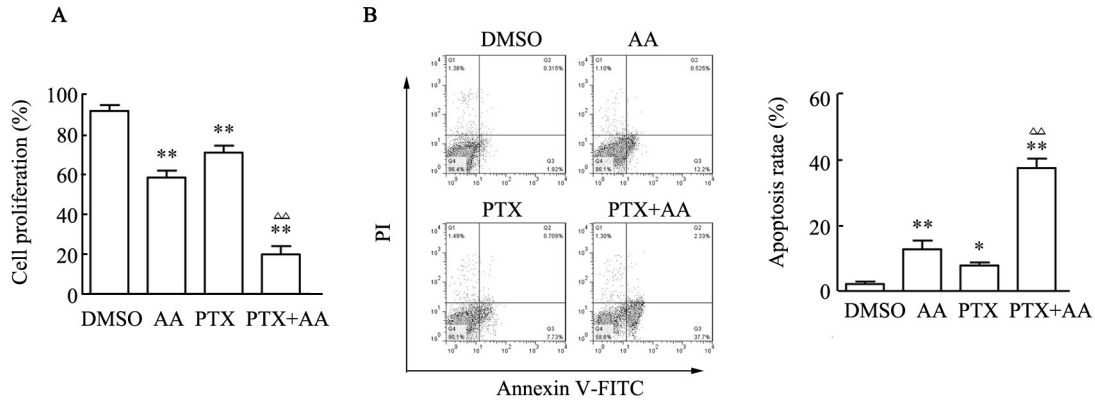
图 2 PTX 诱导构建耐药性细胞株 PR-U87MG

Fig. 2 Establishment of drug resistant cell line PR-U87MG by PTX induction

2.3 AA对PR-U87MG细胞增殖和凋亡的影响

CCK-8 实验检测结果(图3A)显示,与AA或PTX单独处理组相比,AA联合PTX组PR-U87MG细胞增殖活力最低($P < 0.01$);流式细胞术检测结果(图

3B)显示,AA与PTX两者联合处理胶质瘤耐药细胞,其凋亡率较AA或PTX单独处理组显著升高 [$(37.47 \pm 2.86)\%$ vs $(12.88 \pm 2.65)\%$ 、 $(7.97 \pm 0.89)\%$, $P < 0.05$]。



A: CCK-8 was used to determine the effects of AA (5 $\mu\text{mol/L}$), PTX (1 $\mu\text{mol/L}$) and their combination on the viability of PR-U87MG cells; B: Flow cytometry for the effects of AA (5 $\mu\text{mol/L}$), PTX (1 $\mu\text{mol/L}$) and their combination on PR-U87MG cells.

* $P < 0.05$, ** $P < 0.01$ vs DMSO treatment group; $\Delta\Delta P < 0.01$ vs AA or PTX group

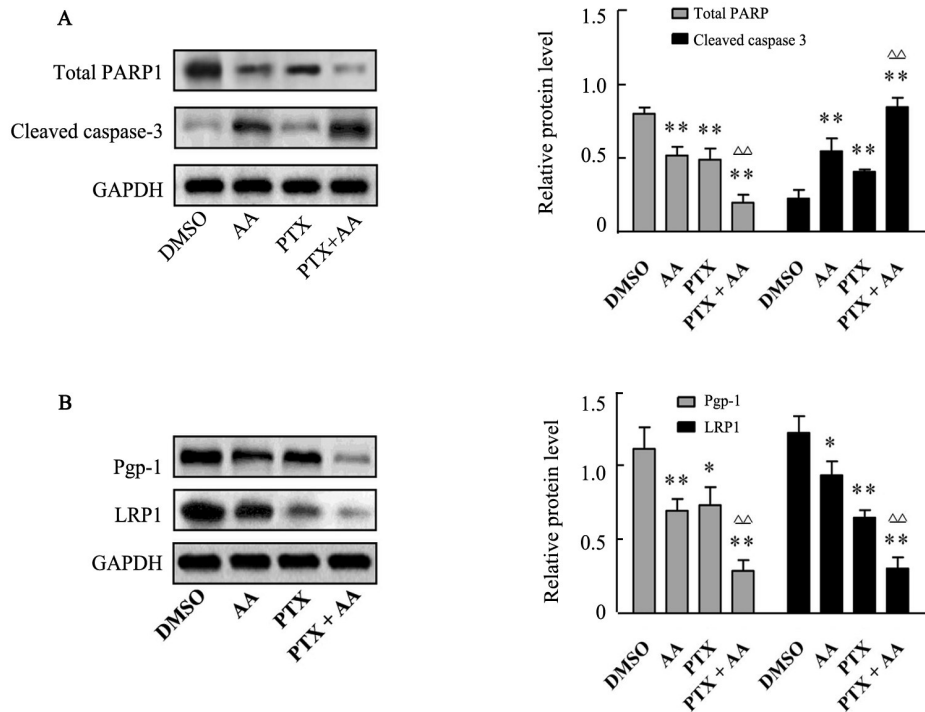
图3 AA对PR-U87MG细胞增殖和凋亡的影响

Fig. 3 Effect of AA on proliferation and apoptosis of PR-U87MG cells

2.4 AA对PR-U87MG细胞凋亡相关蛋白的影响

Western blotting 检测结果(图4)显示,在AA与PTX联合使用组总PARP1的蛋白水平较单独使用对照组明显减少,而caspase 3在两者联合作用后的裂

解水平较单一药物处理组显著增加(均 $P < 0.01$)。联合组Pgp-1和LRP1表达水平较单一药物处理组显著减少(均 $P < 0.01$)。



A: Western blotting analysis of PARP1 and caspase 3 cleavage; B: Western blotting analysis of expression of drug-resistant proteins

Pgp-1 and LRP. * $P < 0.05$, ** $P < 0.01$ vs DMSO group; $\Delta\Delta P < 0.01$ vs AA or PTX group

图4 AA对PTX耐药性胶质瘤细胞PR-U87MG中PARP1表达和caspase 3裂解的作用

Fig. 4 Effect of AA on the expression of PARP1 and cleavage of caspase 3 in PR-U87MG cells

3 讨论

胶质瘤是在我国目前临床上最常见的颅脑肿瘤,约占颅脑肿瘤的30%以上,对人们健康有着很大危害,给社会带来了巨大的经济压力^[3]。近年来,关于AA以及PTX对胶质瘤作用的相关研究^[1-2]已经成为热点。有研究^[4]发现,AA通过诱导胶质瘤细胞凋亡从而抑制其细胞增殖。药物通过作用于癌细胞的蛋白质及DNA,也可抑制癌细胞增殖^[5]。也有相关文献^[6]报道,在临床应用中PTX作为化疗治疗肿瘤的有效药物之一,对脑胶质瘤有着较好疗效。但是关于AA能否增强胶质瘤细胞对PTX的敏感性的研究在国内还尚未报道。在本研究中初步发现,AA对胶质瘤细胞株U87MG细胞凋亡起着促进作用,AA及PTX联用组中耐药性U87MG细胞凋亡相关分子表达较对照组增加,耐药相关蛋白较对照组有所减少。可以看出,AA增强了PTX对U87MG胶质瘤细胞株的活力抑制作用和凋亡促进作用。

AA对U87MG细胞凋亡起着促进作用,AA及PTX联用组中PR-U87MG细胞凋亡相关分子较对照组增加,这与以往学者的研究结果^[7-9]相一致,进一步证实AA增强U87MG胶质瘤细胞株对PTX的敏感性。根据目前研究报道,AA的抗肿瘤活性的机制可能涉及MAPK^[10]、PI3K/Akt^[11]等肿瘤内异常激活的信号通路,这些信号的抑制会导致肿瘤细胞有丝分裂减弱和凋亡增强。另外,通过恢复eNOS/iNOS平衡、内质网应激和炎症水平,AA也会诱导肿瘤细胞凋亡从而发挥抑癌作用^[12-13]。然而,目前关于AA对肿瘤细胞耐药性的影响的相关研究还鲜有报道,其具体机制更不明确。

研究^[14-15]发现,当肿瘤耐药性增加后,多药耐药相关蛋白MRP1表达量显著增加,说明MRP1通过将细胞内药物向细胞外转运直接参与了肿瘤细胞耐药性的形成。LRP分布于体内各种正常组织中,通过阻止以胞核为效应点的药物转运到胞质内以及将进入到胞质的药物转运到运输囊泡隔绝药物作用,以胞吐方式排出药物的方式发挥作用^[16-18]。本研究发现,MRP1和LRP都参与了PR-U87MG细胞耐药性形成,而加AA处理后,这两种蛋白的水平均显著下调,细胞增殖活力减弱,细胞凋亡水平增加。这些结果说明,AA增强U87MG胶质瘤细胞株对PTX的敏感性机制很可能和AA抑制胶质瘤细胞中具有药物排出功能的蛋白(MRP1和LRP)表达有关。然而,AA是通过何种机制来抑制MRP1和LRP蛋白表达的尚不得知。但经查阅相关文献报道^[19-20]后,可在此提出一种猜测为进一步研究提供思路:AA通过某些信号通

路(如ERK MAPK和PI3K/Akt通路)间接下调MRP1和LRP表达。

综上所述,本研究初步发现AA显著促进了耐药胶质瘤细胞中caspase 3酶的激活,抑制了PARP的裂解,同时阻碍了耐药相关蛋白MRP1和LRP的表达,从而加速了U87MG细胞的凋亡,抑制了其增殖活力,最终表现为AA增强了胶质瘤细胞株对PTX的敏感性,其机制还需要进一步从细胞水平和分子水平进行研究探讨。

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