PIBB 生物化学与生物物理进展
Progress in Biochemistry and Biophysics
2008, 35(10): 1161~1167
www.pibb.ac.cn

酪丝亮肽荧光标记物的合成及其在肿瘤治疗靶点研究中的应用 *

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摘要 酪丝亮肽是一种具有抗肿瘤活性的小分子三肽，它可以诱导造成肝癌细胞发生凋亡坏死，从而杀伤肿瘤细胞，但是酪丝亮肽在肝癌细胞的亚细胞定位尚不十分明确．为了达到对酪丝亮肽进行示踪进而观察其亚细胞定位的目的，使用荧光物质(5(6)- 羧基四甲基罗丹明琥珀酰亚胺酯，5(6)-TAMRASE)对酪丝亮肽进行了标记，应用非变性聚丙烯酰胺凝胶电泳、毛细管电泳和荧光分光光度法对标记酪丝亮肽进行纯化和鉴定．并在激光扫描共聚焦显微镜下观察了荧光标记酪丝亮肽在人肝癌 BEL-7402 细胞中的分布．结果显示，合成的酪丝亮肽荧光标记物性质稳定，标记的酪丝亮肽在人肝癌 BEL-7402 细胞的胞浆中呈聚集分布．

关键词 酪丝亮肽，肝癌，荧光标记，激光扫描共聚焦显微镜
学科分类号 R341.6，R979.1，R329.2

自然界存在着大量具有生物学活性的多肽物质，它们在机体的生理与病生理过程中发挥着重要作用，涉及分子识别、信号转导、细胞分化及个体发育等诸多领域．多肽类药物因具有分子质量小、无免疫原性、结构简单等特点，其抗肿瘤活性的研究近年来受到国内外学者的广泛关注[1,2]．酪丝亮肽(tyoserleutide, YSL)是一种具有抗肿瘤活性的小分子三肽，由酪氨酸、丝氨酸、亮氨酸组成，分子质量为 381.42 u，结构式为：

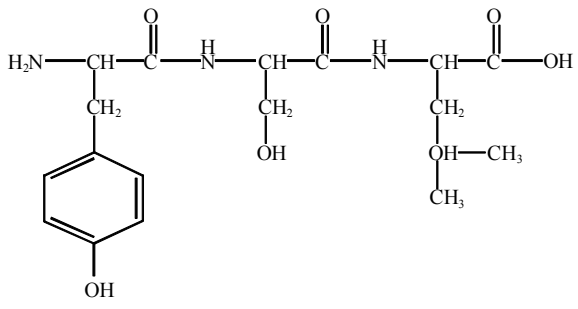

Fig. 1 Molecular structure of YSL

先前研究表明，YSL 对人肝癌 BEL-7402 裸鼠移植瘤具有明显的抑制作用[3]，同时在体外可以明显抑制肝癌 BEL-7402 细胞的生长，造成细胞发生凋亡和坏死[4]．为深入研究 YSL 的作用机制，本文从对 YSL 进行荧光标记示踪的角度出发，应用激光扫描共聚焦显微镜(laser scanning confocal microscopy, LSCM)观察 YSL 在人肝癌 BEL-7402 细胞中的分布，以期为进一步揭示其抗肿瘤的作用机制提供有利的证据．

1 材料和方法

1.1 细胞株及细胞培养

人肝癌细胞株 BEL-7402 购自中国医学科学院肿瘤研究所．细胞用含 10%FBS 的 RPMI-1640 培养基在 37℃，5% CO_2 细胞培养箱中培养．

1.2 YSL 荧光标记物的制备与纯化

将 100 μg 的 YSL(深圳康哲药业有限公司)溶于 200 μl 100 mmol/L 的 Tris-HCl(pH 7.0)，再加入 40 μl 终浓度为 10 mmol/L的 5(6)- 羧基四甲基罗丹

* 国家高技术研究发展计划(863)(2004AA2Z3170, 2005AA2Z3D40)，国家重点基础研究发展计划(2003CCA04300)和教育部重点项目(03007)资助.
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收稿日期：2008-01-27，接受日期：2008-05-31

明琥珀酰亚胺酯(5(6)-TAMRASE, Biotum 公司)荧光试剂，4℃避光过夜反应．收集标记物用无去垢剂的非变性聚丙烯酰胺凝胶电泳(Native-PAGE)进一步纯化．凝胶浓度 20%，电泳缓冲液为 30 mmol/L Tris- 甘氨酸(pH 8.3)，初始电压为 250 V，电泳 2 h 后调整为 180 V 继续电泳 2 h．Native-PAGE 电泳后，用刀片切下三肽区带的凝胶，置于电泳缓冲液中浸泡．泡出液用 0.45 μm 微孔滤膜过滤，冻干备用．

1.3 标记 YSL 纯度和稳定性的鉴定

1.3.1 毛细管电泳鉴定标记 YSL 纯度和稳定性．将 Native-PAGE 后的标记 YSL 采用未涂层熔融石英毛细管(内径 75 μm，河北永年光纤厂)在毛细管电泳仪(TH-2000 型，保定市高新区天惠分离科学研究所) 上进行电泳，并与未标记的 YSL、5(6)-TAMRASE 及 YSL 和 5(6)-TAMRASE 混合物进行毛细管电泳的结果进行比较．毛细管电泳的运行缓冲液采用 50 mmol/L的 NaH_2PO_4-NaOH 缓冲体系(pH 8.0)．电泳前先用 0.1 mol/L氢氧化钠溶液，蒸馏水和运行缓冲液分别冲洗毛细管各 2 min，2 次进样之间再用缓冲液冲洗 2 min．进样压力 4 kPa，进样时间 3 s，检测波长为 200 nm，柱温设为 25℃，分离电压为 14 kV．缓冲液和所有进样溶液在临用前均用 0.45 μm 微孔滤膜过滤．

1.3.2 荧光分光光度法鉴定标记 YSL 稳定性．将标记 YSL 纯化后的 5 个样品每个样品分成 3 份，除其中 1 份在标记纯化后立即进行荧光强度和荧光光谱的测定外其余的 2 份在–20℃条件下分别保存 1 周和 2 周．而后分别在荧光分光光度计(HTIACHI F-4500 型，日本)上测定荧光强度和荧光光谱，观察其随时间变化的情况．选择的激发波长为 545 nm，发射波长为 565 nm.

1.4 标记 YSL 的定量

将荧光标记试剂 5(6)-TAMRASE 的标准品用无血清 RPMI-1640 进行一系列的稀释，在荧光分光光度计上进行荧光强度的测定，选择的激发和发射波长与稳定性鉴定相同．根据测定的结果绘制荧光强度 - 浓度标准曲线，计算出标准曲线的方程．同时，在荧光分光光度计上测出标记 YSL 的荧光强度，按标记试剂 5(6)-TAMRASE 与 YSL 等摩尔反应的关系，将荧光强度代入标准曲线方程计算出待测标记 YSL 的摩尔浓度．

1.5 激光共聚焦显微镜观察标记 YSL 进入细胞情况

将处于对数生长期的人肝癌细胞株 BEL-7402，调整细胞浓度为 1×10^5/ml，接种于圆形盖玻片上，37℃，5%CO_2 细胞培养箱中培养 24 h 后，弃去培养液．药物处理组加入浓度为 26.2 μmol/L 的标记 YSL(相当于 10 mg/L 浓度的未标记 YSL)，对照组加入相同摩尔浓度的游离荧光试剂 5(6)-TAMRASE，37℃，5% CO_2 条件下分别孵育 5、15、30 min 以及 1、2 和 3 h，以激光扫描共聚焦显微镜(Radiance 2000 型 Bio-Rad 公司，美国)检测细胞中的荧光分布．检测条件：激发光为 543 nm，发射滤片为 E570LP，设置一个荧光信号通道和一个普通透射光信号通道，并即时将这 2 个通道通过计算机合成输出．

2 结　果

2.1 YSL 的荧光标记物的制备与纯化

荧光标记 YSL 经 Native-PAGE 纯化后可以与游离的荧光试剂明显地区分开来(图 2).

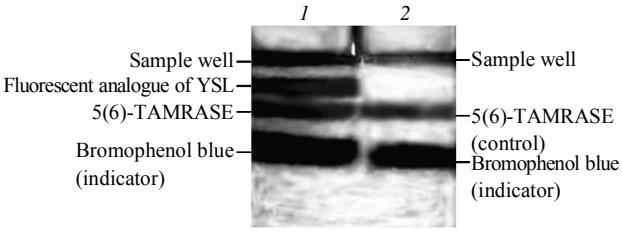

Fig. 2 Native-polyacrylamide gel electrophoresis of YSL fluorescent analogue

The mixture of YSL and 5(6)-TAMRASE, 5(6)-TAMRASE were loaded on the 20% native polyacrylamide gel. The running buffer was 30 mmol/L pH 8.3 Tris-glycine. The YSL and 5(6)-TAMRASE can be separated by native-polyacrylamide gel electrophoresis under 250 V for 2 h continued with 180 V 2 h. *1*: Mixture of YSL and 5(6)-TAMRASE; *2* : 5(6)-TAMRASE.

2.2 毛细管电泳鉴定标记 YSL 的纯度和稳定性

在相同条件下，对纯化后的标记 YSL、游离荧光及未经标记的 YSL 分别进行毛细管电泳．结果表明，电泳图谱的出峰时间分别为 4.95 min (图 3a)、3.92 min(图 3b)和 6.85 min(图 3c)，并且都呈现单一的峰形．对荧光标记 YSL 的反应体系进行毛细管电泳，其中Ⅰ，Ⅱ，Ⅲ 3 个峰的出峰时间分别为 3.64，5.55 和 6.60 min(图 3d)．经过与图 3 对照可得出，荧光标记 YSL 反应体系与单独组分进行的毛细管电泳的出峰时间基本一致，可见图 3d 中Ⅰ峰为游离荧光，Ⅱ峰为荧光标记的 YSL，Ⅲ峰为未标记 YSL．将图 3c、a 和 b 比较可见纯化后的标记 YSL 中基本不含有游离荧光和未标记 YSL．同时将用于图 3c 的荧光标记 YSL 样品在纯化即刻、–20℃的保存条件下保存 1 周、2 周后分

别进行毛细管电泳，结果 3 个时间点的电泳图中未出现出峰时间的变化也未出现纯 YSL 和游离荧光的峰(图 4)．可见纯化后的标记 YSL 在–20℃保存 2 周内是稳定的．

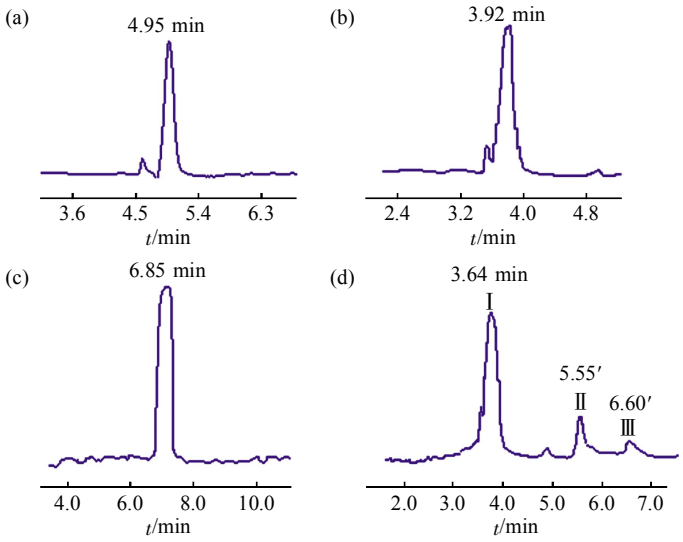

Fig. 3　The analysis results of the retention time by capillary electrophoresis

The purified YSL fluorescent analogue, 5(6)-TAMRASE, YSL and the mixture of YSL and 5(6)-TAMRASE were analyzed by capillary electrophoresis under the condition of running buffer 50 mmol/L NaH_2PO_4-NaOH(pH 8.0), 25℃ 14 kV. The retention time were recoreded at 200 nm .The retention time of YSL fluorescent analogue (a), 5(6)-TAMRASE (b), YSL (c) were 4.95, 3.92, 6.85 min respectively. Among the mixture of YSL and 5(6)-TAMRASE (d) the retention time of 5(6)-TAMRASE, fluorescent analogue and YSL were 3.64 min(Ⅰ), 5.55 min(Ⅱ) and 6.60 min(Ⅲ) respectively which were accordance with the results of (a) to (c).

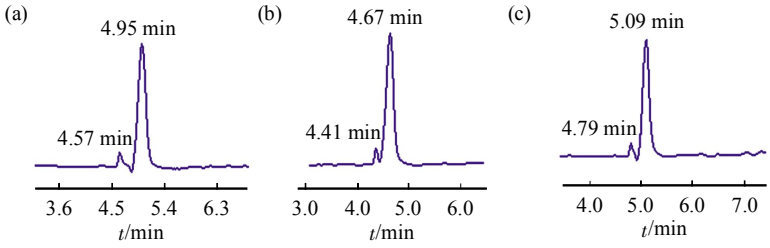

Fig. 4　The retention time changes of the YSL fluorescent analogue at different storage time by capillary electrophoresis

YSL fluorescent analogue was stored at –20℃ for 1 week and 2 weeks respectively after it was synthesized and purified instantly. Then they were analyzed by the capillary electrophoresis under the condition of running buffer 50 mmol/L NaH_2PO_4-NaOH(pH 8.0), 25℃ 14 kV at the end of each storage time point. The retention time of the YSL fluorescent analogue was recoreded at 200 nm. Compared with the retention time of the YSL fluorescent analogue labeled instantly, the retention times of the YSL fluorescent analogue which had been stored for 1 week and 2 weeks had no great changes. (a) Labeled instantly (b) Stored for 1 week (c) Stored for 2 weeks.

2.3　荧光分光光度法鉴定标记 YSL 荧光稳定性

选择 5 例纯化后的标记 YSL 样品在纯化后即刻、纯化后 1 周、2 周检测样品的荧光强度，结果未见荧光强度的明显降低，各组间荧光强度比较未见明显统计学差异($P > 0.05$)(图 5)．标记 YSL 在 545 nm 处激发，荧光光谱出现 2 个峰，分别为 541 nm 和 572 nm，在保存 2 周内未见峰值发生明显的位移，结果见图 6.

2.4　标记 YSL 的定量

经荧光分光光度计分析绘制出的荧光试剂标准曲线回归方程为 $y = 0.0177x - 0.1911$ ($r = 0.995$, $P < 0.01$)．其中 y 为标记荧光试剂的摩尔浓度，x 为该浓度荧光试剂在激发光 545 nm，发射波长为 565 nm 条件下测定的荧光强度，线性范围为 0.1×10^{-3}～5×10^{-3} mmol/L．将标记好的 YSL 在荧光分光光度计上以同样的激发和发射波长测定其荧光强度，根据标准曲线计算出标记 YSL 的浓度．游离荧光试剂的浓度也根据标准曲线计算得出．

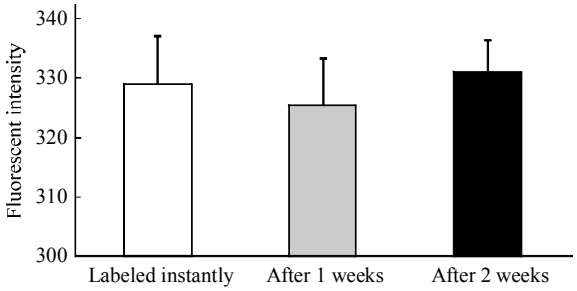

Fig. 5　Fluorescent intensity changes of YSL fluorescent analogue at three different storage time points

The fluorescent intensity of YSL fluorescent analogue was measured by the fluorescent spectrophotometer (HITACHI F-4500) at the excitation wavelength 545 nm after it was synthesized and purified instantly. Then YSL fluorescent analogue were stored at –20℃ for 1 week and 2 weeks respectively. The fluorescent intensities were measured at the end of each store time. Compared with the fluorescent intensity of the YSL fluorescent analogue labeled instantly, the intensities of the YSL fluorescent analogue which had been stored for 1 week and 2 weeks had no obviously decreased.(n=5, P > 0.05).

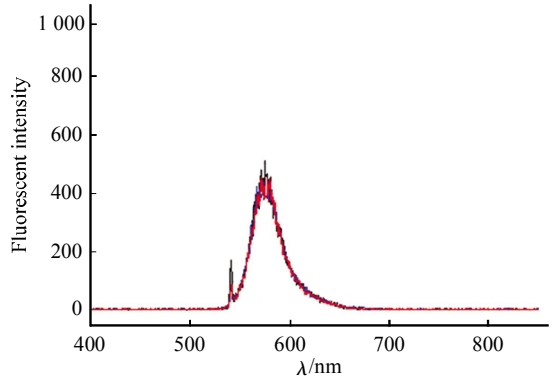

Fig. 6　Fluorescent spectrum changes of YSL fluorescent analogue at three different storage time points

The fluorescent spectrum of YSL fluorescent analogue was recorded by the fluorescent spectrophotometer (HITACHI F-4500) at the excitation wavelength 545 nm after it was synthesized and purified instantly. Then YSL fluorescent analogue were stored at –20℃ for 1 week and 2 weeks respectively. The fluorescent spectrums were recorded at the end of each store time and compared the spectrums with that of the YSL fluorescent analogue labeled instantly. There was no significant changes between these three spectra. Red line: Labeled instantly; Black line: Stored for 1 week; Blue line: Stored for 2 weeks.

2.5　激光扫描共聚焦显微镜观察实验

在 LSCM 观察到标记的 YSL 与单纯的 5(6)-TAMRASE 作用于肝癌 BEL-7402 细胞荧光出现的时间和分布的方式均有所区别. 标记 YSL 与细胞作用 15 min 后即在细胞内可见红色荧光，作用细胞 30 min 后，细胞内的荧光强度有所增强. 作用 1 h 的时候在细胞内可见明显的红色荧光并且呈集中的颗粒状分布，2 h 荧光达到最大值，3 h 后荧光逐渐减弱，但仍在细胞内可见. 单纯的 5(6)-TAMRASE 作用组荧光强度随着作用时间的延长而增加，并且红色荧光均匀弥散地分布在整个细胞中，这与标记 YSL 作用组的分布方式有明显的不同(图 7, 8).

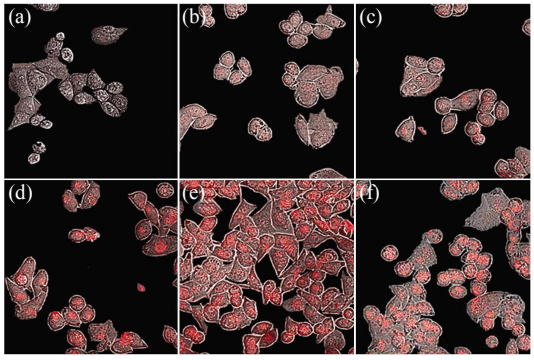

Fig. 7　The distribution of YSL fluorescent analogue within the hepatocellular carcinoma bel-7402 cells observing under the laser scanning confocal microscopy

26.2 μmol/L YSL fluorescent analogue were incubated with human hepatocellular carcinoma bel-7402 cells at 37℃, 5% CO_2 for 5 min, 15 min, 30 min, 1 h, 2 h, 3 h, respectively. The fluorescent signals were collected by the Aron laser 543 nm and the emission filter 570LP under the laser scanning confocal microscopy (Radiance 2000) at the end of each incubation time point. The images were collected by the software Laser sharp 2000 (×600). Each image was merged by the fluorescent image (which represented the YSL fluorescent analogue and the transmission image (which represented the cells outline). (a) 5 min drug exposure. (b) 15 min drug exposure. (c) 30 min drug exposure. (d) 1 h drug exposure. (e) 2 h drug exposure. (f) 3 h drug exposure.

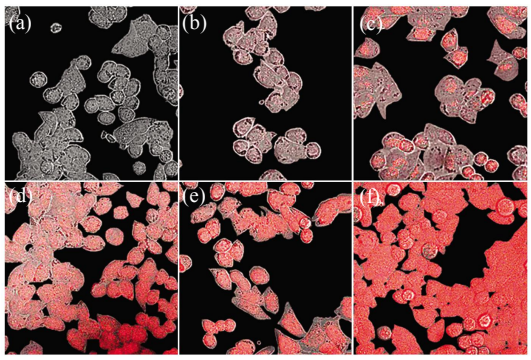

Fig. 8　The distribution of 5(6)-TAMRASE within the hepatocellular carcinoma bel-7402 cells observing under the laser scanning confocal microscopy

26.2 μmol/L 5(6)-TAMRASE were incubated with human hepatocellular carcinoma bel-7402 cells at 37℃, 5%CO_2 for 5 min, 15 min, 30 min, 1 h, 2 h, 3 h, respectively. The fluorescent signals were collected by the Aron laser 543 nm and the emission filter 570LP under the laser scanning confocal microscopy (Radiance 2000) at the end of each incubation time point. The images were collected by the software Laser sharp 2000 (600×). Each images were merged by the fluorescent image (which represented the 5 (6)-TAMRASE and the transmission image (which represented the cells outline). (a) 5 min drug exposure. (b) 15 min drug exposure. (c) 30min drug exposure. (d) 1 h drug exposure. (e) 2 h drug exposure. (f) 3 h drug exposure.

3 讨　论

现今对蛋白质类药物的研究，多集中在将大分子蛋白质变成分子质量较小的多肽，从中寻找活性中心，以达到更好的药效．在生物活性肽的作用机制研究中，一个重要的手段就是对多肽进行标记示踪．多肽的标记示踪方法主要有放射性核素标记和荧光标记两大类[5~8]．相对放射性标记而言，荧光标记对人无害而且实验操作简单、易于开展．目前，国内外在利用荧光标记探针进行多肽的示踪方面已经进行了大量研究工作并且取得了一定的进展．如利用有机荧光染料探针 FITC，AMCA 对小肽进行标记示踪以研究小肽被机体摄取的情况以及小肽转运蛋白质底物的筛选[9, 10]，使用一种新型的荧光标记探针纳米半导体量子点与多肽的巯基结合后用于 G 蛋白偶联受体的研究[11]等．

本研究中的酪丝亮肽(YSL)是一种具有抗肿瘤活性的 3 个氨基酸组成的超短肽，分子质量只有 381.42 u 并且结构中没有复杂的侧链，因此需要寻找可以与小肽游离氨基结合的荧光染料．据文献报道琥珀酰亚氨酯可以和氨基酸的氨基稳定结合[12]．四甲基罗丹明琥珀酰亚氨酯属于罗丹明类的有机荧光染料，具有光稳定性好、对 pH 不敏感、较宽的波长范围和较高的荧光量子产率等优点．因而选用该物质来标记 YSL 的游离氨基以达到示踪的目的．

本研究尝试使用无去垢剂的非变性聚丙烯酰胺凝胶电泳(native-PAGE)对标记的 YSL 进行了分离纯化．Native-PAGE 是在不加入 SDS、疏基乙醇等变性剂的条件下，对保持活性的蛋白质进行分离纯化的方法．未加 SDS 的天然聚丙烯酰胺凝胶电泳可以使生物大分子在电泳过程中保持其天然的形状和电荷，在保证较高分辨率的同时，仍能保持蛋白质和酶等生物大分子的生物活性，能够满足本研究中在分离纯化标记 YSL 的同时需要保持标记 YSL 活性的要求．由于酪丝亮肽含有酚羟基，属于弱酸性，native-PAGE 分离酸性蛋白质时需要使用高 pH 值缓冲液，因此我们选择了 pH 8.3 Tris- 甘氨酸的缓冲体系．实验结果表明，经过 native-PAGE 得到了 YSL 和游离荧光标记试剂 2 条电泳条带从而达到了标记 YSL 与过量的游离荧光试剂分离的目的．

毛细管电泳法是根据被分离物质的荷质比进行分离的方法，因此适用于肽类化合物的分析．本研究应用毛细管电泳法对分离纯化后的标记 YSL 进行了鉴定．由于酪丝亮肽呈弱酸性，而毛细管电泳时电渗流对于 pH 的改变很敏感，且 pH 值的变化亦可引起峰形以及分辨率的改变，因此较高 pH 值的缓冲液对分离酪丝亮肽有利．但是高 pH 值条件(pH > 9.0)会导致样品的快速氧化，故选择电泳时的 pH 值为 7< pH < 9. 电泳的缓冲体系选择了 pH 8.0 NaH_2PO_4-NaOH 缓冲体系，在此体系中电泳电流稳定，而且对分离体系有很好的分离检测效果，这与吕海鸿等[13]的报道结果相一致．综合考虑速度与分离度对运行时间的影响，运行缓冲液浓度选用 50 mmol/L 为最佳浓度，在该浓度条件下出峰时间快，且混合物分离效果良好．

本研究发现，标记的 YSL 在–20℃ 条件下保存 2 周后未发生荧光素与 YSL 解离和荧光强度的下降，这进一步表明我们的标记方法是稳定可靠的．因为 YSL 只有一个游离的氨基，标记荧光物质与 YSL 结合的比例是 1∶1，根据这个性质建立了定量检测标记 YSL 的荧光分光光度法．本研究得出的荧光标准曲线线性范围为 0.1×10^{-3}～5×10^{-3} mmol/L，在浓度超过 7×10^{-3} mmol/L 时荧光强度反而会随浓度的增加而逐渐下降，推测这是由于荧光强度增强到一定程度会出现荧光猝灭的情况而导致的结果．

LSCM 是一种先进的细胞生物学分析仪器，它具有分辨率高、可对活细胞进行显微动态追踪观察的特点．将药物进行荧光标记后可以在 LSCM 下直接观察到药物在细胞内的分布及其与细胞器的特殊结合情况，并可以动态追踪药物在细胞内的代谢过程[14]．我们使用激光扫描共聚焦显微镜进行动态追踪观察到与单纯的荧光试剂 5 (6) -TAMRASE 相比，标记 YSL 能够更快地被肝癌 BEL-7402 细胞摄取并且在细胞内呈现一种聚集分布．因此推测 YSL 可能是在细胞内发挥抗肿瘤作用的，而且最终定位到某个细胞器．目前研究认为多肽的跨膜转运主要涉及以下几种机制[15~18]：a．高特异性受体介导的多肽的内吞；b．带正电荷多肽与细胞表面带负电荷的部位结合后导致细胞膜结构发生改变而引起多肽的内吞；c．由肽转运蛋白等载体介导的多肽的跨膜转运；d．非特异性的细胞对多肽的内吞．本研究观察到了 YSL 最终被转运到了细胞内部，但是 YSL 究竟是采用哪种方式进入细胞还有待于进一步的实验加以确证．

本研究建立的荧光标记 YSL 的方法不仅可以应用于 YSL 抗肿瘤作用机制的进一步深入研究，同时还为其他类似的小分子肽类物质的荧光标记示

踪研究提供了一个有利的工具.

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Synthesis of Tyroserleutide Fluorescent Analogue and Its Application on The Target Research of Antitumor Therapy*

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Abstract Tyroserleutide (YSL) is a new anticancer polypeptide developed recently. It can induce hepatocellular carcinoma cells apoptosis and necrosis. However, the subcellular targets of YSL in the hepatocellular carcinoma cells are not known very well. Therefore, a fluorescent bioconjugate of 5-(and-6)-carboxytetramethylrhodamine, succinimidyl ester (5 (6)-TAMRASE)-YSL) was synthesized and purified by native polyacrylamide gel electrophoresis and capillary electrophoresis. After stability was assessed by capillary electrophoresis and fluorescent spectrophotometric assay, concentration of this conjugate was quantitated by fluorescent spectrophotometric assay. Then subcellular distribution of 5(6)-TAMRASE labeled YSL were investigated by laser scanning confocal microscopy to gain a better understanding of the anticancer mechanism of YSL *in vitro*. The experimental results showed that the 5(6)-TAMRASE can conjugate with YSL stably. When incubated with human hepatocellular carcinoma BEL-7402 cells, 5 (6)-TAMRASE labeled YSL could entered the cells in 15 min, and mainly concentrated in the cytoplasm of BEL-7402 cells. Then the fluorescent intensity of 5(6)-TAMRASE labeled YSL which indicated the concentration of them reach the maximum until incubated with the cells for 2 h. After that it began to go down. The 5(6)-TAMRASE could also enter the hepatocellular carcinoma BEL-7402 cells, but it distributed in the whole cell and the intensities had no decrease within 3 h. The results suggest that the distribution pattern of fluorescent labeling YSL is different from that of 5 (6)-TAMRASE. The subcellular distribution pattern of 5 (6)-TAMRASE labeled YSL was ascribed to YSL and could represent that of YSL. In conclusion, YSL located at the cytoplasm of hepatocellular carcinoma BEL-7402 cells and distributed intensively.

Key words tyoserleutide, hepatocellular carcinoma, fluorescence labeling, laser scanning confocal microscopy

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*This work was supported by grants from Hi-Tech Research and Development Program of China(2004AA2Z3170, 2005AA2Z3D40), National Basic Research Program of China (2003CCA04300) and Important Project of Ministry of Education of China(03007).

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Received: January 27, 2008 Accepted: May 31, 2008