DNA 纳米技术在凝血酶适配体分子设计中的应用进展

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2018-08-28 收稿, 2018-10-07 修回, 2018-10-09 接受, 2018-11-23 网络版发表

摘要 DNA纳米技术是纳米生物技术的一个重要研究领域, 近年来发展迅猛. 通过合理的设计, 利用DNA或其他核酸的分子性质构建出可操控的新型纳米尺度的结构或机器, 在生物医学应用领域中有广泛应用. 本文介绍了利用DNA纳米技术对凝血酶适配体结构合理设计的相关进展.

关键词 DNA 纳米技术, 凝血酶, 可逆调控, 核酸适配体, 二价适配体

抗凝治疗在预防和治疗血栓栓塞性疾病中发挥重要作用. 目前常用的抗凝剂存在以下主要缺点: 非胃肠道给药、容易导致出血、需要实验室监测以调整剂量、肝素诱导血小板减少症及骨质疏松症等等. 除肝素过量时可用鱼精蛋白对抗外, 其他抗凝药物无特异性的对抗药物, 不利于不良反应的消除. 对低分子肝素引起的出血, 鱼精蛋白仅部分有效^[1]. 新一代抗凝药中, 口服直接凝血酶抑制在使用过程, 依然存在着一一定的出血副作用, 尤其在使用剂量较大时, 出血和凝血功能障碍都是不可忽视的严重问题^[2]. 由于缺乏针对这些药物的解毒药, 在严重出血情况下, 将无法迅速逆转服药患者的凝血功能障碍, 甚至会威胁到他们的生命. 这些缺点限制了上述药物的临床应用, 增加了病人的发病率和死亡率. 帮助使用口服抗凝药患者恢复生理性止血的途径有4种: (1) 加快抗凝药物的清除速度; (2) 置换出由抗凝药物所抑制的凝血因子; (3) 使用广谱的止血药物; (4) 使用中和抗凝药物活性的特殊解毒药^[3]. 因此, 开发新的抗凝药物, 既能更有效抗凝, 又能克服现有药物的缺点一直是该领域的重点和难点. 近年来, 随着对凝血的潜在分子机制的更多了解、重组DNA技术的进步、

药物化学合成技术的提高等, 已经加速了抗凝新药的研究步伐. 直接凝血酶抑制剂(direct thrombin inhibitor, DTI)和Xa因子(FXa)抑制剂是其中最具希望和代表性的抗凝新药, 成为近年来研究的热点.

凝血酶是一种多功能丝氨酸蛋白酶, 在凝血过程中起到关键性作用, 通过特异性水解血纤维蛋白原为纤维蛋白而发挥凝血作用^[4,5]; 另一方面, DTI^[6]是一组不需要辅助因子参与直接抑制凝血酶活性的小分子, 其既能抑制游离的也能抑制与血凝块结合的凝血酶. 而且, 它们不与血浆蛋白结合, 使其药理学及药动学特征具有可预测性. 它们不与血小板第4因子(PF4)发生中和反应, 因而无血小板减少的危险. 它们不仅有抗凝血功能, 还能抑制血小板的活化. 因此从理论上讲, DTI与其他抗凝药相比, 其药理作用及药动学特点更优越^[7,8].

在凝血酶抑制剂的研究中, 核酸适配体的应用取得了一定进展. 核酸适配体(aptamer)是一种可以通过体外筛选技术(指数富集的配基系统进化技术, SELEX)得到的^[9-12], 对特定靶标物质具有强亲和力和高特异性的一小段寡核苷酸序列或者短的多肽. 核酸适配体的靶分子范围广, 可以识别包括离子、无

引用格式: 唐林林, 黄承志, 左华, 等. DNA 纳米技术在凝血酶适配体分子设计中的应用进展. 科学通报, 2019, 64: 1018–1026

Tang L L, Huang C Z, Zuo H, et al. DNA nanotechnology in molecular design of thrombin aptamers (in Chinese). Chin Sci Bull, 2019, 64: 1018–1026, doi: 10.1360/N972018-00882

机和有机小分子、蛋白质和活细胞等靶标,具有化学稳定性好、易保存等优点^[13].核酸适配体与对应的靶标物质的识别结合是凭借氢键作用、范德华力、静电作用或者碱基间的互补配对作用而形成特殊的空间结构^[14],这种空间结构可以稳定地与靶标物质结合^[15~17],其结合强度高,解离常数(K_d)最高可达到 $\mu\text{mol/L} \sim \text{pmol/L}$ 级^[18~24].因此,核酸适配体能特异性地对相应的靶标物质产生高的亲和力并影响其生物活性.至今已产生了很多适配体,部分已经处于临床研究或者作为药物上市^[17,25].

目前, α -凝血酶的适配体主要有2种,它们分别结合在凝血酶的不同位点.一种含有15个特异性碱基(TBA15: GGT TGGTGTGGTTGG)的序列(图1(a)),它与凝血酶的纤维蛋白原识别位点特异性结合,结合作用弱(K_d , $\sim 100 \text{ nmol/L}$),但抗凝作用强^[26~28];另一种是含有29个特异性碱基(TBA29: AGTCCGTG GTAGGGCAGGTTGGGGTGACT)的序列(图1(b)),它与凝血酶的肝素识别位点相互作用,结合作用强(K_d , $\sim 0.5 \text{ nmol/L}$),但抗凝作用弱^[29,30].Pagano等人^[31]对TBA15的序列进行修饰得到mTBA15(3'GGT5'-5'TGGTGTGGTTGG3'),发现其与凝血酶的纤维蛋白原识别位点也能特异性结合,并且提高了适配体与凝血酶结合的亲和力和稳定性.

1 一价适配体作为凝血酶可逆抑制剂的研究

凝血酶一价适配体TBA15(GGT TGGTGTGGTTGG)是由体外筛选技术(SELEX)筛选而得到的.由于

它与凝血酶的纤维蛋白原识别位点特异性结合,可以抑制凝血酶的活性,因此传统凝血酶适配体结构的设计主要是围绕TBA15进行结构修饰,而构建出基于一价适配体TBA15的可逆抑制剂.

凝血酶适配体(TBA15)在一价金属钾离子的作用下可形成G-四联体(G-quadruplex),与 α -凝血酶结合并抑制其活性.此外,在适配体的互补链等辅助链或某些小分子的加入后,凝血酶活性能得以恢复^[32~34].比如,Dittmer等人^[35]尝试用修饰过的TBA15形成的分子机器,结合和释放凝血酶,达到可逆抑制凝血酶活性的目的.如图2所示,I中红色部分的15个碱基序列是能亲和识别凝血酶的核酸适配体,在一价金属钾离子的作用下,形成G-四联体结构从而与凝血酶结合. II中蓝色的碱基序列与适配体的碱基序列部分互补(在红色碱基序列的3'端修饰荧光基团,蓝色碱基序列的5'端修饰荧光猝灭基团),在只有凝血酶存在的情况下,加入蓝色碱基序列,此时溶液中DNA之间的结合状态逐渐变为III,荧光基团和猝灭基团接近,荧光被猝灭.向溶液中加入绿色的碱基序列时,其能完全与蓝色碱基序列互补,从而置换出单链的红色碱基序列的适配体,在一价金属钾离子的作用下,适配体重新形成G-四联体的结构并与凝血酶结合,此时荧光基团与荧光猝灭基团远离,荧光再次出现.这样通过荧光的猝灭与恢复去指示适配体结合凝血酶和释放凝血酶之间的可逆循环过程. Kim等人^[36]通过偶氮苯小分子修饰的核酸探针,利用偶氮苯分子在可见光和紫外光照射下的顺式-反式分子构型变化,可逆地结合TBA15,从而对凝血酶产生可逆的抑制作用.将偶氮苯小分子修饰在与TBA15适配体互补的cDNA序列内,然后通过聚乙二醇(PEG)将偶氮苯修饰的cDNA与TBA15相连接(图S1).利用偶氮苯分子的顺式-反式光异构化,在可见光照射下,由于TBA15与反式偶氮苯构象中的cDNA杂交,使TBA15不能结合凝血酶.然而,当紫外光照射时,通过反式-顺式的构象转换而诱导TBA15和cDNA杂交双链解链,使TBA15暴露并重新结合凝血酶并抑制其活性.可以看出Dittmer等人^[35]和Kim等人^[36]分别巧妙地通过分子机器和光敏的分子结构,实现了可逆调控凝血酶的活性,在保留TBA15适配体功能的基础上,又增加了新的调控功能. TBA29几乎对凝血酶活性无抑制作用,然而Shiang等人^[30]通过实验发现将TBA29修饰在金纳米颗粒上,形成的

图1 (网络版彩色)TBA15(a)和TBA29(b)分子的二级结构,两者在钾离子的作用下均形成G-四联体

Figure 1 (Color online) The secondary structures (G-quadruplex) of TBA15 (a) and TBA29 (b) formed in the presence of potassium ions

图2 在凝血酶存在下, 基于TBA15 适配体的分子机器的工作循环图. AP: 修饰过的TBA15 适配体, 在 3'端带有荧光基团; TB: α -凝血酶; AP-TB: 适配体与凝血酶结合的复合物; Q: 与AP部分互补配对, 5'端带有荧光淬灭基团; R: 与Q能完全互补配对

Figure 2 Representation of the operation cycle of the aptamer-based molecular machine in the presence of thrombin. AP: the modified TBA15 aptamer with fluorescent group at 3'-DNA; TB: α -thrombin; AP-TB: the aptamer-thrombin complex; Q: partially complementary to AP with a fluorescence quenching group at the 5'-DNA; R: completely complementary to Q

TBA29-AuNPs能高效地对凝血酶活性产生抑制, 从而抑制纤维蛋白原转化为纤维蛋白. 加入cTBA29-AuNPs (cTBA29是TBA29的互补链)后使cTBA29-AuNPs与TBA29-AuNPs相结合, 从而释放出凝血酶, 恢复凝血酶活性(图S2).

2 二价或多价适配体作为凝血酶抑制剂的 研究

传统凝血酶适配体结构的设计主要是围绕一价适配体, 但是由于调控凝血酶的活性需要适配体同时具有很好的亲和力和特异性, 这使基于一价适配体的技术的应用受到较大限制. 一方面, 依赖现有 SELEX 技术做更多筛选工作; 另一方面, 如何适宜利用现有适配体尤为重要. 因此, 可选择具有独立稳定结构的多个适配体代替一价适配体, 以蛋白作为识别对象, 从而可以产生多种适配体的作用. 多价作用与一价作用的不同之处在于多价作用涉及与多个配体结合, 比如小分子、低聚糖、蛋白质、核酸、脂类; 或与这些分子聚集; 与靶标的多个结合口袋或者受体结合, 如蛋白、病毒或者细菌. 在生物系统中, 多价随处可见, 比一价有更多优势^[37]. 多价作用比一价作用具有更强的结合力^[38,39], 多价可以产生构型的协同作用, 当其中1个单体从靶标上解离后,

另1个同样能结合到同一靶标的单体能增加它再次与这一靶标结合的机会. 因此, 目前凝血酶适配体结构的设计主要是基于二价适配体的结构修饰.

Kim等人^[40]用二价适配体作为凝血酶抑制剂, 并同时加入辅助链恢复凝血酶的活性. 作为潜在的抗凝剂, 只有TBA15对凝血酶具有酶抑制作用, 因为它占据纤维蛋白原结合位点. 然而, TBA15与凝血酶位点的结合力很弱, 导致对它抗凝作用的研究受到很大局限. 为了使适配体对凝血酶具有更好的亲和力和选择性, 通过亚磷酸胺连接TBA15(或15 Apt)和TBA29(或27 Apt, 解离常数只有0.7 nmol/L左右), TBA29与凝血酶的肝素识别位点相互作用, 结合作用强, 可以使TBA15与凝血酶解离扩散到溶液中后受到TBA29制约, 迅速回到与凝血酶结合的状态. 加入与TBA15互补的链, 可以恢复凝血酶的活性, 作为解决抗凝副作用的解毒剂(图S3). 在Kim等人^[40]的基础上, Tian等人^[41]利用偶氮苯小分子衍生物作为光触发的分子开关, 与TBA15和TBA29之间起连接作用的端粒DNA进行可逆地识别结合, 进而实现可逆调节凝血酶的活性. 在可见光的照射下, 此偶氮苯小分子为反式构象, 与端粒DNA相结合并压缩端粒DNA的形态, 使TBA15和TBA29之间的距离减小, 不能起到同时结合凝血酶两个识别位点的作用, 从而对凝

血酶活性的抑制效果显著减弱. 在365 nm紫外光的照射下, 此偶氮苯小分子为顺式构象, 小分子不与端粒DNA结合, 使端粒DNA从压缩状态变为伸展状态, TBA15与TBA29之间的距离增大, 可以同时结合凝血酶的2个识别位点, 大大增强了对凝血酶活性的抑制效果(图3). 此外, 袁荃课题组^[42,43]结合纳米金和近红外光的应用可逆调控凝血酶活性. 将巯基标记的TBA15修饰在金纳米棒上, 金纳米棒被固定在磁珠表面, 随着TBA29和凝血酶的加入, TBA15和TBA29可以分别结合凝血酶的2个结合位点, 此时位于TBA15的3'端配体1与TBA29的5'端配体2相互靠近, 邻近表面杂交在一起, 凝血酶、AuNRs-TBA15

和TBA29三者形成闭环结构, 能高效地抑制凝血酶的活性, 阻止纤维蛋白原转化为纤维蛋白. 近红外光(808 nm)照射时, 金纳米棒能有效地将入射光转化成热, 从而干扰配体1与配体2的杂交, 破坏闭环结构, 导致捕获的凝血酶被释放, 凝血酶的活性得以恢复, 可以重新将纤维蛋白原转化为纤维蛋白(图4).

二价适配体的合理设计, 不仅仅包括选择适配体本身, 另外的重要之处在于正确选择两个适配体之间的连接部分. 连接部分的作用之一在于使2个适配体不重叠, 保持与蛋白结合的合理距离, 另一方面, 可以使两个适配体维持适宜的空间取向, 最终有利于降低适配体与蛋白结合的解离常数(K_d), 因此适

图3 (网络版彩色)基于二价适配体的小分子光控可逆抑制凝血酶活性. Telomere DNA序列为GGGTTAGGGTTAGGGTTAGGG

Figure 3 (Color online) Small-molecule-triggered and light controlled reversible regulation of thrombin activity. Telomere DNA sequence: GGGTTAGGGTTAGGGTTAGGG

图4 (网络版彩色)结合纳米金生成闭环结构以及利用近红外光可逆调控凝血酶活性

Figure 4 (Color online) Thrombin inhibition by the closed-loop Au-aptamer structure and subsequent release of thrombin by NIR light stimulation

配体的连接部分在调控蛋白活性过程中发挥重要作用。作为二价适配体的连接部分,与polyA相比^[44],polyT柔性更大,5个碱基T的序列长度和柔韧性使该二价适配体能同时和凝血酶的2个不同识别位点(2位点距离约为3.4 nm)结合(图S4)^[45]。

配体和受体之间高的结合力的产生,不是因为2个适配体中的任意1个适配体的亲和力提高,也不是因为结构单元与蛋白之间直接作用的增强,而是因为框架连接的适配体在空间方向上调整到了各自结合位点的最佳位置,这样可以有效地提高结合的亲和力^[46]。随着DNA纳米技术的发展,人们发现可以通过构建DNA纳米结构在纳米尺度空间范围内实现对凝血酶活性的精确控制,这样可以有效地提高二价适配体的功能性。Rinker等人^[47]利用DNA砖块(DNA tile)自组装DNA纳米结构,将凝血酶的2个一价适配体TBA15和TBA29整合到此纳米结构上,使TBA15与TBA29刚性连接,最终证实当TBA15与TBA29刚性连接距离为5.3 nm时得到的DNA纳米结构与凝血酶的结合能力最强(图5)。随后,在Rinker等人^[47]的基础上,Chao等人^[48]利用DNA双交叉砖块(double crossover motif)构建出外形类似“钳子”的DNA机器,凝血酶的2个适配体TBA15和TBA29分别修饰在“钳子”的两端,可以通过加入燃料链(fuel)将“钳子”张开,TBA15和TBA29相互远离,释放出结合的凝血酶。随着去燃料链(antifuel)的加入,“钳子”开

始收拢使TBA15和TBA29相互靠近,重新开始结合凝血酶。这样就能通过“钳子”的张开和收拢可逆地调控凝血酶活性(图6)。

3 DNA纳米技术用于凝血酶的最新进展

随着DNA纳米技术的不断发展,DNA折纸术(DNA origami)这一新颖的DNA自组装方法也广泛被应用研究^[49]。利用此自组装方法,Li等人^[50]用M13噬菌体基因组DNA构建出一种自组装的矩形DNA折纸层(rectangular DNA-origami sheet),并将导致血液凝固的凝血酶连接到这种DNA折纸层上。随后,他们将DNA紧固件(DNA fastener)连接到这种矩形DNA折纸片的长边上,从而形成一种内表面上携带着凝血酶的管状纳米机器人(tubular nanorobot)。当这些DNA紧固件识别并结合到核仁蛋白(肿瘤血管细胞表面上存在的一种特异性蛋白)上时,它们就会脱落下来。管状纳米机器人打开,从而暴露它携带的凝血酶,凝血酶会导致此部位的血液凝结,形成的血凝块会导致肿瘤细胞坏死,从而起到抗癌作用(图7)。

4 总结与展望

关于凝血酶研究尽管取得了相关进展,同时亦展现了DNA用于抗凝作用的应用前景,但该领域的研究尚存在诸多问题。(1)对于一价适配体,DNA对凝血酶的亲和力和选择性不够高;(2)已有研究中,

图5 DNA砖块上间隔不同距离的二价适配体与凝血酶的结合。(a) 刚性DNA瓦片(蓝色)上的TBA29和TBA15适配体(红色和绿色)在空间上分离,与凝血酶(橙色)进行二价结合。(b) TBA29(红色)和TBA15(绿色)从DNA砖块螺旋中突出,并分别相距不同距离(2, 3.5, 5.3和6.9 nm)

Figure 5 DNA tile based self-assembled divalent aptamers for protein binding. (a) A cartoon showing a rigid DNA tile (blue) that can spatially separate two ligands (red and green) at a controlled distance with each ligand attached to a different part of thrombin (orange) for bivalent binding. (b) 5-Helix-bundle (5HB) DNA structure with TBA29 (red) and TBA15 (green) protruding out of the tile helices and being separated at a distance of 2, 3.5, 5.3, and 6.9 nm

图6 DNA分子机器介导的二价适配体可逆结合凝血酶

Figure 6 Reversible regulation of thrombin binding affinity by a DNA machine

部分DNA机器使用数条DNA链,其成本高、结构大而且复杂;(3)在引入其他金属材料过程中,未考虑金属材料在体内的毒性和蓄积;(4)部分设计需要引入紫外光才能恢复凝血酶活性,长时间的紫外照射会

对人体造成伤害;(5)对于已有的二价适配体,连接具有很大柔性,尽管这种结构很简单,但是导致大量熵损失,并且当使用刚性的连接时,又没有考虑到适配体的空间排列方向而产生熵损失,所以亲和力也不会有明显提高。

多价适配体构建的结构所产生的亲和力和特异性的提高是通过多价适配体连接配体的结构单元的有效性来实现的,其决定了配体的位置和空间方向来结合靶标分子。然而目前没有通用规则来设计通过多价配体构建的配体,如何提高适配体的亲和力是适配体研究领域的首要问题,而使用预先组织的,刚性多价的结构是一种潜在的解决方法。

可以预见,随着对凝血酶多价适配体的研究和合理构建,针对抗凝药物和解毒药的研究将逐渐从结构上的设计扩展为功能与应用,我们期望在该领域的研究能为开发新的抗凝药物,其既能更有效抗凝,又能克服现有药物的缺点奠定理论基础。

图7 凝血酶功能化DNA纳米机器人的设计与表征。(a) DNA折纸构建装载凝血酶的纳米机器人,并将其重新配置成1个矩形的DNA层以响应核仁素结合。(b) 原子力显微镜表征DNA纳米机器人结构关闭状态(左)和张开状态(右)

Figure 7 Design and characterization of thrombin-functionalized DNA nanorobot. (a) Construction of thrombin-loaded nanorobot by DNA origami, and its reconfiguration into a rectangular DNA sheet in response to nucleolin binding. (b) DNA nanorobots were examined by atomic force microscopy (AFM) and representative images of closed (left) and opened states (right) are shown

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补充材料

图 S1 基于 TBA15 适配体的光敏 DNA 纳米结构可逆抑制凝血酶活性

图 S2 TBA29-AuNPs 可逆抑制凝血酶活性的示意图

图 S3 一价适配体和二价适配体的工作原理

图 S4 通过 5 个碱基 T 相连的凝血酶适配体

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Summary for “DNA 纳米技术在凝血酶适配体分子设计中的应用进展”

DNA nanotechnology in molecular design of thrombin aptamers

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The field of DNA nanotechnology has had a remarkable impact on nanobiotechnology, and has been developed rapidly. New manipulatable nanoscaled structures or machines constructed by DNA molecules are widely used in biomedical field. An interesting goal of DNA nanotechnology is to assemble DNA nanostructures to display multivalent interactions, which are characterized by simultaneous binding of multiple ligands on one biological entity to multiple receptors on another. With well-established DNA modification methods, multivalent aptamers targeting binding entities have been developed.

Thrombin is a coagulation protein that plays a rather vital role in the coagulation cascade—converting soluble fibrinogen into insoluble strands of fibrin. Therefore, thrombin is usually considered an important target when searching for anti-coagulants and antithrombotics to interfere with blood coagulation. Among thrombin inhibitors, aptamers have shown great advantages. Aptamers are short DNA or RNA strands that can specifically bind to proteins and inhibit their activities. They can be selected using a technique known as SELEX (systematic evolution of ligands by exponential enrichment). Aptamers specifically recognize and bind to various target molecules (ions, small organic, inorganic molecules, proteins or living cells) with dissociation constants (K_d) in the picomolar to nanomolar range by folding into unique secondary or tertiary structures that accommodate the target's molecular structure. Two thrombin aptamers bind to two different sites of thrombin. The 15-mer thrombin-binding aptamer (TBA15: GGTGGTGTGGTTGG) having a G-quadruplex structure in the presence of potassium ions can recognize the fibrinogen-binding exosite of thrombin *via* a T-loop and inhibit the thrombin activity but the binding affinity is modest (K_d , ~100 nmol/L). The 29-mer thrombin-binding aptamer (TBA29: AGTCCGTGGTAGGGCAGTTGGGGTGACT) recognizes the heparin-binding exosite of thrombin and exhibits weak inhibitory capability toward thrombin, but has strong binding affinity (K_d , ~0.5 nmol/L). Traditionally, the molecular design of thrombin aptamers focuses on the structural modification of TBA15, and in some designs, the activity of thrombin can be recovered by introduction of complementary strand of aptamers or some small molecules. Therefore, many reversible inhibitors have been constructed based on aptamer TBA15. However, the application of monovalent aptamers-based technology is limited due to the low affinity of monomers. With the quest to develop new antithrombotic agents stimulated by clinical needs and advances in biotechnology, multivalent aptamers with independent stable structures are developed to improve the binding capabilities. In multivalent interactions, when one of the monomers dissociates from the target, another monomer, which can also bind to the same target, can increase its opportunity of binding to the target again. Present molecular designs of thrombin aptamer are mainly based on the structural modifications of the divalent aptamers. With the development of DNA nanotechnology, the precise control of thrombin activity can be achieved by constructing new DNA nanostructures, which can effectively improve the functionality of the bivalent aptamers. It can be predicted that the studies on anticoagulants will gradually expand from structural design to practical applications. In this review, monovalent and divalent thrombin aptamers are discussed, and finally, a perspective on the future directions of this field is presented. We hope this review may help researchers to improve techniques to develop ideal anticoagulants, with potential benefits including high efficacy, safety, rapid onset of action and no requirement for therapeutic monitoring.

DNA nanotechnology, thrombin, reversible regulation, aptamer, bivalent aptamer

doi: 10.1360/N972018-00882