Biochemical-activity studies of NGR-peptide-drug conjugates for targeted tumour therapy

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Introduction

Chemotherapy has a great importance in the treatment of cancer patients. However, the vast majority of chemotherapeutic drugs used today can cause serious side effects. Targeted tumour therapy may offer an opportunity to overcome this problem by conjugating chemotherapeutic drugs to tumour specific homing peptides. NGR (Asn-Gly-Arg) peptides received particular interest when phage display libraries were used to identify non-RGD integrin binding motifs. Among the non-RGD peptides the NGR motif was the most frequent one that showed integrin binding properties.[1] However, it has been found that peptides containing the NGR motif, are not integrin ligands, but specifically recognize Aminopeptidase N (CD13) receptor isoforms that are overexpressed in tumour vasculature and on some tumour cells.[2] In addition, these sequences prone to spontaneous decomposition through a succinimide-ring formation, forming isoAsp and Asp derivatives which is strongly influenced by the structure. The resulting isoaspartyl (isoDGR) derivatives are recognized by the members of RGD-integrin family that are essential for tumour metastasis.[3] Hence NGR peptides are of high interest, due to their simultaneous and selective targeting of CD13 and RGD-integrin receptors, therefore potential applications in dual drug targeting.

Results and Discussion

Based on the literature and our earlier work, we have chosen seven small cyclic NGR peptides as targeting molecules: c[KNGRE]-NH₂, c[NleNGRE]-NH₂, c[GNSarRGK], Ac-c[CNGRC]-NH₂ and H-c[CNGRC]-NH₂, and also the thioether bond containing c[CH₂-CO-KNGRC]-NH₂ and c[CH₂-CO-NGRC]-NH₂. [4,5,6] Using these small cyclic NGR derivatives as homing moieties, we designed, synthesised and characterized novel cyclic NGR peptide-drug conjugates with a well-known chemotherapeutic agent, daunomycin (Dau). Since sequences of certain peptides allowed different conjugation sites for Dau, where it was possible, not only the C-terminal elongated, but also branched variants were prepared. Because the efficient internalization of the target receptors is questionable, therefore an extracellular MMP-2 enzyme labile spacer (GPLGVRG) was incorporated between the homing peptide and drug molecule to ensure selective drug release. It is believed that the released drug or its active metabolite can enter the cells by diffusion afterwards, that make them suitable for targeted tumour therapy. The following conjugates were prepared (compound 1-9).

Figure 1

Linear peptides were synthesized on Rink Amide MBHA resin by Fmoc chemistry. To avoid side reactions the aminoxyacetyl group was blocked with isopropyliden protection. The cyclization through thioether bond formation was carried out in TRIS buffer (0.1 M, pH: 8.1), followed by deprotection of the aminoxyacetyl

group with 1 M methoxylamine in NH₄OAc buffer (0.2 M, pH: 5.0). The cyclization of the KNGRE, NleNGRE and GNSarRGK containing peptides were done in solution, through in situ active ester formation. Finally, the daunomycin was conjugated *via* oxime bond formation in NH₄OAc-buffer in all cases.

The cytotoxicity effect of the novel cyclic NGR peptide-Dau conjugates were examined *in vitro* on CD13 positive HT1080 (human fibrosarcoma) and KS (Kaposi's sarcoma) and, as a CD13 negative control, on HT-29 (human colon adenocarcinoma) cell lines using MTT assay. All the three cell lines express RGD integrins and MMPs.

We theorised that the metabolite generated from the MMP cleavable spacer enters the cells *via* diffusion. This is supported by the fact, that both the spacer (Dau=Aoa-GPLGVRG-OH) and metabolite (Dau=Aoa-GPLG-OH) show high activity on all cell lines. Also, a clear difference could be detected in conjugates with different conjugation sites: conjugates built through the side chain of Lys (5,8) have better anti-tumour activity and the longer treatment time resulted in a better CD13 selectivity, compared to the conjugates which were elongated through their C-terminal (4,6). Moreover, it was reported, that N-acetylation of CNGRC would impair the biological activity [7]. However, in our case (Compound2 and 3) acetylation highly enhanced the *in vitro* effect on all cell lines.

Nevertheless, more metabolism and cell uptake studies are needed and are in progress to determine the exact mechanism of action of the conjugates.

Table 1

Stability		Compounds	24 h IC ₅₀ (μM)			72 h IC ₅₀ (μM)		
24 h	72 h		KS (CD13+)	HT1080 (CD13+)	HT-29 (CD13-)	KS (CD13+)	HT1080 (CD13+)	HT-29 (CD13-)
(Asn/isoAsp/Asp)	(Asn/isoAsp/Asp)							
0/0/100	0/0/100	1	>>50	>>50	>>50	>>50	37.8 ± 0.7	>>50
0/0/100	0/0/100	2	2.9 ± 1.2	2.7 ± 1.0	2.9 ± 0.9	3.5 ± 1.3	2.7 ± 1.1	5.0 ± 1.2
0/0/100	0/0/100	3	22.5 ± 0.5	14.0 ± 0.2	7.5 ± 0.7	26.8 ± 0.1	14.0 ± 0.2	15.8 ± 0.4
0/100/0	0/100/0	4	11.3 ± 0.3	10.5 ± 0.3	12.6 ± 0.4	14.0 ± 0.3	7.9 ± 0.8	28.7 ± 0.4
12/0/88	0/0/100	5	7.3 ± 0.1	5.7 ± 0.7	11.2 ± 0.5	11.8 ± 0.2	6.4 ± 0.9	17.6 ± 0.8
100/0/0	100/0/0	6	12.5 ± 0.9	13.1 ± 0.1	16.2 ± 0.9	18.9 ± 0.1	10.7 ± 0.2	16.3 ± 0.6
84/0/16	58/0/42	7	>>50	>>50	>>50	>>50	>>50	>>50
75/0/15	52/0/48	8	3.4 ± 1.7	6.9 ± 1.7	5.8 ± 0.8	2.6 ± 1.5	4.7 ± 1.9	16.5 ± 2.3
100/0/0	100/0/0	9	23.2 ± 0.6	6.5 ± 1.1	31.0 ± 0.7	14.0 ± 0.6	3.4 ± 0.9	>>50
-	-	Daunomycin	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
-	-	Dau=Aoa-GPLGVRG-OH	4.1 ± 1.0	2.6 ± 0.8	8.9 ± 0.6	3.6 ± 1.0	10.5 ± 1.2	12.0 ± 1.1
-	-	Dau=GPLG-OH	1.5 ± 0.3	1.1 ± 0.7	0.9 ± 0.1	1.2 ± 0.5	2.0 ± 1.0	2.0 ± 0.7

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