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Characterisation of a novel, high affinity and selective αvβ6 integrin RGD-mimetic radioligand

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ABSTRACT

The alpha-v beta-6 (αvβ6) integrin has been identified as playing a key role in the activation of transforming growth factor-β (TGFβ) that is hypothesised to be pivotal in the development of cancer and fibrotic diseases. Therefore, the αvβ6 integrin is an attractive therapeutic target for these debilitating diseases and a drug discovery programme to identify small molecule αvβ6 selective arginyl-glycinyl-aspartic acid (RGD)-mimetics was initiated within GlaxoSmithKline. The primary aim of this study was to pharmacologically characterise the binding to αvβ6 of a novel clinical candidate, compound **1**, using a radiolabelled form.

Radioligand binding studies were completed with [^{3}H]compound **1** against the human and mouse soluble protein forms of αvβ6 to determine accurate affinity estimates and binding kinetics. The selectivity of compound **1** for the RGD integrin family was also determined using saturation binding studies (αvβ1, αvβ3, αvβ5, αvβ8, α5β1 and α8β1 integrins) and fibrinogen-induced platelet aggregation (αIIbβ3 integrin). In addition, the relationship between divalent metal cation type and concentration and αvβ6 RGD site binding was also investigated.

Compound **1** has been demonstrated to bind with extremely high affinity and selectivity for the αvβ6 integrin and has the potential as a clinical tool and therapeutic for investigating the role of αvβ6 in a range of disease states both pre-clinically and clinically. In addition, this is the first study that has successfully applied radioligand binding to the RGD integrin field to accurately determine the affinity and selectivity profile of a small molecule RGD-mimetic.

1. Introduction

Integrins are heterodimeric, transmembrane glycoproteins that act as adhesion receptors and signalling proteins in mammals [1]. They are made up of an α and β-subunit (of which in mammals there are 18 and 8 variants, respectively) bound in a noncovalent complex, that can make up to 24 heterodimers [2]. The alpha-v beta-6 (αvβ6) integrin is a member of the arginyl-glycinyl-aspartic acid (RGD) sub-family of integrins that share an amino acid binding motif (arginine (R), glycine (G) and aspartic acid (D)) in their endogenous ligands [1]. αvβ6 was first identified in the 1990s [3] and is an epithelially-restricted integrin that demonstrates a high affinity for latency associated peptide-1 (LAP_1) [4], among other ligands. This integrin is expressed in relatively low levels in the epithelia of healthy human tissue, however is observed to be upregulated upon injury or inflammation and in a number of diseases including acute lung injury [4], fibrosis [5–7] and a wide range of carcinomas [8].

It is via the engagement with LAP_1 that αvβ6 has been shown to activate transforming growth factor-β1 (TGFβ1) from its latent form [4] and it is the dysregulation of this potent cytokine that has been implicated in cancer and fibrotic disease [8,9]. Therefore, the αvβ6 integrin is a promising potential therapeutic target for cancer and fibrosis with clinical experiments proposed or ongoing to test the αvβ6/TGFβ1 inhibition hypothesis in these diseases [10,11]. Compound **1** is a small molecule RGD-mimetic currently in Phase I trials that was identified as part of a medicinal chemistry programme [12]. In this study compound **1** has been radiolabelled ([^{3}H]compound **1** (Fig. 1)) to fully characterise its

Abbreviations: αvβ6, alpha-v beta-6; RGD, arginyl-glycinyl-aspartic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DPU, Discovery Performance Unit; DMSO, dimethyl sulphoxide; ELISA, enzyme-linked immunosorbent assay; FCS, foetal calf serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; LAP_1, latency associated peptide-1; LS, liquid scintillation; NSB, non-specific binding; TGFβ1, transforming growth factor-β1; tLAP, truncated LAP.

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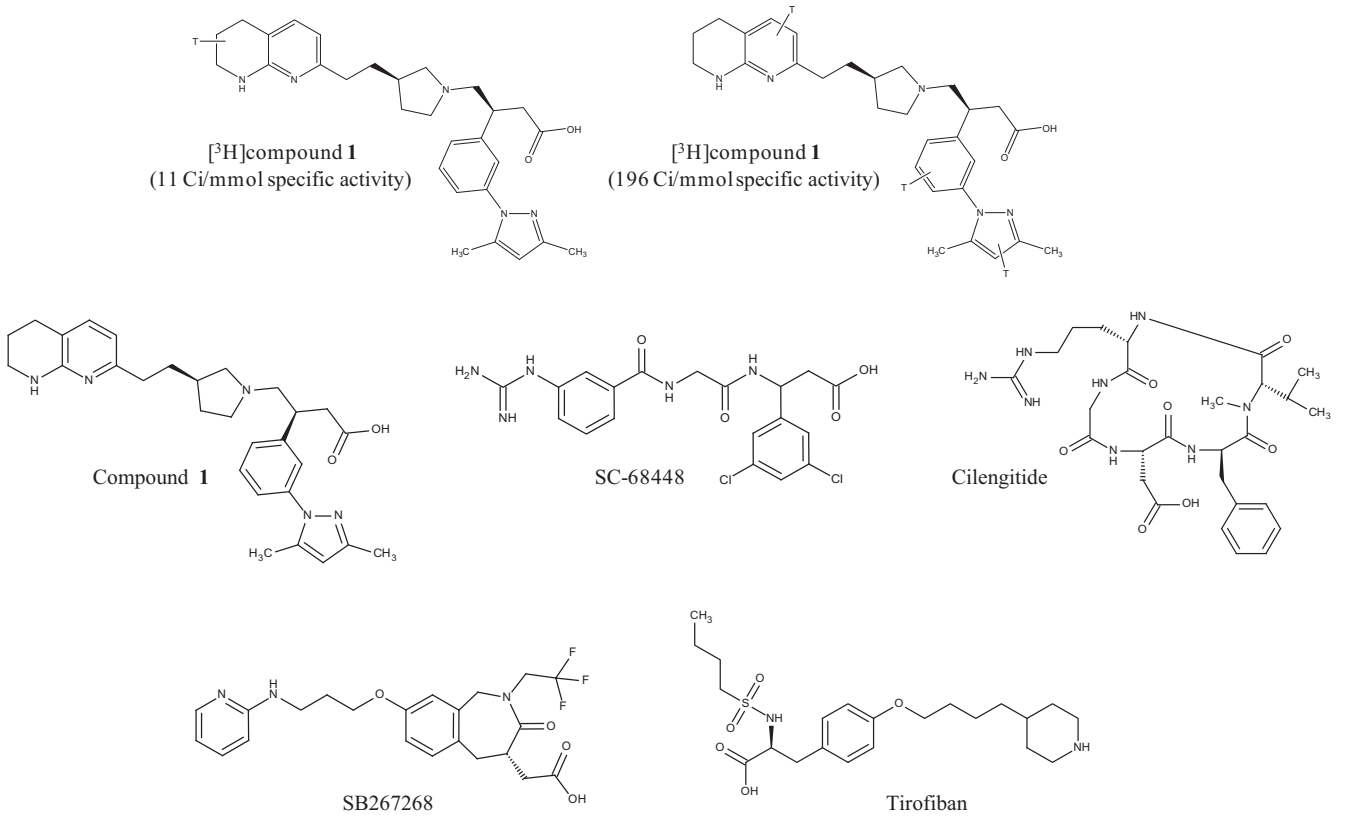

Fig. 1. The chemical structures of the small molecule RGD-mimetics used in this study.

αvβ6 binding against the human and murine form of the receptor as well as compared with the integrin endogenously expressed in a human colorectal adenocarcinoma cell line. In addition, it has been used to determine an accurate selectivity profile for this novel chemical entity against the RGD integrin family. Historically, the determination of the ability of an integrin ligand to compete with an endogenous ligand has been measured in either a cell adhesion assay [13,14] or competitive binding enzyme-linked immunosorbent assay (ELISA) [15]. The IC_{50} values generated via these techniques measure the ability of the integrin ligand to inhibit the binding of an endogenous ligand to the integrin that is either recombinantly expressed in a cell line (cell adhesion) or a soluble integrin preparation (ELISA). Although efficient in being able to differentiate the rank order of IC_{50} values of a set of ligands against a particular integrin and also being high throughput, one of the limitations of both these assay types is that a true affinity cannot be calculated and therefore accurate selectivity profiling between integrins is not possible. In addition, these assay types do not allow a full characterisation of the type of binding of the ligands in terms of being competitive and reversible, and the kinetics of the ligand-integrin interaction cannot be ascertained. Interestingly, radioligand binding has not been used in characterisation of the binding of ligands with the αvβ6 integrin and has only been used sparingly in the integrin field [16,17].

Therefore, in this study we have characterised [^{3}H]compound **1** against the RGD integrins and have shown it to have unprecedented high affinity and selectivity for the αvβ6 integrin for a small molecule RGD-mimetic, with a comparable profile observed between the human and murine form of the protein. This will serve as an extremely useful pre-clinical tool at the αvβ6 integrin, as well as the other αv RGD integrins, for determining functional activity in both *in vitro* and *in vivo* disease models. In addition, it has the potential as a clinical tool and a therapeutic agent for testing the role of αvβ6 in TGFβ activation in a number of diseases in multiple organs that includes cancer and fibrosis.

2. Materials and methods

2.1. Materials

Compound **1**, SC-68448 [18], SB267268 [19] and cilengitide [20] (Fig. 1) were synthesised by the Fibrosis and Lung Injury Discovery Performance Unit (DPU) Medicinal Chemistry group at GSK Medicines Research Centre (Stevenage, UK). Truncated LAP_1 GRRGDLATIHG ($tLAP_1$), truncated LAP_2 YTSGDQKTIKS ($tLAP_2$), truncated LAP_3 HGRGDLGALKK ($tLAP_3$) and the αvβ6 selective peptide NAVPNLRGDLQVLAQKVART (A20FMDV2) [15] (Fig. 1) were synthesised by Cambridge Research Biochemicals (Cleveland, UK). Compound **1** was radiolabelled with [^{3}H] via catalytic reduction by Quotient Bioresearch (Radiochemicals) Ltd. (Cardiff, UK) with a specific activity of 11 or 196 Ci/mmol (Fig. 1). All other chemicals and reagents were purchased from Sigma-Aldrich Co. Ltd. (Gillingham, UK) unless otherwise stated. All cell culture media and reagents were obtained from Thermo Fisher Scientific (Waltham, MA, USA). All tissue culture flasks and plates were purchased from Greiner Bio-One (Firckenhausen, Germany).

2.2. Cell culture

The human colorectal adenocarcinoma cell line HT-29 (HTB-38™ obtained from ATCC® (Manassas, VA, USA) and authenticated using the Promega Cell ID™ System (Fitchburg, WI, USA)) endogenously expressing the αvβ6 integrin [21] was maintained in culture in T175 tissue culture flasks at 37 °C in a 95% O2/5% CO2 atmosphere in growth medium (Roswell Park Memorial Institute (RPMI) 1640) containing 10% heat inactivated foetal calf serum

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