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Novel analogs of antitumor agent calixarene 0118: Synthesis, cytotoxicity, click labeling with 2-[^{18}F]fluoroethylazide, and *in vivo* evaluation

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ABSTRACT

Calixarene 0118 is a potent anti-angiogenic agent that effectively inhibited tumor growth in preclinical studies, and is currently being evaluated in a phase I clinical trial. We have designed two close mimetics of calixarene 0118 containing a terminal alkynyl-functional group, and developed an optimized semi-automated procedure for radiolabeling with 2-[^{18}F]fluoroethylazide using click chemistry. Following semi-preparative HPLC purification and formulation, the lower-rim modified analog [^{18}F]**6** and the equatorially labeled [^{18}F]**13** were obtained in >97% radiochemical purity and overall decay-corrected isolated radiochemical yields of 18.7 ± 2.7% (n = 4) and 10.2 ± 5.0% (n = 4), respectively, in a total synthesis time of about 2 h. Preliminary *in vivo* studies in nude mice bearing human tumor xenografts revealed highest accumulation of both tracers in the liver, followed by spleen, kidney, lung and bone, with no substantial uptake in the tumor. Still, these first-in-class radiotracers are a valuable tool for pharmacokinetic profiling and improvement of calixarene-based anti-angiogenic therapeutics in the future, as similar radiolabeling strategies may be applied to other compounds in the calixarene series. The cold reference compounds of the radiotracers were characterized in terms of cytotoxicity and anti-proliferative effects on HUVEC cells and on MA148 human ovarian carcinoma cells, along with the respective precursors, a small series of 0118 analogs modified with short-chain linear alkyl substituents, and a PEG_3-spaced calixarene dimer. While all of the new analogs proved at least equipotent to parent 0118, some of them inhibited HUVEC and MA148 cell growth almost 4- and 10-fold more effectively, rendering these analogs promising candidates for further evaluation in anti-angiogenic cancer therapy.

1. Introduction

In the past decades, drug-based cancer therapy has evolved from general cytotoxic chemotherapy to increasingly complex personalized treatment regimens using drugs matching the specific targets of a particular cancer. Beside new generations of anti-proliferative cytotoxic drugs and anti-hormone therapies, an increasing number of anti-angiogenic agents – small molecules, peptides and therapeutic antibodies – have been developed and

Abbreviations: *n*-BuLi, *n*-butyl lithium; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; HSQC, heteronuclear single quantum coherence; HUVEC, human umbilical vein endothelial cells; %ID/g, percentage of injected dose per gram of tissue; KO*t*Bu, potassium *tert*-butoxide; NaOMe, sodium methoxide; PBS, phosphate buffered saline; PEG, polyethylene glycol; PET-CT, positron emission tomography – computed tomography; SAR, structure–activity relationship; TFA, trifluoroacetic acid; VOI, volume of interest.

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many of those have progressed to the clinic [1]. Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is a crucial prerequisite for primary tumor growth above a size of about 1–2 mm^3, and has also been found essential for development of metastases [2–4].

In the early years, most anti-angiogenic agents discovered were endogenous proteins that inhibit EC growth, e.g. platelet factor-4 (PF4), plasmin fragment angiostatin, collagen XVIII fragment endostatin, and bactericidal-permeability-increasing protein (BPI). Containing numerous hydrophobic and cationic residues, and a common antiparallel β-sheet motif, these proteins are characterized by a high level of compositional and structural similarity [5]. Based on these findings, a new generation of synthetic β-sheet forming peptide 33-mers have been designed, one of which, named anginex, showed particular potent anti-angiogenic activity *in vitro* and *in vivo* [6]. Anginex was found to inhibit tumor growth and reduce microvessel density both in murine melanoma and breast sarcoma models, and in athymic mice bearing different types of human tumor xenografts, among others LS174T colon adenocarcinoma, and MA148 and SKOV-3 ovarian carcinoma [7–10]. Extensive structure–activity studies established five hydrophobic residues in β-strands 1 and 2 on the same face of the amphipathic anti-parallel β-sheet structure of anginex as being essential for anti-proliferative activity, and formed the foundation for the design of a series of partial peptide mimetics containing a β-sheet-inducing dibenzofuran (DBF) turn, some of which more effectively inhibited tumor growth in mice than anginex [11].

Although anginex and its partial peptide mimetic 6DBF7 have shown promising anti-tumor effects *in vivo*, non-peptidic compounds are generally known to be superior drugs, mainly because they potentially allow oral administration, typically lack an immune response, and can be optimized in terms of chemical and metabolic stability, resulting in a better pharmacokinetic profile. With the overall backbone dimensions of key residues in the two-stranded beta-sheet of anginex roughly matching those of the calix[4]arene scaffold, calix[4]arene derivatives containing basic and hydrophobic substituents on the upper and lower rim, respectively, were envisioned as potential non-peptidic analogs of anginex. Indeed, from a library of 23 substituted calix[4]arene analogs, two candidates, termed compound 0118 and 1097, proved to be potent inhibitors of angiogenesis *in vitro*, and were found to reduce tumor growth and microvessel density in B16 murine melanoma and MA148 xenografts in athymic mice [12]. While since its discovery, calixarene 0118 (Fig. 1) was known to display multimodal activities similar to anginex, such as inhibition of endothelial cell proliferation, migration, and induction of apoptosis [12], it was only recently that both calixarene 0118 [13] and anginex [14] were discovered to also target the same receptor, galectin-1 (gal-1). Interestingly, SAR-data from anginex and HSQC mapping studies with calixarene 0118 indicate that both ligands interact with gal-1 via their hydrophobic surfaces [11,13]. This is very different from the majority of galectin-1 antagonists presently available, which are β-galactoside analogs and glycomimetics targeting the canonical β-galactoside carbohydrate binding site [15–17]. In fact, calixarene 0118 was established to act as a noncompetitive, allosteric inhibitor of gal-1, with its binding site being located at the back face of gal-1 opposite to the β-galactoside binding site [13]. In the meantime, after successful completion of extensive preclinical studies [12,18], calixarene 0118 (also known as PTX008, OTX008) is currently being evaluated by OncoEthix in a phase I clinical trial in patients with malignant advanced solid tumors (ClinicalTrials.gov: NCT01724320) [17], and ongoing early discovery research has led to the identification of an even more potent analog, PTX013 [19].

Despite the promising *in vitro* and *in vivo* data collected for lead compound 0118 and others in the series, radiolabeled analogs have not been reported so far, although they may prove highly valuable, both as a research tool and companion diagnostic to study target engagement *in vivo*, and for pharmacokinetic profiling of this class of compounds. Designing radiolabeled 0118 analogs without introduction of major structural changes is an intrinsically difficult task, particularly in view of the stringent structural constraints established in previous SAR-studies [12,13] indicating that only minor modifications are tolerated at the hydrophobic upper rim of the calixarene, and the symmetric nature of the lower rim substituents, which is introduced already at a very early stage in the previously reported chemical syntheses of these compounds. We

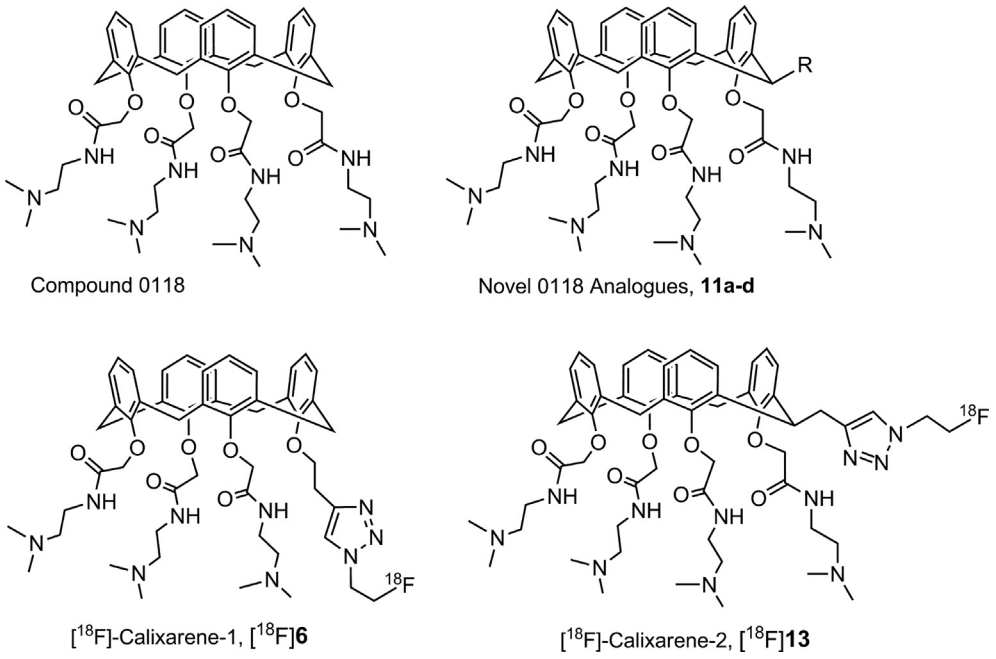

Fig. 1. Chemical structure of compound 0118, novel 0118 analogs **11a**–**d**, and radiotracers [^{18}F]**6** and [^{18}F]**13**.