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Astatine-211 radiolabelling chemistry: from basics to advanced biological applications

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Abstract

Background: ^{211}At-radiopharmaceuticals are currently the subject of growing studies for targeted alpha therapy of cancers, which leads to the widening of the scope of the targeting vectors, from small molecules to peptides and proteins. This has prompted, during the past decade, to a renewed interest in developing novel ^{211}At-labelling approaches and novel prosthetic groups to address the diverse scenarios and to reach improved efficiency and robustness of procedures as well as an appropriate in vivo stability of the label.

Main body: Translated from the well-known (radio)iodine chemistry, the long preferred electrophilic astatodemetallation using trialkylaryltin precursors is now complemented by new approaches using electrophilic or nucleophilic At. Alternatives to the astatoaryl moiety have been proposed to improve labelling stability, and the range of prosthetic groups available to label proteins has expanded.

Conclusion: In this report, we cover the evolution of radiolabelling chemistry, from the initial strategies developed in the late 1970's to the most recent findings.

Background

Astatine-211 is currently considered as one of the most promising alpha particle emitters for application in targeted alpha therapy (TAT) of cancers (Eychenne et al. 2021), a modality that is experiencing an increasing interest and is expected to have a strong impact on cancer treatment in the coming years (Jang et al. 2023; Miederer et al. 2024). TAT relies on the high energy deposited on a limited volume around the radionuclide decay to destroy targeted cancer cells with limited impact on surrounding healthy tissue. On the one hand, the direct physical effect on cell nucleus material, in particular DNA double strand breaks, impairs cell repair, leading to their demise, while indirect effects such as generation of highly toxic reactive oxygen species (ROS), or activation of immune response (also known as bystander effect) participate in treatment efficacy (Gorin et al. 2014; Pouget and Constanzo 2021). Several features distinguish ^{211}At from other available α emitters, particularly its intermediate half-life of 7.2 h, a simple decay scheme leading to one emitted α particle unlike the cascade of α and β^- particles emitted by other α emitters, and a main production process relying on irradiation of a cheap

material, ^{209}Bi, by a cyclotron (Fig. 1A). Irradiation must be precisely controlled as concomitant production of ^{210}At ($t_{1/2}$ = 8.1 h), which decays to highly toxic ^{210}Po, is observed if α beam energy is too high. This is why an α beam energy of ~ 28–29 MeV is considered as optimal (Fig. 1B) (Feng and Zalutsky 2021).

These attractive characteristics have led to several preclinical studies since the 1990's up to today, with clinical trials reported, in progress, or in preparation (Guérard et al. 2013; Lindegren et al. 2020). While initially mainly monoclonal antibodies (mAb) and their derivatives were used as targeting vectors to transport ^{211}At to the tumour cells, small molecules are now increasingly considered. However, ^{211}At-labelling chemistry has long been restricted to few reactions that limited the scope of compounds to be labelled. Astatine is the rarest of all natural elements on Earth and exists only as short-lived isotopes. Knowledge on its physical and chemical behaviour is incomplete and much remains to be understood to exploit fully its properties for the development of radiopharmaceuticals. It is however clearly established that ^{211}At belongs to the halogen family and exhibits many similarities with iodine that were exploited in radiolabelling approaches. On the other hand, alternative approaches relying on the metallic character reported for some ^{211}At species may also be considered (Guérard et al. 2021). In this article, an exhaustive review of the radiolabelling approaches reported in the literature is provided, with a first part focusing on the available radiolabelling reactions, and a second part focusing on the main ^{211}At-labelled prosthetic groups developed that are required to label large molecules such as antibodies. ^{211}At-labelled nanoparticles are out of the scope of this report.

Main text

^{211}At-radiolabelling reactions

Production of [^{211}At]astato(hetero)aryl compounds by electrophilic approaches

Aromatic electrophilic substitution (SEAr) Electrophilic halo-deprotonation is one of the first strategies that was used for the astatination of (bio-)molecules. This approach derives inspiration from established techniques previously set up in radioiodine-based procedures. The crux of this approach lies in the well-known direct coupling of iodine in its oxidized form (I^+) onto the tyrosine residues embedded within proteins that found applications in a number of biological assays up to the approval of radioiodinated mAbs for clinical use such as ^{131}I-Tositumomab (Bexxar) for CD20 antigen on B-cell non-Hodgkin's lymphoma (Friedberg and Fisher 2004). A preliminary step involves the in-situ

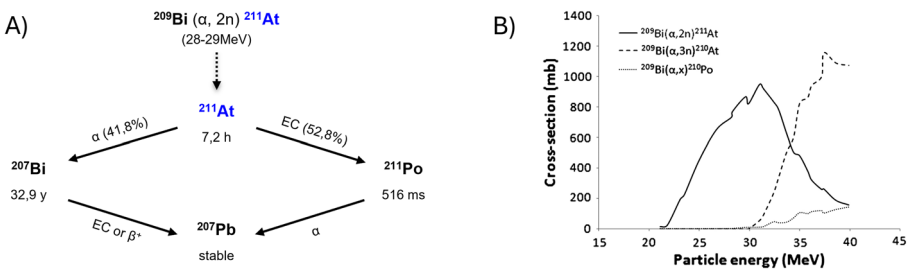


Fig. 1 **A** Nuclear reaction for production of ^{211}At from ^{209}Bi and decay scheme. **B** Cross section of the irradiation of ^{209}Bi by α particles (reproduced from Guérard et al. 2013)

addition of an oxidant to produce the reactive halogen species. Notable oxidants in this context include chloramine-T, Iodogen®, *N*-chlorosuccinimide (NCS) and hydrogen peroxide (H_2O_2). Subsequent substitution by iodine onto one of the carbons occurs in *ortho* position relative to the tyrosine phenol group. Initial attempts to introduce ^{211}At onto protein tyrosine groups were conducted using either the Hunter and Greenwood method (NCS, 23 °C, 5 min) or through oxidation with hydrogen peroxide (H_2O_2, pH 7.4, 60 min), as noted by (Aaij et al. 1975). In both cases, unsatisfactory outcomes were observed, with radiochemical yields (RCY) ranging from 5 to 30% and notable in vivo instability of the resulting radioconjugates. This indicated a significant difference between iodine and astatine's ability to form bonds with sufficient stability for biological use. Attempts by (Vaughan and Fremlin 1977) to synthesize astatotyrosine from tyrosine to optimize the reaction parameters showed that no reaction occurred at pH lower than 9, contradicting Aaij's previous assumption of ^{211}At labelling on tyrosine that was conducted at pH 7.4. Other research groups have explored the instability associated with the purportedly formed astatotyrosine moiety, putting forth multiple hypotheses to unravel its underlying mechanisms. Notably, in his comprehensive investigations into halogens, Coenen provided insights demonstrating the lower chemical stability of the phenyl-astatine bond compared to phenyl-iodine bond in a general trend within the halogen series (Table 1). This observation, coupled with the recognized natural instability of iodotyrosine, represents another stride toward comprehending the greater instability of astatotyrosine, if formed, when introduced in vivo (Coenen et al. 1983). Along the same line, Visser et al. has also demonstrated that astatotyrosine produced by electrophilic demetallation was not stable at blood pH (Visser et al. 1979).

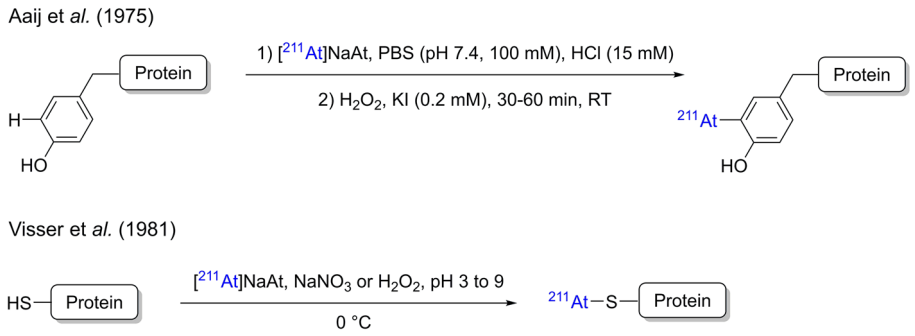
In follow up reports, Visser demonstrated that the conditions designated as "direct labelling" in earlier studies led to the binding of electrophilic ^{211}At onto protein cysteine rather than tyrosine residues by forming a labile ^{211}At-sulfur bond (Scheme 1) (Visser et al. 1981a; Visser and Kaspersen 1980). Consequently, to attain a level of stability deemed satisfactory for in vivo applications, it appeared necessary to consider indirect tagging approaches using pre-labelled prosthetic groups.

A limited number of instances showcased successful application of ^{211}At SEAr reaction (Scheme 2). Besides above attempts to label proteins, Vasaros reported the labelling of benzene and halobenzenes. A high RCY of 89.1 ± 1.5% of [^{211}At]astatobenzene could be obtained when performing the reaction with the substrate as solvent at elevated temperature above 170 °C in the presence of sulfuric or perchloric acid. In the halobenzene series, RCY decreased from 89.4 to 2.5% when halogen substituent was changed from

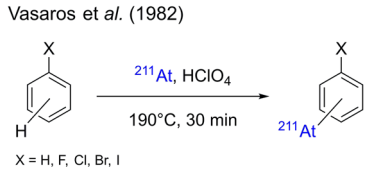
Table 1 Carbon-halogen bond energies (data from Coenen et al. 1983; Yssartier et al. 2024)

Halogen (X)	$E_{phenyl\text{-}X}$ (kJ/mol)	$E_{alkyl\text{-}X}$ (kJ/mol)
F	523	444
Cl	398	339
Br	335	285
I	268	222 ± 12
At	197 ± 20 (195.4)[a]	163 ± 12

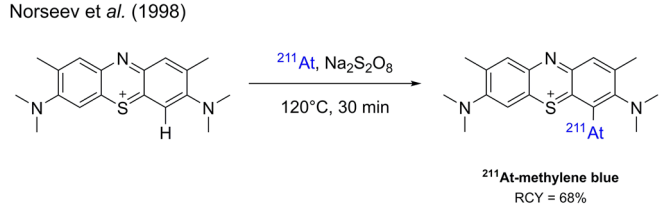
[a] Value in parenthesis was calculated (Yssartier et al. 2024)



Scheme 1 Initial hypothesis of an electrophilic labelling of tyrosine residues of proteins by Aaij et al. (1975), and revisited hypothesis by Visser et al. (1981a) of binding of electrophilic ^{211}At at cysteine residues



Halogen (X)	^{R}o-,m-,p-, AtC_6H_4X (%)	Isomeric distribution (%)		
		ortho	*meta*	*para*
H	89.1±1,5	NA	NA	NA
F	89.4±2.0	25.0±0.2	5.0±1.2	70.0±1.2
Cl	72.0±3.0	30.0±0.8	20.0±1.8	49.8±2.6
Br	8.1±0.2	33.2±0.4	21.0±0.8	45.8±0
I	2.5±0.1	30.0±1.2	26.8±1.5	43.2±1.5



^{211}At-methylene blue
RCY = 68%

Scheme 2 Repoted examples of SEAr with ^{211}At

F to I, and astatination was favoured in *para* position, followed by *ortho* and then *meta* position, confirming the SEAr nature of the reaction (Vasaros et al. 1982). A rare example of biologically relevant compound labelled by this approach is ^{211}At-methylene blue that demonstrated a propensity to accumulate in melanoma (Norseev 1998). Initial conditions reported by (Vasaros et al. 1982) were not compatible due to thermal decomposition of the precursor at elevated temperature. However, addition of sodium persulfate as oxidizer proved efficient to attain a satisfactory RCY of 68% within 30 min at 120 °C. Separation from unbound ^{211}At was performed by ion exchange chromatography. Starting methylene blue could not be removed by this approach which may cause issues related to molar activity (A_m), underscoring the need for better approaches to produce ^{211}At-labelled radiopharmaceuticals.

Halo-demetallation The efficacy of halo-demetallation through electrophilic substitution surpasses halo-deprotonation, which is primarily attributable to the carbon–metal bond's heightened polarization. This polarization facilitates the substitution reaction under milder conditions. Notably, this method addresses the regioselectivity challenges commonly encountered in halo-deprotonation reactions, as the reaction can occur specifically at the carbon bearing the metal. Metals such as mercury, silicon, germanium,

boron, and tin have been reported for their suitability as proficient leaving groups (Moerlein 1987).

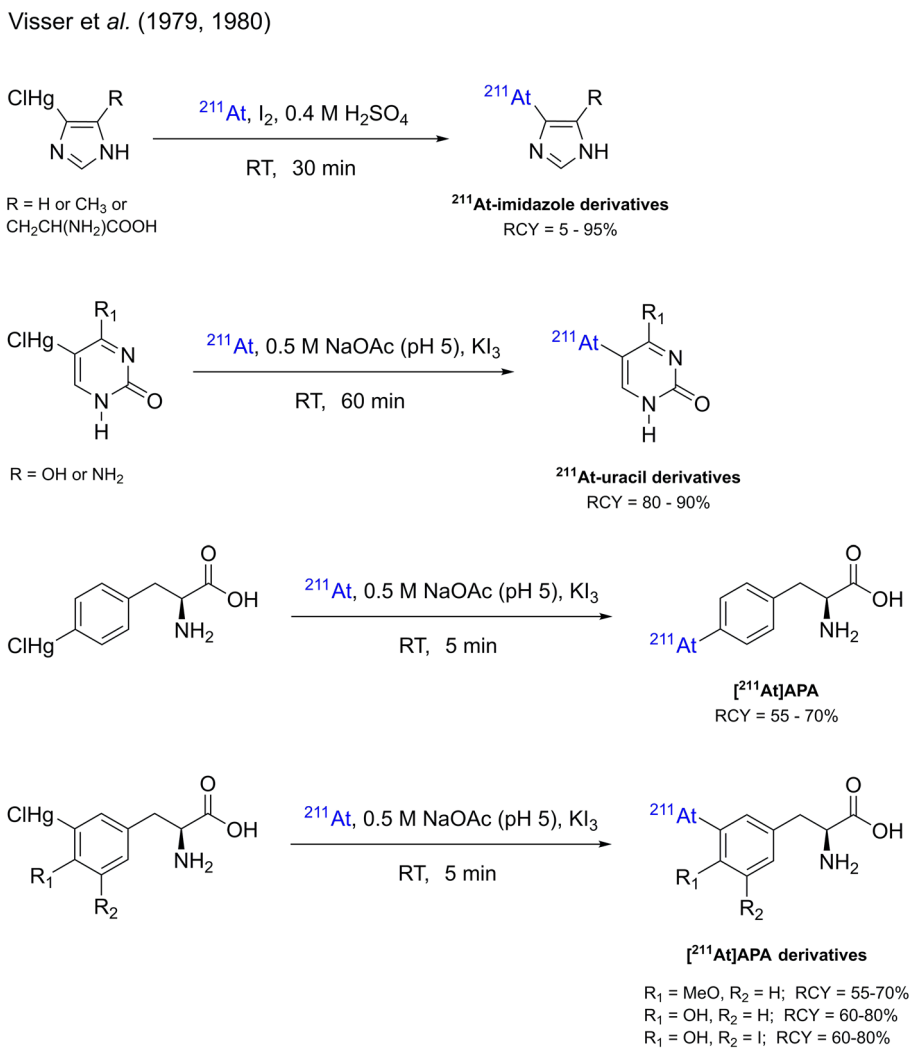
Arylmercuric precursors In early studies ^{211}At-labelled compounds have been prepared by demercuration of arylchloromercuric derivatives easily obtainable through various indirect reaction routes. Astatodemercuration can be performed under mild conditions, making them potentially suitable for a broad spectrum of compounds. The first studies reporting the synthesis of astatinated molecules were conducted by Visser to produce astatotyrosine (Visser et al. 1979) and astatoimidazole (Visser et al. 1980a) derivatives from chloromercuric precursors in an acidic environment at room temperature in the presence of I_2 or KI_3 as oxidizing agent and followed by the addition of KI to precipitate released Hg^{2+} ions. The RCYs were variable (5–95%), depending on the selected imidazole derivatives. These compounds exhibited good stability at room temperature on a large pH range (0–14). However, in contrast to their iodinated counterparts, their stability in reducing or oxidizing conditions was low, contributing valuable insights into the mechanisms of electrophilic astatination of proteins and their stability. The same research group then explored the synthesis of aryl astatide compounds using the same labelling procedure from chloromercuric precursors (phenol, aniline, nitrobenzene, uracil, phenylalanine). Astatination RCY were quite high (70–95%) however mercuration required more stringent conditions with less activated substrates such as phenylalanine derivatives (phenylalanine, 4-methoxyphenylalanine) (Scheme 3) (Visser et al. 1980a, 1980b, 1979).

Two reports rapidly followed, applying this chemistry to the ^{211}At-radiolabelling of biomedical compounds (steroids and pyrimidines), yielding results that supported previous studies and validated the potential application of this labelling method to molecules of interest in the field of nuclear medicine, despite the low in vivo stability of the ^{211}At-labelled pyrimidine observed (Visser et al. 1981b) (Scheme 4). The synthesis of 6-[^{211}At]-astato-2-methyl-1,4-naphthoquinol bis (disodium phosphate) which is of interest in the treatment of melanoma was also reported using a 5-step radiolabelling procedure (Brown 1982). The length of the procedure (about one ^{211}At half-life), led however to the selection of other approaches to access to this compound.

The application of this chemistry has not been reported in recent studies, most likely due to the constraints imposed by modern radiotracers as approaches using less hazardous compounds have been described since then. For instance, in a comparative study trialkylstannyl leaving groups were preferred over dichloromercuric (Wilbur et al. 1989).

Arylstannane precursors Because of their significantly lower toxicity compared to arylmercuric compounds, aryltrialkylstannane have been considered and have become by far the preferred class of precursors for astatination over the following decades. Tributyltin and trimethyltin groups do not exhibit discernible reactivity differences (Vaidyanathan et al. 1994a) but both are equally frequently reported despite the significantly lower toxicity of tributyl derivatives that should favour their use. First labelling was reported by Milius in 1986 to produce ^{211}At-astatobenzoic acid from the oxazoline precursor, and ^{211}At-tamoxifen (Milius et al. 1986) (Scheme 5).

Shortly after, Zalutsky and Wilbur concomitantly reported the synthesis of *N*-succinimidyl-[^{211}At]astatobenzoate ([^{211}At]SAB) prosthetic group, derived from the organotin precursor differing only by the position of ^{211}At, *meta* or *para* to the ester (Scheme 6)

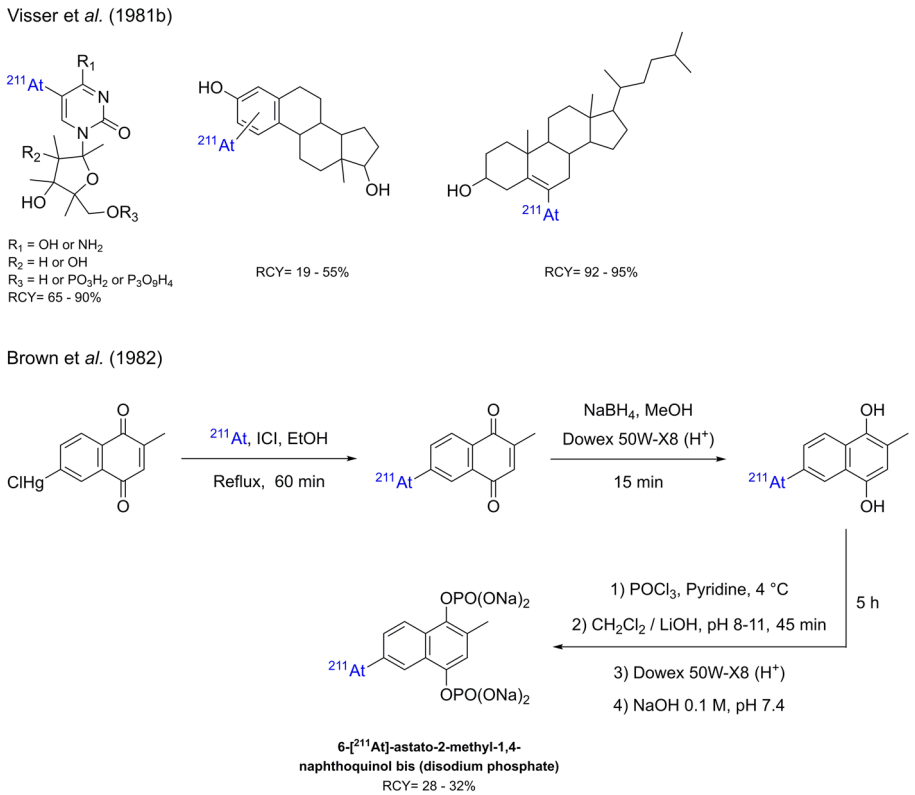


Scheme 3 Initial studies on the ^{211}At-labelling by electrophilic astatodermercuration

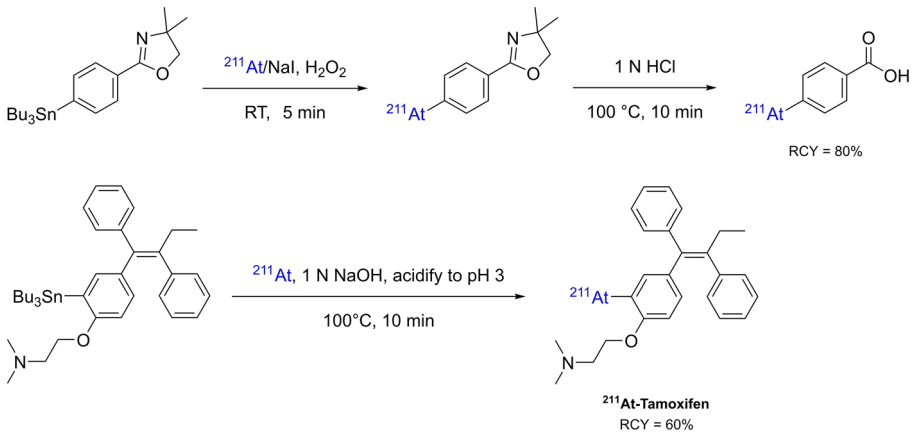
(Wilbur et al. 1989; Zalutsky and Narula 1988). [^{211}At]SAB was then efficiently conjugated to mAb via their lysine residues, providing the first stable and reliable approach, considered as a landmark in the development of ^{211}At-labelled conjugates (Zalutsky et al. 1989).

It remains until today, the gold standard method used in most preclinical therapeutic studies and in clinical trials. [^{211}At]SAB production process has evolved and improved over time, which is discussed in detail in the second part of this review.

The labelling process is typically conducted at ambient temperature in an acidic organic solvent (chloroform or methanol combined with 1–5% acetic acid) in the presence of an oxidizing agent. Following this step, purification through either high-performance liquid chromatography (HPLC) or solid phase extraction (SPE) is undertaken. The resultant [^{211}At]SAB is ready to be subsequently conjugated to the lysine residues of the corresponding proteins. A final purification step ensues, utilizing a gel filtration chromatography with phosphate-buffered saline (PBS) as the mobile phase, as outlined



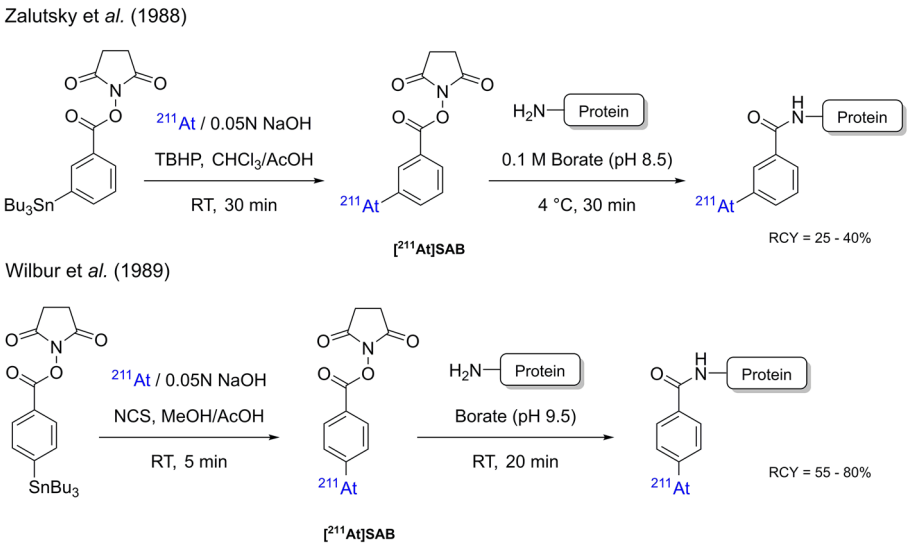
Scheme 4 Biologically relevant compounds labelled with ^{211}At by electrophilic astatodemercuration



Scheme 5 First astatodestannylation reactions reported by Milius et al. in 1986

by Zalutsky et al. The stability of the labelled proteins was evaluated in vitro and in vivo and showed superior results compared to hydrogen substitution with conservation of protein affinity (Zalutsky et al. 1989).

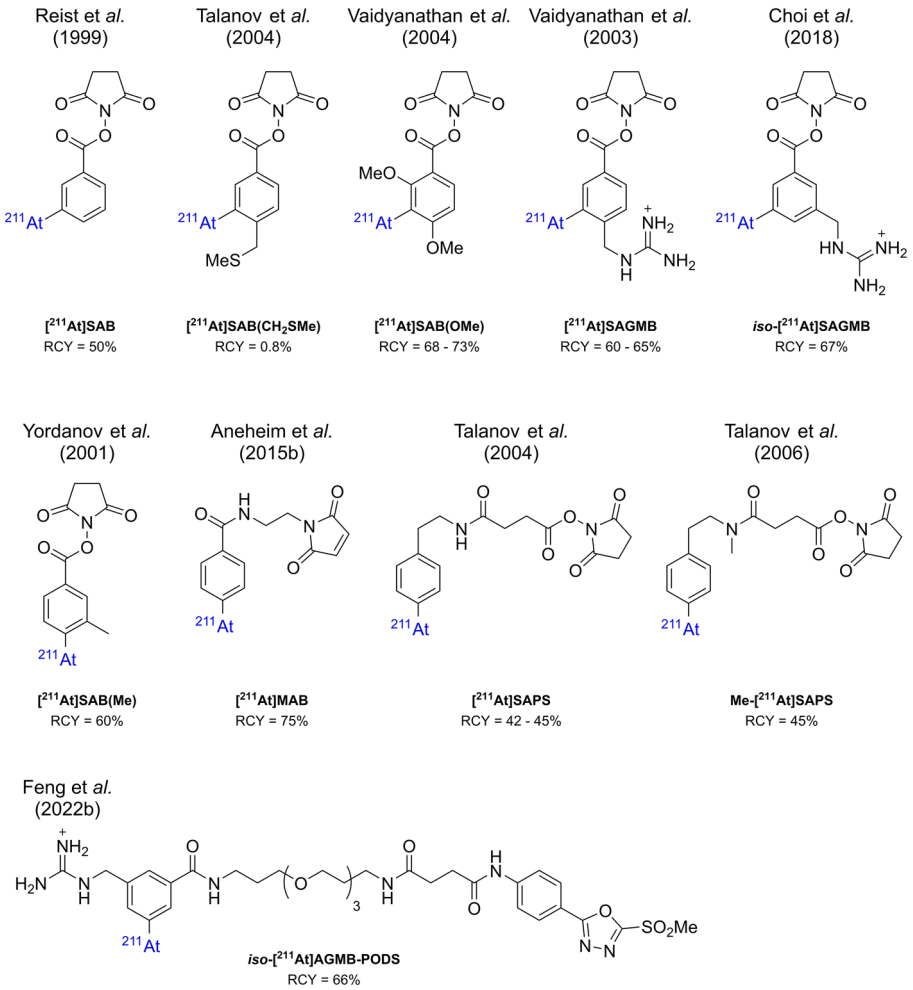
Organotin chemistry was then applied as the main approach to form At-aryl compounds in the 3 following decades with several examples of ^{211}At-labelled compounds, from small molecules to peptides and proteins labelled via a prosthetic group. Some studies have focused on variations around the SAB pattern to modulate the biological



Scheme 6 Synthesis of [^{211}At]SAB by electrophilic destannylation followed by conjugation to a mAb according to Zalutsky or Wilbur

behaviour of prosthetic groups, particularly their in vivo stability. Several papers have discussed the lower in vivo stability of the astatoaryl moiety for proteins internalized within targeted cells and most small molecules when compared to the iodinated counterpart. (Guérard et al. 2021; Teze et al. 2017; Wilbur 2008; Yssartier et al. 2024). This led to the production of alternatives to SAB, mainly from Zalutsky and Brechbiel's groups (Scheme 7). Changes to the [^{211}At]SAB motif essentially consisted in adding substituents at the *ortho* position of ^{211}At to modify electronic density of the ring and steric hindrance close to At position (Vaidyanathan et al. 1994b; Yordanov et al. 2001), evaluating chelating ability of (thio)ethers to improve At retention on the prosthetic group (Talanov et al. 2004), inserting a spacer between the conjugating carboxyl moiety and the aryl ring ([^{211}At]SAPS/ Me-[^{211}At]SAPS) (Yordanov et al. 2001; Talanov et al. 2006) and using a pyridine moiety (Reist et al. 1999). Among all reported attempts, only *N*-succinimidyl-3-[^{211}At]astato-4-guanidinomethylbenzoate ([^{211}At]SAGMB) provided significant improvement and has become the subject of further interest (Choi et al. 2018; Vaidyanathan et al. 2003). This specific prosthetic group is discussed in the second part of the review. Almost all reports describe the use of activated ester compounds for bioconjugation at lysine residues, except two, which describe compounds for coupling to cysteine thiol. This was done either via a maleimide group (Aneheim et al. 2015b) or more recently via an approach based on phenyloxadiazolyl methylsulfone that are deemed to be more stable in vivo (Feng et al. 2022b), as discussed in second section of this review.

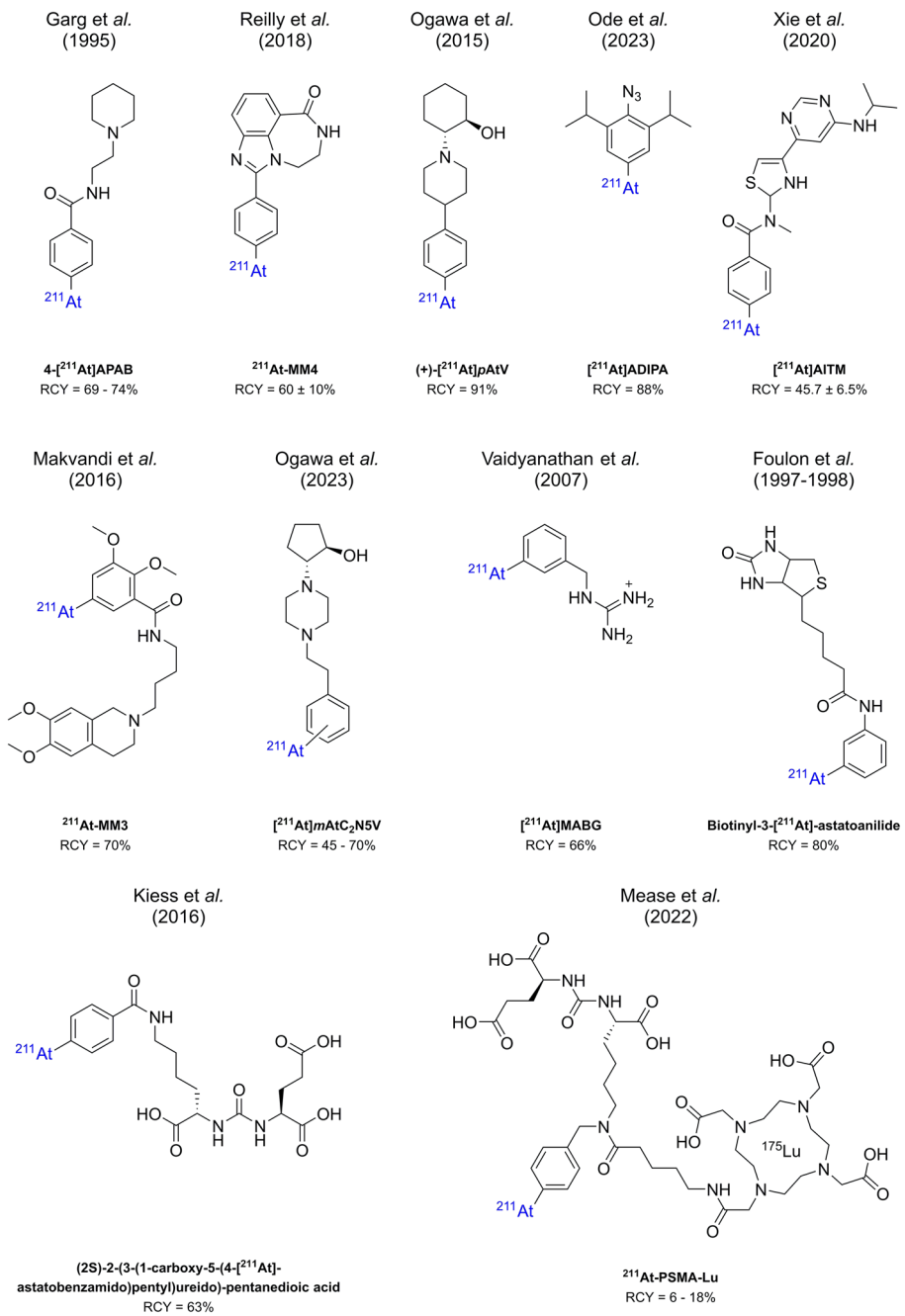
Earlier interest focused on using mAbs as main carriers for ^{211}At and small molecules were investigated to a lesser degree (Garg et al. 1995; Foulon et al. 1997, 1998). However, in the past few decades there has been an increase in the development of small molecule-based ^{211}At-radiopharmaceuticals (Kiess et al. 2016; Makvandi et al. 2016; Mease et al. 2022; Ogawa et al. 2015, 2023; Xie et al. 2020), for which tin chemistry remains a commonly used strategy (Scheme 8).



Scheme 7 SAB related prosthetic groups produced from corresponding tin precursors. RCYs are reported for isolated radiolabelled prosthetic group

A limited number of astatination from heteroaryl stannane precursors is reported. These include the pyridine and pyrimidine-based derivatives. For instance, bisphosphonates are active molecules used as inhibitors of osteoporosis and protectants against skeletal complications in cancer. A first-generation of ^{131}I-labelled bisphosphonate had demonstrated elevated uptake in bone metastases and significant pain relief. Due to their high radiobiologic effectiveness and limited tissue range (2–5 cells penetration), alpha-particle emitters may effectively deliver concentrated radiation doses to osseous metastases while reducing bone marrow toxicity. Astatinated bisphosphonate derivatives were successfully synthesized by conjugating radiolabelled intermediates SAB/SIB and *N*-succinimidyl 5-[^{211}At]astato-3-pyridinecarboxylate ([^{211}At]SAPC)/*N*-succinimidyl 5-[^{131}I]iodo-3-pyridinecarboxylate ([^{131}I]SIPC) to 3-amino-1-hydroxypropylidene bisphosphonate in borate buffer (pH 9.0) for 15 min (Scheme 9). The final products were purified by HPLC, and their biodistribution was evaluated in mice (Larsen et al. 1999).

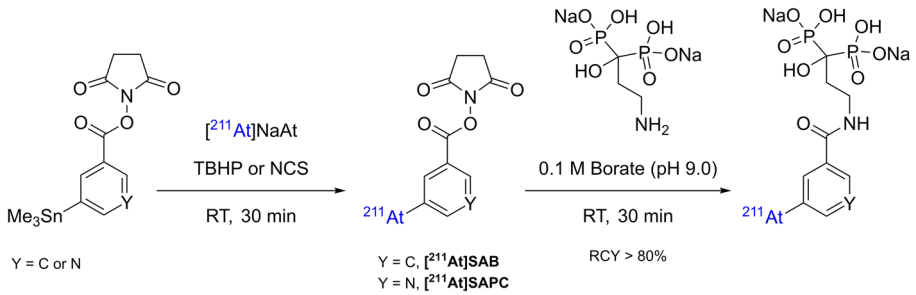
Biodistribution of these ^{211}At-labelled agents demonstrated low in vivo dehalogenation in general for small At-aryl-based molecules. Of note, using [^{131}I]SIPC and [^{211}At]



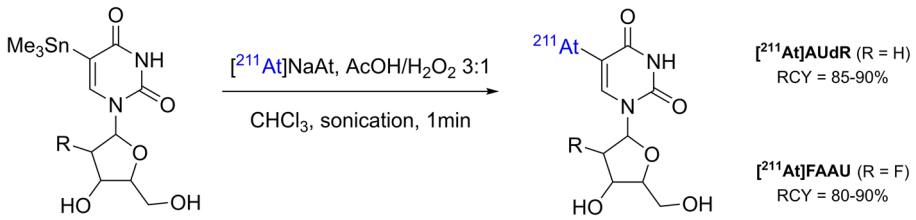
Scheme 8 Small ^{211}At-labelled molecules produced by electrophilic astatodestanylation

SAPC did not show a notable improvement in stability when compared to [^{211}At]SAB and [^{131}I]SIB. Similar studies (Reist et al. 1999; Vaidyanathan et al. 2021) with mAb or small prostate-specific membrane antigen (PSMA) targeting molecules reached similar conclusions regarding the impact of the ^{211}At-pyridine group on in vivo stability.

Thymidine analogues, such as [*I]IUdR, labelled with the Auger electron emitter ^{125}I or with ^{131}I have previously demonstrated efficacy as radionuclide therapy agents in human glioma cell lines. The ^{211}At-labelled thymidine analogue was considered as a



Scheme 9 Preparation of ^{211}At-bisphosphonates reported by Larsen et al.

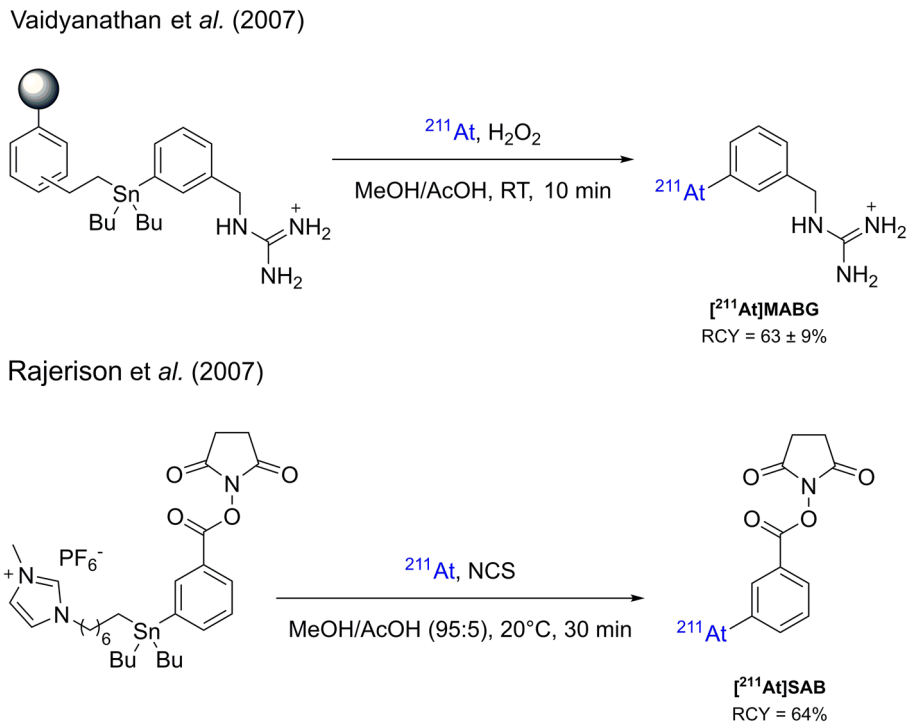


Scheme 10 Preparation of [^{211}At]AUdR and FAAU by electrophilic destannylation (Vaidyanathan and Zalutsky 1998)

potentially better agent due to the higher linear energy transfer of α particles than Auger electrons and beta particles. [^{211}At]AUdR was successfully synthesized by Vaidyanathan using the 5-(trimethylstannyl)-2'-deoxyuridine precursor (TMSUdR) in the presence of H_2O_2 (Vaidyanathan 1996). This synthesis yielded 85–90% [^{211}At]AUdR within 15–20 s after sonication of the heterogeneous solution and was followed by HPLC purification of the crude mixture (Scheme 10). Comparative study between ^{211}At and radioactive iodine analogues demonstrated the superior efficacy of alpha particles in killing human glioma cells in in vitro study. However, given that [^{211}At]AUdR, like [*I]IUdR, was prone to dehalogenation, it was suggested that this agent may be useful only if applied loco-regionally.

To circumvent this instability issue, the same authors reported shortly afterward a 2'-fluorinated analogue, [^{211}At]FAAU (Scheme 10) aiming at preventing enzymatic cleavage at the *N*-glycosidic bond (Vaidyanathan and Zalutsky 1998). This strategy indeed prevented the enzymatic cleavage, resulting in higher blood stability. However, release of astatine remained nearly unchanged (46% vs 47.7% for [^{211}At]AUdR) unlike with the radioiodinated analogues that exhibited a significant reduction of iodine release (2.6% for [*I]FIAU vs 26.1% for [*I]IUdR).

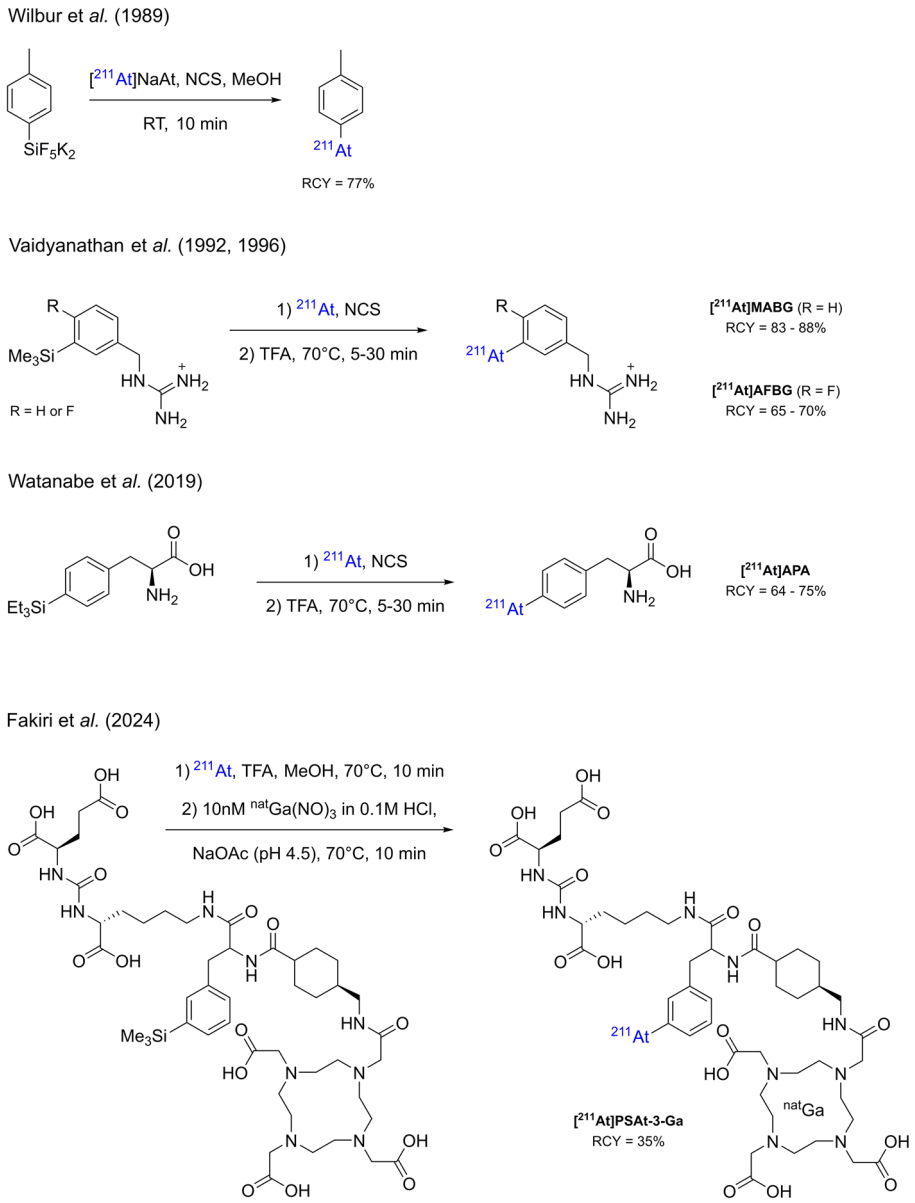
The purification step is crucial in the synthesis of ^{211}At-labelled compounds from their organotin precursors. In most cases HPLC purification is imperative to eliminate free astatine species, oxidizing reagents and their byproducts, and potentially hazardous tin-containing compounds. However, this conventional purification method introduces challenges, including prolonged production times and losses of ^{211}At-labelled product within the HPLC system (column, tubing) and during the evaporation step, lowering RCY and A_m. This is why supported tin chemistry approaches were reported with the promise of a facilitated purification step (Scheme 11). The first example was the



Scheme 11 Synthesis of ^{211}At-labelled compounds from supported tin precursors

development of a kit for the synthesis of [^{211}At]MABG based on its precursor bound to a polystyrene matrix via one of the tin alkyl chains. While chromatographic purification through a C18 cartridge provided unsatisfactory recovery yields due to important loss during evaporation of the eluted methanol fraction, the process was optimized by using a cation exchange resin cartridge, providing a RCY of 63 ± 9% and RCP > 90% with relevant starting activities (74–666 MBq) for clinical use (Vaidyanathan et al. 2007). Later, a similar strategy was employed, this time for [^{211}At]SAB preparation, utilizing an ionic liquid supported tin precursor that was efficiently labelled under mild conditions, improving the recovery of the ^{211}At-prosthetic group by using a rapid filtration over a silica cartridge, which could then be efficiently conjugated to the anti-CD138 9E7.4 mAb (Rajerison et al. 2016).

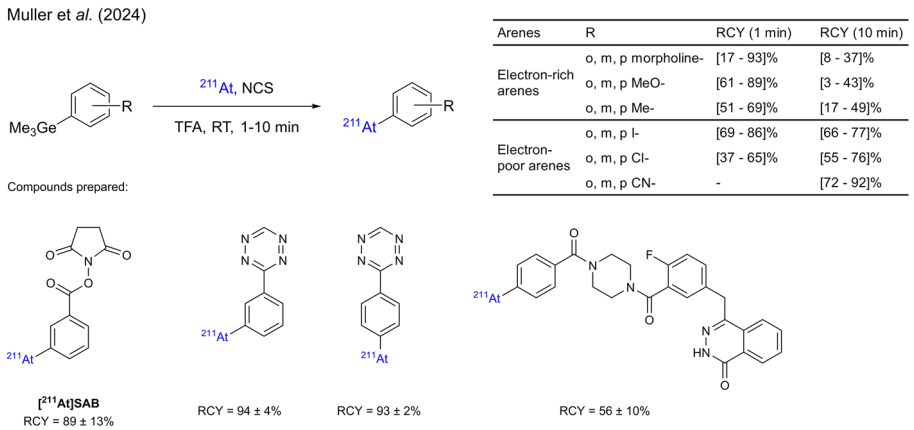
Arylsilicon precursors and arylgermanyl precursors Due to their lower toxicity, arylsilicon compounds are attractive surrogates of organotin precursors. They also exhibit stronger C-M bond energy, leading to an increased stability of intermediates, facilitating subsequent reaction steps such as deprotection prior to radiolabelling step and storage. These advantages come however with the drawback of lower reactivity with electrophiles. Except a rare example of pentafluorosilicate leaving group in Wilbur's comparative study (Wilbur et al. 1989) (Scheme 12), synthesis of astatoaryl molecules were set up from trimethylsilicon precursors as initially reported by the Zalutsky group in 1992. While astatodestannylation approach required a two-step procedure leading to low RCY (13%) to form [^{211}At]MABG, a high RCY of 85% was obtained using a silicon precursor and in a single step after optimizing labelling conditions, such as the presence of water, choice of acid and oxidant and temperature, which strongly impacted reaction efficiency



Scheme 12 Examples of ^{211}At-labelling by electrophilic desillylation

(Vaidyanathan and Zalutsky 1992). Subsequently, the preparation of the 4-fluorinated analogue (Scheme 12, [^{211}At]AFBG]) was reported using the same conditions (Vaidyanathan, 1996). A related report aiming at studying the potential of [^{211}At]AFBG to treat neuroblastoma evidenced an increased retention in SK-N-SH cells; however, a significantly decreased stability was observed when compared with MABG (Vaidyanathan et al. 1997).

It was only recently that silicon derivatives reemerged as viable alternatives to stannylated compounds with the production of 4-[^{211}At]astato-L-phenylalanine ([^{211}At]APA). This radiopharmaceutical, synthesized from oxidized astatine in a methanol/NCS solution (70 °C—10 min), exhibited competitive RCY (65–85%) and excellent radiochemical purity (RCP) > 99% when compared to traditional methods like Cu^{+}-catalysed



Arenes	R	RCY (1 min)	RCY (10 min)
Electron-rich arenes	o, m, p morpholine-	[17 - 93]%	[8 - 37]%
	o, m, p MeO-	[61 - 89]%	[3 - 43]%
	o, m, p Me-	[51 - 69]%	[17 - 49]%
Electron-poor arenes	o, m, p I-	[69 - 86]%	[66 - 77]%
	o, m, p Cl-	[37 - 65]%	[55 - 76]%
	o, m, p CN-	-	[72 - 92]%

Scheme 13 Electrophilic ^{211}At-labelling using trimethylgermanyl precursor. RCYs determined by TLC analysis of crude product

nucleophilic halogen exchange reaction or tin chemistry that provided only 10–17% RCY (Watanabe et al. 2019). This method shows potential for synthesizing various phenylalanine derivatives, including α-methyl phenylalanine and astatinated peptides containing aromatic residues.

Another recent example is the labelling of the PSMA radiopharmaceutical [^{211}At]PSAt-3-Ga, which was produced in two steps from the silyl precursor. The first step consisted of the astatodestannylation that was carried out in MeOH/AcOH with NCS as oxidizing agent and heating for 10 min in the presence of TFA, leading to a radiochemical conversion of 75%. After complexation with ^{nat}Ga and HPLC purification, the product was isolated with a 35% RCY (Fakiri et al. 2024). Interestingly, the radiolabelled product was found stable in vivo unlike other PSMA derivatives previously reported that exhibited the typical behaviour of small ^{211}At molecules bearing an At-aryl moiety (uptake of activity in thyroid and stomach). The authors speculated that this is due to the shielding against oxidative dehalogenation by burying the ^{211}At-region of the molecule deep inside the PSMA lipophilic pocket.

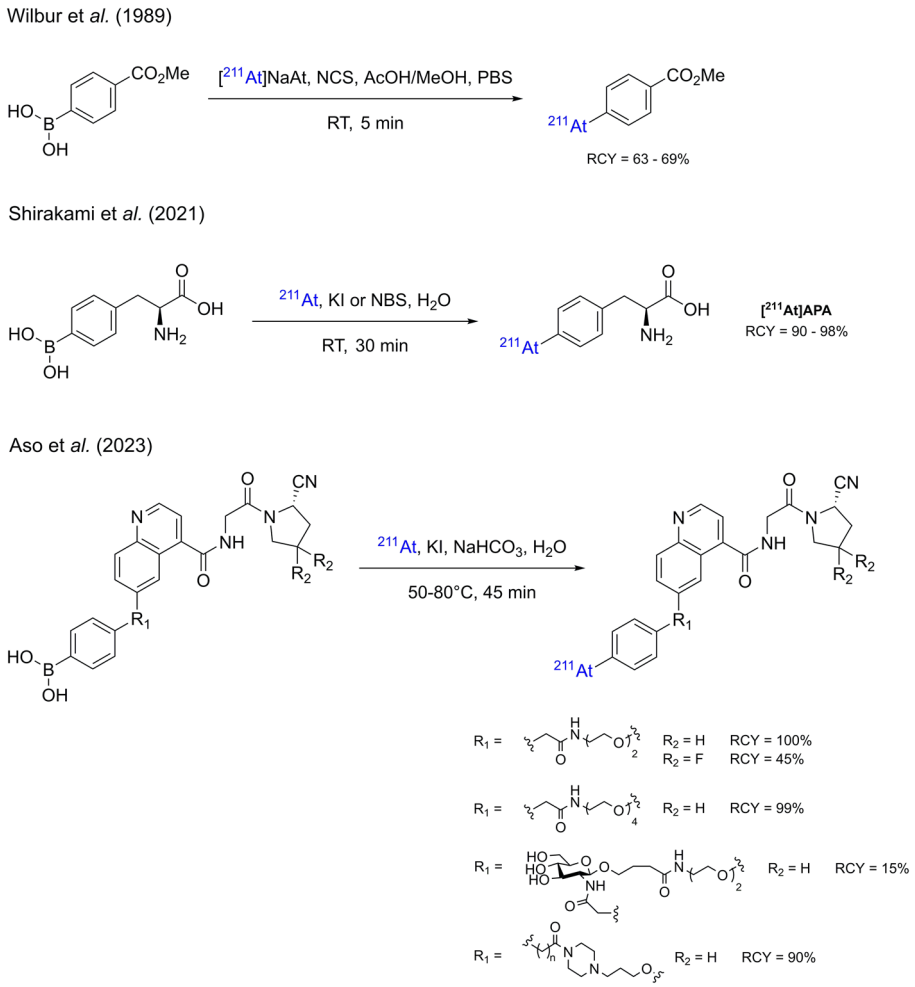
In a search for an intermediate situation with an improved balance of toxicity, stability, and reactivity compared to tin and silicon reagents, Müller explored the trimethylgermanyl leaving group (Muller et al. 2024). Based on previous radioiodination investigations (Moerlein 1985; Moerlein et al. 1987), the ^{211}At-labelling of electron-rich and electron-deficient arenes was studied in trifluoroacetic acid in the presence of NCS for 10 min at room temperature. Contrary to expectations for an electrophilic substitution, better RCY were observed with electron-deficient arenes (55–80%, *vs.* 5–49% for electron rich arenes). Further investigation showed that improved RCY could be obtained with electron rich compounds when using a shorter reaction time (1 min), thus indicating decomposition of product over longer reaction times. The method could be efficiently applied to the preparation of [^{211}At]SAB, ^{211}At-tetrazines and a PARP inhibitor (Scheme 13).

Arylboron precursors Although arylboron precursors have mostly been studied recently for nucleophilic astatination (vide infra), these precursors were considered for electrophilic labelling in an early comparative study by Wilbur and colleagues wherein 4-[^{211}At]astato benzoate was produced with moderate RCYs (Wilbur et al. 1989). As

recently as in 2021, electrophilic labelling of an arylboron precursor was again reported. Shirakami developed an improved method to produce 4-[^{211}At]astato-L-phenylalanine from 4-borono-L-phenylalanine (BPA) precursor (Shirakami et al. 2021). *N*-bromosuccinimide (NBS) or KI could be used as redox agents to produce electrophilic At and the reaction was performed at room temperature in water for 30 min. Interestingly, while KI is not an oxidizing agent, it led to better results than NBS. Authors suggested that KI acts as a reducing agent for overoxidized species and could stabilize At(I) as AtI and/or AtI_2^- species. Crude RCY was > 99% and RCY after SPE cartridge purification was > 90%. (Aso et al. 2022) employed a similar chemical approach for labelling dihydroxyboryl Fibroblast Activation Protein Inhibitor (B-FAPI) derivatives. They successfully astatinated two derivatives, B-FAPI-1 and B-FAPI-2, using ^{211}At, KI, and $NaHCO_3$ at 80 °C for 45 min, resulting in RCYs of 79% (100 µg of precursor) and 81% (10 µg of precursor), respectively. In a follow-up study, 3 new FAPI derivatives differing by their linker nature were reported by a similar approach (Aso et al. 2023). No discernible decomposition was observed within 24 h at room temperature in saline solution, indicating the efficient electrophilic substitution of ^{211}At for a dihydroxyboryl group on the aromatic ring of the FAPI derivatives (Scheme 14).

Production of [^{211}At]astato(hetero)aryl compounds by nucleophilic approaches

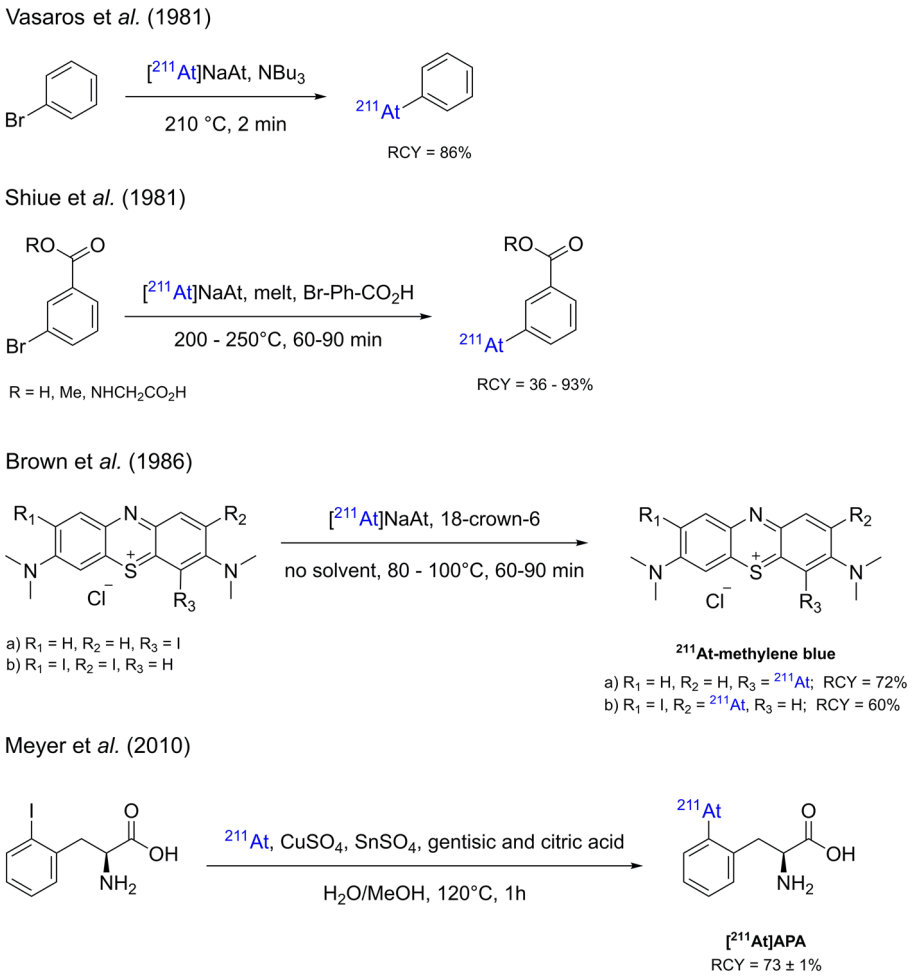
Halogen exchange Halogen exchange, in particular bromine or iodine displacement by astatide in haloaryl precursors, is one of the earliest astatination reactions investigated, based on already known halogen or isotope exchange for radioiodination (Scheme 15). Vasaros was the first to report a systematic comparative study on the substitution of bromide by [^{211}At]astatide and [^{131}I]iodide in bromobenzene (Vasaros et al. 1981). The reaction was investigated at 210 °C in ethanol, acetone and a series of alkylamines. While all conditions studied resulted in good to high ^{131}I-iodination RCY, only reactions performed in the presence of amines led to astatination. This suggests the formation of a phenylamonium intermediate that facilitates nucleophilic attack by astatide. The hypothesis was confirmed by kinetic studies evidencing an induction period for radioiodination in the presence of trialkylamine that was not observed in ethanol or acetone. In addition, activation energy for astatination decreased with decreasing steric hindrance of the amine, from 26.7 kcal/mol for triethylamine to 5.2 kcal/mol for diethylamine. The same year, Shiue et al. reported the bromine to astatine exchange by the melt method, which provided RCY from 36 to 64% with bromobenzoic acid-based compounds for reactions conducted at 200–250 °C according to gas radiochromatographic analyses (Shiue et al. 1981). Later on, Brown et al. optimized the approach by performing the reaction in a crown ether melt to enhance astatide reactivity (Brown et al. 1986). This approach performed better than astatodediazotation reaction or electrophilic astatodeprotonation. This allowed for the preparation of ^{211}At-labelled methylene blue by iodine exchange with high RCY by heating at only 80–100 °C. Another method to facilitate halogen exchange was reported by Meyer et al. using copper(I) catalysis to prepare [^{211}At]astato-L-phenylalanine ([^{211}At]APA) from the iodinated precursor (Meyer et al. 2010). Apart from [^{211}At]APA that was recently prepared by this method and used in the treatment of multiple myeloma (Yan et al. 2022), the halogen exchange reaction has found limited applications,



Scheme 14 Electrophilic astatodeboronation reported in the literature

mainly due to the difficulty to separate starting bromo- or iodoaryl precursor from product that limits A_m.

Halodediazotation The nucleophilic astatodediazotation reaction of aniline precursors is the first approach that was developed to stably label proteins with ^{211}At. Starting from *p*-aminobenzoic acid, Friedman designed a three-step approach consisting in the formation of the diazonium salt using sodium nitrite and sulfuric acid followed by reaction with [^{211}At]NaAt. After extraction of the formed [^{211}At]astatobenzoic acid, activation as a mixed anhydride was performed using *iso*butylchloroformate and tributylamine in dioxane. Conjugation to lysines was then performed on bovine serum albumin in dioxane/water (pH = 9) with a RCY of 12% (Friedman et al. 1977). A similar approach was later reported for the labelling of antibodies (Harrison and Royle 1984; Vaughan 1979) or other proteins such as concanavalin A (Vaughan et al. 1981), with RCYs ranging from 10 to 30% and a good retention of vectors' properties (Scheme 16). An alternative approach was also reported that consisted in generating a bis aryldiazonium salt, one diazonium group serving as a leaving group for astatination, the

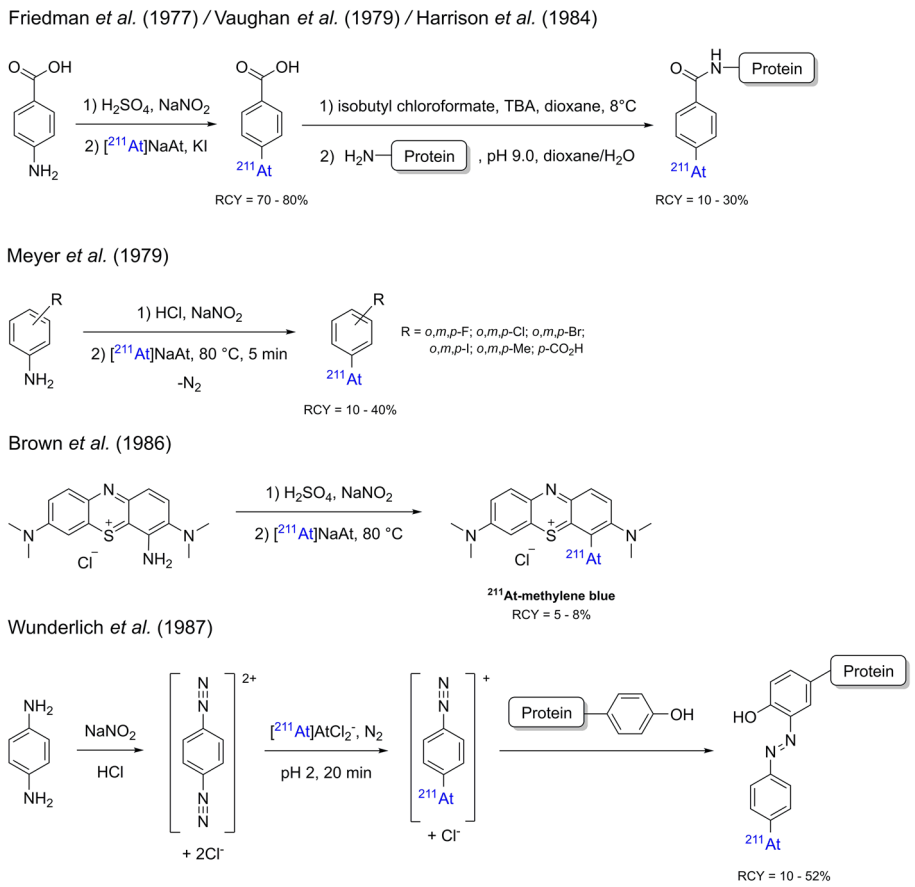


Scheme 15 ^{211}At-labelling by halogen exchange

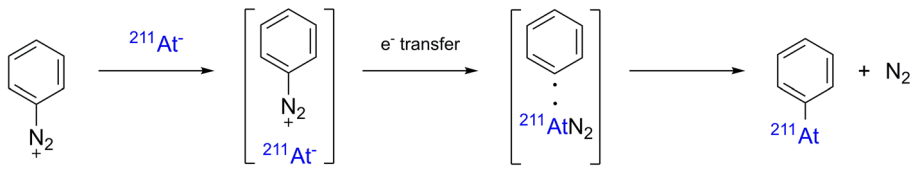
second one for conjugation to tyrosine residues of proteins by electrophilic substitution (Wunderlich et al. 1987). The advantage is that the activation step after labelling is no longer necessary. The whole procedure is reported to yield approximately 25% of ^{211}At-labelled rabbit immunoglobulin. No biological studies were however reported.

A mechanistic study was conducted by (Meyer et al. 1979) to explain the unexpected efficiency and selectivity of the reaction given the low astatide concentration in competition with a high hydroxide concentration. The proposed mechanism is based on the favoured formation of a complex between the phenyldiazonium and astatide followed by an electron transfer due to the high polarizability of the heavy halogenide, a pathway that is unlikely for lighter halogenides sur as F^- and Cl^-, or for HO^-. The obtained radical pair then recombines to form the astatoaryl product (Scheme 17).

Overall, few examples were reported in the literature due to the harsh conditions required and difficult purification, the latest example being reported by (Brown et al. 1986) for the preparation of ^{211}At-methylene blue which was described as laborious and inefficient (5–8% RCY, Scheme 15).



Scheme 16 ^{211}At-labelling by nucleophilic astatodediazotation

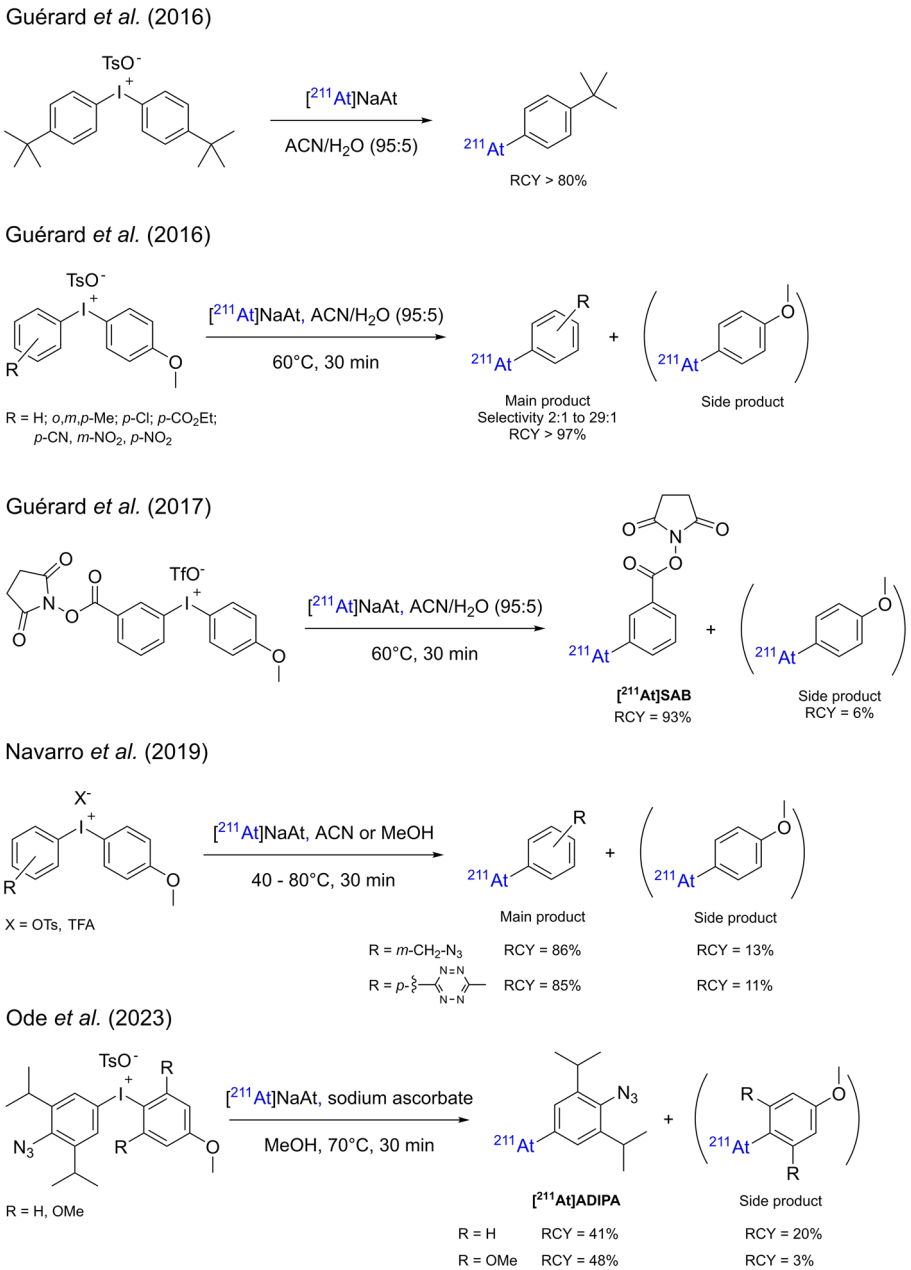


Scheme 17 Astatodediazotation mechanism proposed by (Meyer et al., 1979)

Aryliodonium salts/Ylides Hypervalent iodine compounds and particularly aryliodonium compounds are broadly used in organic chemistry due to their high reactivity in the presence of nucleophile species promoted by the reductive elimination that releases the aryliodide to its monovalent state (Merritt et al. 2009). Their use as precursors in radiolabelling chemistry was first introduced by Pike for the radiofluorination at an aryl position (Pike 2018). The first study of nucleophilic ^{211}At-labelling of diaryliodonium salts was reported in 2016 (Guérard et al. 2016). Reactivity with a model compound in CH_3CN was reported to be significantly higher for astatide compared to iodide with an activation energy of 17.1 kcal/mol versus 23.5 kcal/mol, respectively. This was reflected by a reaction operating at a significantly lower temperature, with a quasi-quantitative reaction within 30 min at 60 °C for ^{211}At-labelling while 120 °C

were necessary to reach the same result for ^{125}I-iodination. In addition, unlike ^{125}I, reaction proceeded efficiently in other solvents such as MeOH, DMF or a 4:1 CH_3CN/H_2O mixture with ^{211}At. Quantum chemical calculations suggested two different pathways: a monomeric iodonium halide intermediate with astatine, and a heterodimeric iodonium halide with iodine that requires additional energy for complex dissociation for the reaction to proceed. Study with various substituents highlighted higher reaction kinetics with electron deficient substrates, confirming a nucleophilic mechanism. In addition, similar to that seen with radiofluorination, the *ortho* effect was observed, which leads to a better chemoselectivity for the most hindered aryl ligand in *ortho* position in line with the transition state that favours the ligand in axial position (Chun et al. 2010). The better reactivity for electron deficient substrates appeared appropriate for the synthesis of [^{211}At]SAB, and a precursor was designed using the anisyl group as electron rich auxiliary ligand, providing high and consistent RCY of [^{211}At]SAB with low amount (6%) of astatoanisole as side product which could be easily eliminated during evaporation step before bioconjugation to a mAb (Guérard et al. 2017). The procedure was then used for the preparation of an anti-mCD138 mAb for a preclinical therapeutic study of multiple myeloma (Gouard et al. 2020) and glioblastoma (Roncali et al. 2024). The aryliodonium salt chemistry was also reported to produce ^{211}At-labelled clickable prosthetic groups that were used to efficiently label antibodies (Navarro et al. 2019) and more recently as a possible approach to access to ^{211}At-labelled 2,6-diisopropylphenyl azide ([^{211}At]ADIPA) for in vivo click chemistry in the potential treatment of lung cancer (Ode et al. 2023) (Scheme 18).

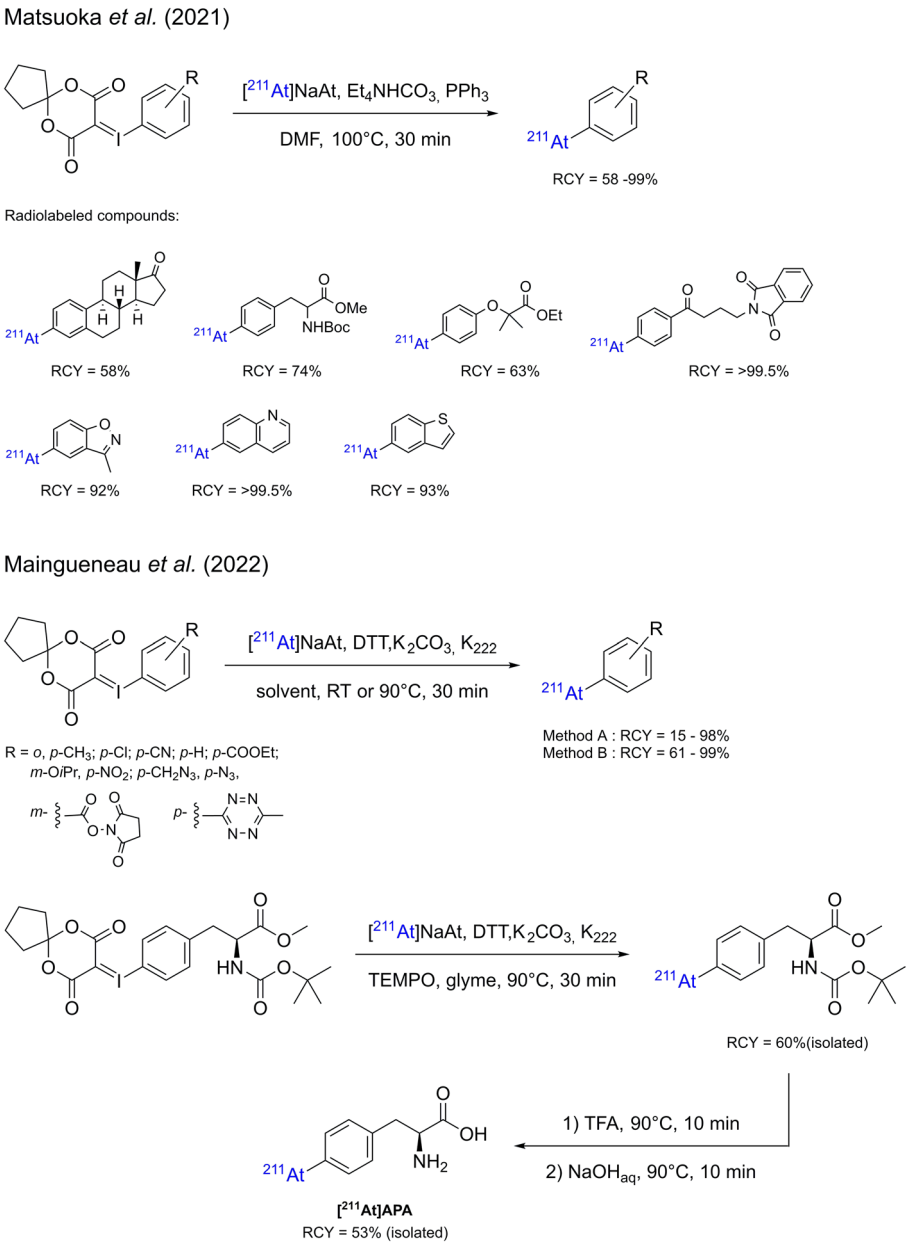
Despite the improvement provided by aryliodonium salts in terms of labelling efficiency and robustness compared to electrophilic destannylation, some limitations related to the lower reactivity and chemoselectivity with electron rich substrates led to the investigation of alternative trivalent aryliodine compounds, namely aryliodonium ylides, which exhibit the advantage of a chemospecificity of nucleophilic substitution at the aryl position, with the auxiliary ligand, a Meldrum's acid derivative, which is non-reactive. Matsuoka et al. and Maingueneau et al. concomitantly investigated the reactivity of these precursors. Matsuoka's study focused on biologically relevant compounds such as the estrone steroid and heteroaryl compounds (Scheme 19). It defined the cyclopentyl Meldrum's auxiliary as the most favourable ligand, PPh_3 as reducing agent, Et_4NHCO_3 as base, with 30 min heating at 100 °C in DMF and 10 mg precursor as optimal conditions, leading to RCYs ranging from 58 to > 99.5% depending on substrate (Matsuoka et al. 2021). On the other hand, the systematic study by Maingueneau et al. optimized the reaction conditions on a set of model (hetero)aryl compounds and showed that the reaction could be quantitative even with electron rich precursors. The cyclopentyl Meldrum's acid auxiliary was also identified as most favourable and two main conditions were defined depending on the reactivity of the substrate. For most reactive (electron deficient) aryl precursors, reaction could be run at room temperature in CH_3CN for 30 min in the presence of K_{222} and K_2CO_3, and only 3 µmol precursor. Less reactive substrates were also labelled quantitatively with an alternative method, using DME as solvent, and heating at 90 °C. At this high temperature, addition of TEMPO was necessary to limit precursor degradation and maintain high RCYs. Several bifunctional precursors for bioconjugation were labelled by this approach and [^{211}At]APA



Scheme 18 Nucleophilic ^{211}At-labelling of diaryliodonium salts

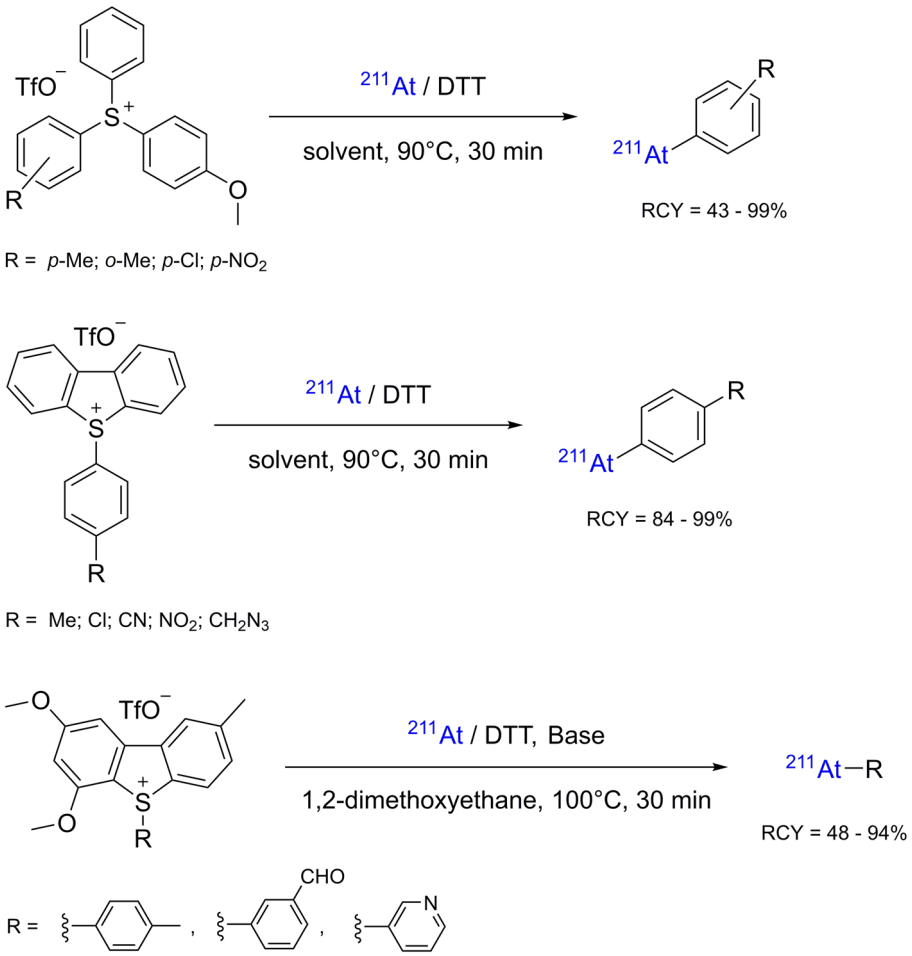
was prepared with a significantly higher molar activity than the conventional halogen exchange approach (Maingueneau et al. 2022a). Overall, aryliodonium ylides can significantly improve RCYs compared to aryliodonium salts. However, they suffer from lower stability that may limit long term storage, and impact molar activity due to the release of cold iodinated analogue upon decomposition.

Sulfonium salts A potential limit of using aryliodide-based substrates in aryliodonium chemistry is the presence of iodinated analogue of the targeted ^{211}At-labelled product that may remain from incomplete purification during preparation of precursor or arising



Scheme 19 ^{211}At-labelling of aryiodonium ylides

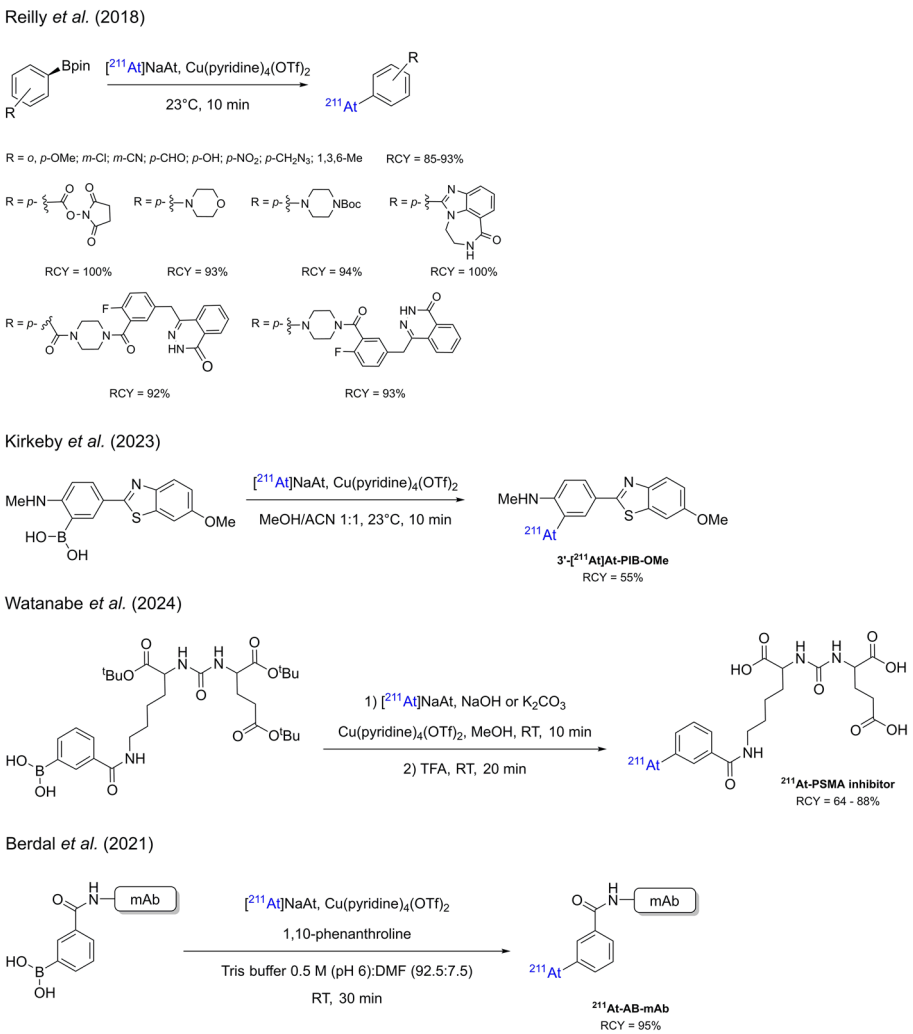
from precursor decomposition on storage or during radiosynthetic steps. Being difficult to separate from product, the resulting A_s may in some case be unsatisfactory. The use of arylsulfonium precursors was recently reported as a potential alternative to solve this issue (Maingueneau et al. 2022b). Triarylsulfonium and dibenzothiophenium salts were evaluated to produce diversely substituted (hetero)aryl compounds (Scheme 20). With 3 potential labelling sites, triarylsulfonium salts appear less attractive although high RCY and selectivity for the labelling site of interest was obtained when targeted compound was substituted by an electron withdrawing substituent such as *p*-Cl or *p*-NO_2. On the other hand, dibenzothiophenium favoured the formation of a single product with high RCY, even when substituted by an electron donor substituent (*p*-Me) and were applied to



Scheme 20 ^{211}At-labelling or sulfonium salts by Maingueneau et al. (2022b)

produce potential prosthetic groups (functionalized by an aldehyde or an azide), as well as ^{211}At-astatopyridine as an example of heteroaromatic compound (Guerard et al. 2023).

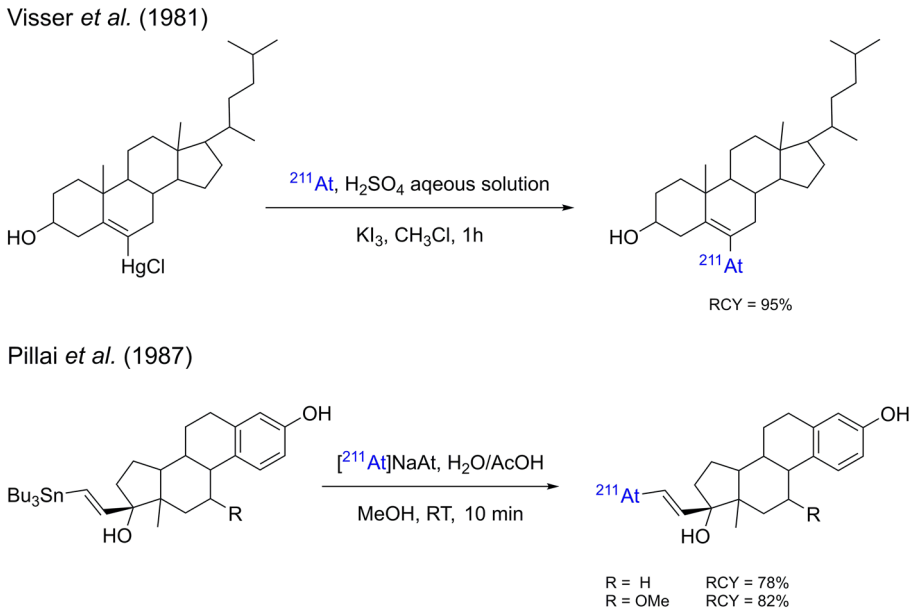
Arylboron The Chan-Evans-Lam cross coupling reaction between arylboronic acids and nucleophiles was recently adapted in radiochemistry, first for ^{18}F-labelling (Tredwell et al. 2014), rapidly followed by radioiodination (Zhang et al. 2016). In 2018, Reilly et al. reported the first method for ^{211}At-labelling by this approach with arylboronic esters as precursors (Reilly et al. 2018). The reaction operates with high efficiency in the presence of a large variety of functional groups with model (hetero)aryl compounds at room temperature in 10 min using tetrakis(pyridine)copper(II) triflate as copper source. Biologically relevant small molecules (such as PARP-1 inhibitors) were radiolabelled but with low RCY. However, the addition of a phenanthroline derivative as ligand improved the reaction to quantitative labelling. Since then, a few examples have been reported from arylboronic acids, including a benzothiazole ([^{211}At]3'-At-PIB-OMe) for amyloid plaque targeting (Kirkeby et al. 2023) and an anti-PSMA inhibitor (Watanabe et al. 2024). The PSMA inhibitor was labelled in a two-step procedure, the second step consisting in the acidic deprotection of carboxylic acids. Interestingly the fraction



Scheme 21 ^{211}At-labelling by copper catalysed nucleophilic astatodeboronation

of ^{211}At bound to the vector was higher after deprotection, reportedly due to conversion of remaining At to its electrophile form in acidic conditions to perform an electrophilic deboronation (Scheme 21).

The high reactivity of the reaction reported in organic media led Berdal et al. to investigate the possibility to run the reaction in aqueous medium (Berdal et al. 2021). Using 1,10-phenanthroline, the reaction was reported to operate efficiently at a pH ranging from 2 to 7. In addition, the arylboronic acid precursor concentration could be decreased to 250 µM, making the approach compatible for the late-stage labelling of proteins in a single step. By this approach, the labelling of an antibody that usually takes two steps (labelling of prosthetic group followed by bioconjugation) was simplified with a single radioactive step (direct labelling on the preconjugated antibody precursor), saving time and improving overall RCY. The biodistribution properties of the labelled mAb were not impacted compared to the 2-step approach and the preconjugated mAb exhibited a shelf life of at least a year, making possible the production of radiolabelling kits.



Scheme 22 Non aryl At-C_{sp2} based ^{211}At-labelled compounds

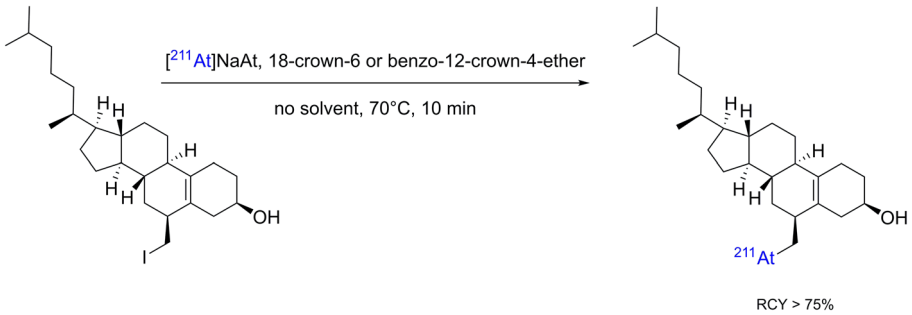
Overall, nucleophilic labelling of arylboronic acid compounds appears as a highly versatile approach that may however find some limitations due to the high amount of copper catalyst generally required (mM concentrations), warranting further research in the nucleophilic astatination reactions.

Other C-At bonds

Non (hetero)aryl C_{sp2}-At based compounds The C-At bond energy has been calculated to be nearly identical in At-vinyl and At-aryl compounds (Amaouch et al. 2016), but few examples of ^{211}At-labelling at a non-aryl C_{sp2} position can be found. Visser employed organomercury chemistry to introduce ^{211}At at the 6-position in chloesterol with high RCY (Visser et al. 1981b). The product was reported to be highly stable in oxidizing (20 mM H_2O_2) or reducing (50 mM sulphite) conditions (Scheme 22). No biological assays were however reported. Later on, Pillai utilized organotin chemistry for the carrier-free synthesis of astatinated vinylsteroid hormones from tri-*n*-butylstannylvinyl steroid precursors (Pillai et al. 1987). This study was carried out in an oxidizing environment (H_2O_2) at room temperature for 10 min, yielding excellent RCYs exceeding 75% and reaching 90% in some cases. Information regarding stability were not also reported.

Compounds labelled via formation of an At-C_{sp3} bond The energy of the carbon-halogen bond plays a crucial role in the stability of the radiolabelled compound. In addition to the observed trend of decreasing bond strength (BS) with the increasing size of the halogen, it is noteworthy that the bond strength also diminishes with increasing hybridization nature of the carbon carrying the halogen (calculated C-At bond enthalpies: alkyne-At > Aryl-At ≈ vinyl-At > alkyl-At) (Amaouch et al. 2016).

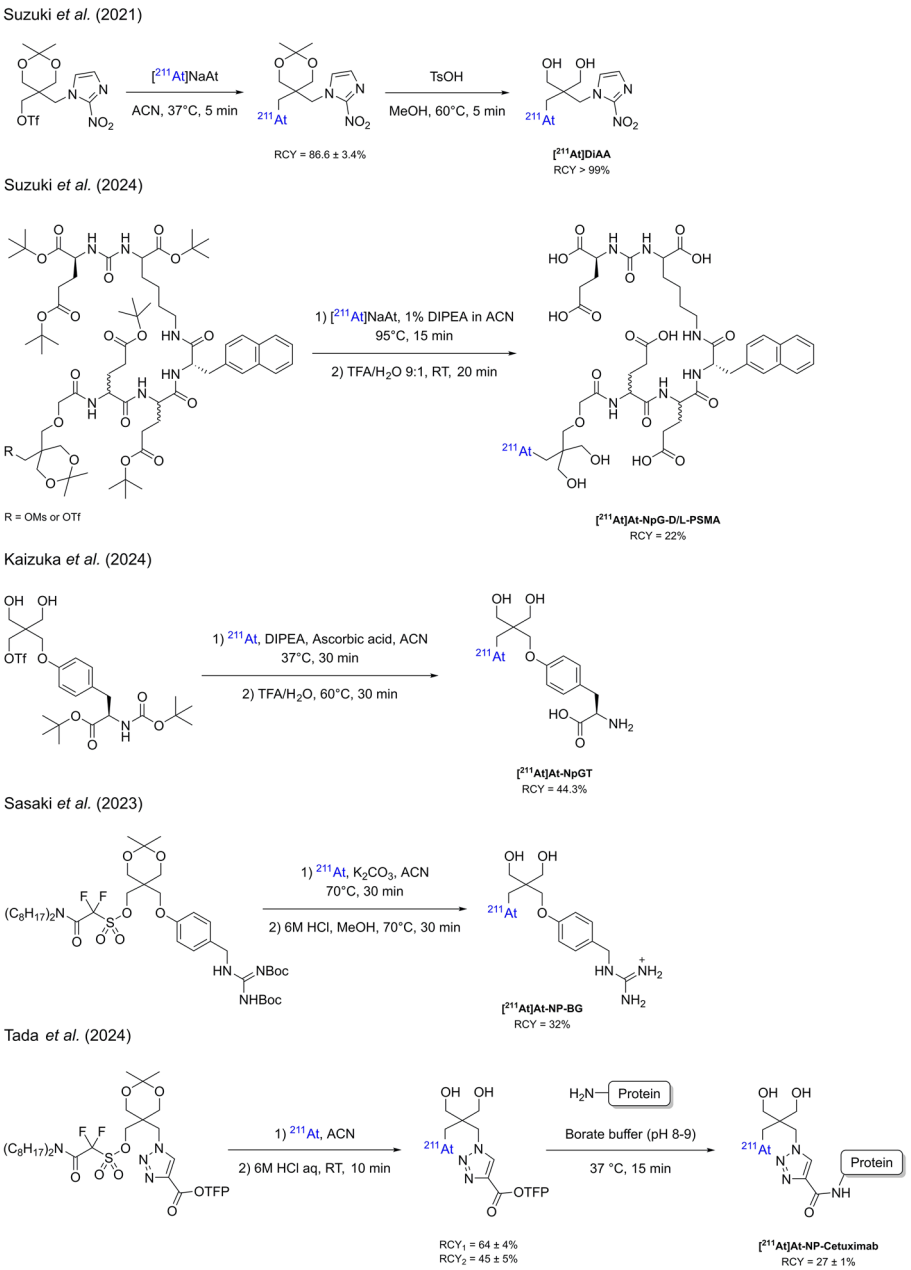
Accordingly, a limited number of molecules astatinated at a C_{sp3} position have been described in early studies (Scheme 23). Liu reported in 1985 the radiolabelling of a cholesterol derivative (NCL-6-I) previously used as a scintiscanning agent for the



Scheme 23 Preparation of 6-[^{211}At]astatomethyl-19-norcholest-5(10)-en-3 beta-ol by Liu et al. (1985)

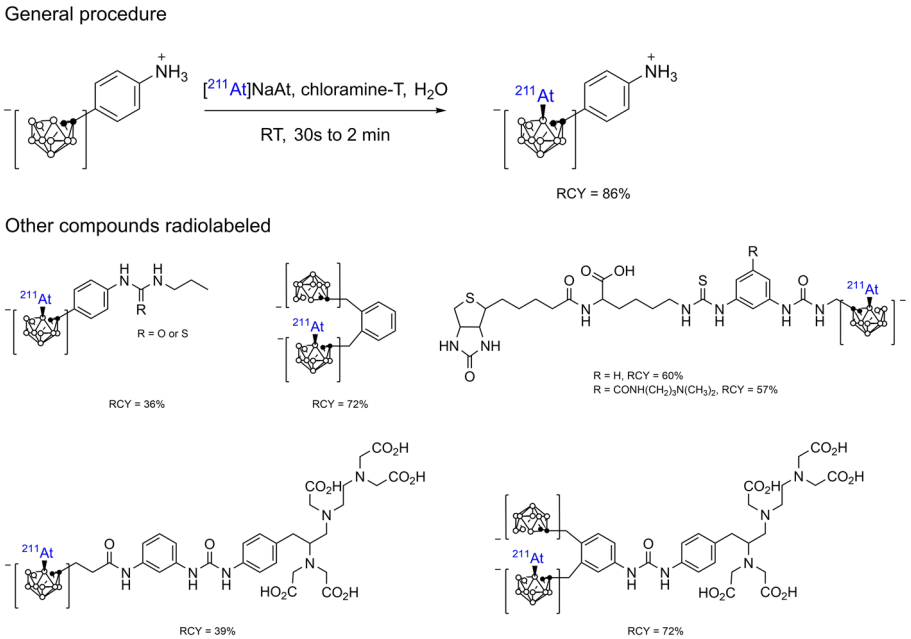
adrenal gland (Liu et al. 1985). Radiolabelling with various heavy radiohalogens (^{211}At / ^{131}I / ^{125}I / ^{82}Br) was achieved through halogen exchange using benzo-12-crown-4 or 18-crown-6 catalysts (Scheme 23). Astatination yields exceeded 80% within 10 min at 70 °C (RCY: ^{211}At > ^{131}I > ^{125}I > ^{82}Br). The better RCY obtained with astatine was attributed to the increased halide reactivity and the enhanced degree of ionic dissociation expected with the astatine anion. Both crown ethers used showed similar exchange efficiencies, with a preference for the 18-Crown-6 ether that provided faster ^{211}At-labelling kinetics. However, biodistribution study indicated that the product was prone to extensive deastatination.

Thirty-five years later, the chemistry of astatination at a sp3 carbon re-emerged in a study reported by Suzuki et al. investigating the usefulness of neopentyl glycol scaffold (NpG), following previous studies reporting the high in vivo stability of ^{18}F-NpG (Scheme 24). The importance of the two hydroxyl groups in NpG was first evaluated with radioiodine by labelling nitroimidazole coupled with NpG moiety, or by analogues exhibiting only one hydroxyl group (the second one replaced by a methyl group), or none (two methyl groups instead). An increasing stability in mouse liver microsomes was observed as the number of hydroxyl groups increased from 0 to 2. Accordingly, the authors selected only the most stable compound to prepare the ^{211}At analogue (Suzuki et al. 2021). [^{211}At]DiAA, the ^{211}At-analogue of 2,2-dihydroxymethyl-3-[^{18}F]-fluoropropyl-2-nitroimidazole ([^{18}F]DiFA) was produced with high efficiency from the triflate precursor by nucleophilic substitution (5 min, 60 °C, CH_3CN, 88%) followed by acidic deprotection of the alcohol groups using TsOH (5 min, quant.). Biodistribution of this compound in non-tumor-bearing mice study indicated that the compound was stable in vivo as the uptake of ^{211}At activity in thyroid and stomach were low and significantly below results obtained with the astatoaryl analogue. The high stability of the carbon-At bond in the NpG group was further confirmed shortly after by applying this scaffold to L-tyrosine as biologically relevant compound (Kaizuka et al. 2024). The biodistribution in mice grafted with C6 glioma tumours of the tyrosine analogue labeled with ^{211}At ([^{211}At]AtNpGT) using this method was nearly identical to the corresponding ^{18}F-labeled analogue ([^{18}F]FNpGT). This finding suggests that this approach could facilitate the development of ^{18}F/^{211}At theranostic probes. This NpG chemistry was also applied to the labelling of NP-BG, the NpG analogue of benzylguanidine (Sasaki et al. 2023),



Scheme 24 ^{211}At- labelled compounds prepared using the NpG scaffold

using 1-*(N,N*-dialkylcarbamoyl)-1,1-difluoromethane sulfonyl (CDf) ester as leaving group instead of triflate to facilitate purification by solid phase extraction. The ^{211}At-labelling step required a temperature of 70 °C and the presence of K_2CO_3 to achieve an optimal RCY of 87%. However, deprotection of acetal and N-Boc groups using 6N HCl resulted in the release of free astatine, highlighting a lack of stability of the protected intermediate or final product under these conditions, leading to a RCY of only 32% for the complete radiosynthetic procedure. No biological results were reported for ^{211}At-NP-BG. In an extension of the NpG group strategy, the same authors developed an activated ester labeled with ^{211}At via the NpG moiety for conjugation to

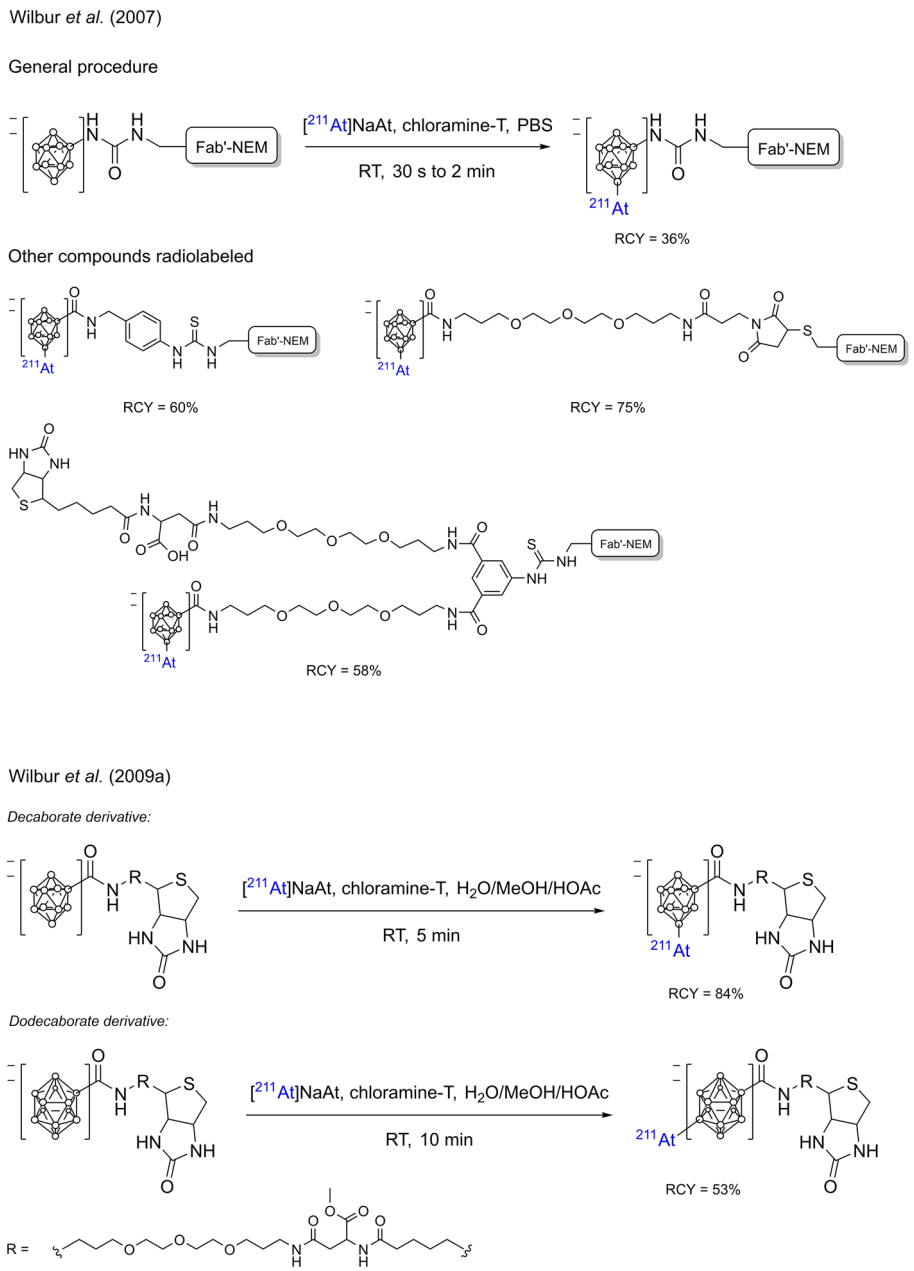


Scheme 25 ^{211}At-labelled nido-carboranyl compounds (Wilbur et al. 2004a, 2004b)

proteins (Tada et al. 2024). A triazole spacer was included to augment the nucleophilic astatination rate. A specific purification procedure was developed that consisted of trapping the labelled compound and precursor on a SPE resin, deprotecting the labelled intermediate directly on the resin using 6 M HCl and selectively releasing the labelled product. This approach provided the prosthetic group with a 45% RCY and 99% RCP, which was then conjugated to Cetuximab mAb with a RCY of 27% in 10 min. Biodistribution in normal mice showed low accumulation of activity in stomach and thyroid, similar to its ^{125}I-labelled analogue, indicative of a good in vivo stability. The NpG scaffold was also applied to labelling a PSMA compound with a RCY of 22%, the resulting radiopharmaceutical exhibiting also a good in vivo stability and promising biodistribution profile for further therapeutic investigations (Suzuki et al. 2024).

Boron-At bonds

Based on the observation that B-I bonds in boron clusters are stronger than aryl C-I bonds, Wilbur hypothesized that the same bond energy trend would also be observed with astatine. In a seminal study testing this approach on a series of simple model compounds, it was reported that nido-carboranyl moiety could be rapidly labelled electrophilically in mild conditions in water by ^{211}At oxidized by chloramine-T with 39–72% RCY (Wilbur et al. 2004a) (Scheme 25). Biodistribution of ^{211}At-labeled nido-carboranyl-bearing compounds did not indicate a strongly improved stability compared to ^{211}At-benzamide model compounds, but the bis-nido-carborane moiety imparted significantly higher stability. This positive result was however at the expense of a strong liver uptake of ^{211}At activity. The same authors reported shortly afterwards



Scheme 26 ^{211}At-labelled decaborates and dodecaborates (Wilbur et al. 2007, 2009a)

the synthesis of ^{211}At-labeled biotin derivatives using anionic nidocarboranyl pendant group (Wilbur et al. 2004b).

The improvement in labelling stability was confirmed compared to astatoaryl analogues but release of free At was still observed, which prompted the authors to search for alternative boron clusters to reach optimal stability. This was done using the decaborate moiety, that, when diversely functionalized and conjugated to a Fab' fragment, proved highly stable in vivo (Wilbur et al. 2007) (Scheme 26). However, this too was associated with an increase in liver uptake compared to the native carrier compound. Further attempts to reduce off-target tissue uptake included evaluation of the dodecaborate

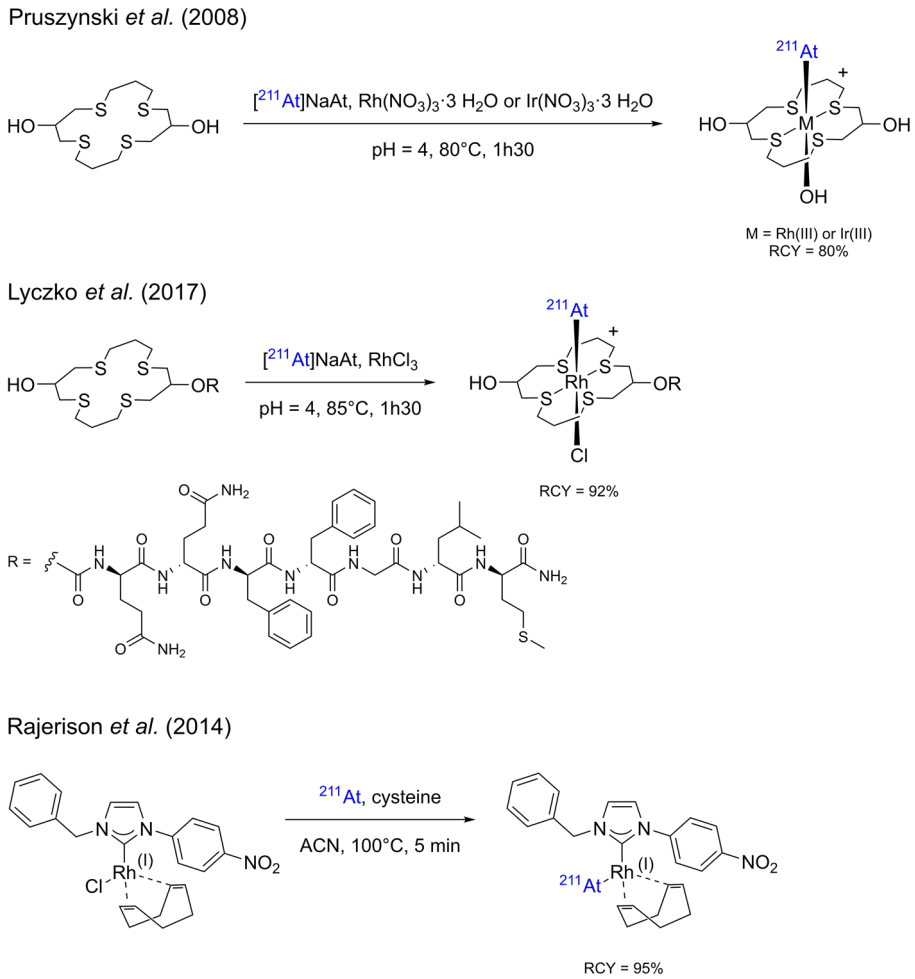
moiety that turned out to be less efficient in ^{211}At-labelling reaction and more retained in kidneys when attached to biotin (Wilbur et al. 2009a). Also, alternative conjugation methods were developed (Wilbur et al. 2009b). An interesting report also evaluated the use of hydrazone, acting as an acid sensitive linker between the carrier (here a Fab') and the labelled decaborate moiety, and expected by the authors to be cleaved in the kidneys, more specifically following glomerular filtration and reabsorption into tubule cells where radiolabelled protein derivatives are exposed to the low pH of lysosomes (Wilbur et al. 2011), with the aim of facilitating the release of radiolabelled catabolites from the kidney and limit long retention of radioactivity in this sensitive organ. While the initial hypothesis was confirmed with the radioiodinated Fab' compounds for which low kidney retention of radioactivity was observed (4.23 ± 1.57 ID/g 24 h post injection), no effect of the hydrazone linker occurred for the ^{211}At-analogue (39.52 ± 15.87%ID/g). While it is not clear what causes the difference in the behaviour between both halogens, it appears clear that the presence of At on the boron cluster has an impact on the degradation of the linker, leading to different catabolites, or that the presence of At alters the retention of these catabolites in kidneys.

Overall, the use of decaborate for labelling with ^{211}At appears as one best alternatives to SAB. In preclinical studies, it was found to be efficient for the labelling of intact antibodies and clinical trial are ongoing as discussed in detail in the second part of this review. However, optimization of this chemistry to be applicable to smaller carrier compounds is needed to minimize off-target irradiation.

Metal-At bond

According to Pearson's Hard and Soft Acids and Bases theory, the astatide anion being a soft base, it is expected to form strong interaction with soft metal ions. The investigation of this strategy was initiated by Pruszyński with Hg^{2+} (Pruszyński et al. 2006). For this, the hydroxide to astatide ligand exchange reaction in the hydrolysed mercury(II) nitrate complex was monitored by electromigration experiments and ion exchange chromatography. In addition to the formation of the expected Hg(OH)At complex, it is interesting to note the significantly higher stability constant measured compared to the Hg(OH)I analogue (log $K_1 = 5.4$ and 4.1 respectively), confirming the trend expected from the HSAB theory. Building on these promising finding, a labelling strategy was developed using rhodium(III) and iridium(III) as metallic centres, astatide as ligand and the macrocyclic 16-S4-diol chelator to stabilize the complex (Pruszyński et al. 2008). It was shown that the complex formed most efficiently in mildly acidic pH within 30 min with microwave irradiation at 75–80 °C. In a follow up study, the biostability was evaluated in human blood serum and biodistribution in normal mice with positive results highlighted by a limited release of free At in mice (Pruszyński et al. 2015). Subsequently, an activated analogue of the 16-S4-diol ligand was conjugated to the substance P peptide with good stability in cerebrospinal fluid, which validated the potential of this approach (Lyczko et al. 2017). No direct comparison with standard astatoaryl chemistry was however performed, which does not allow conclusion on a potential gain in stability provided by this approach (Scheme 27).

On the assumption that Rh(I), a softer metal centre than Rh(III), would exhibit a stronger interaction with astatide, Rajerison et al. studied the formation of a Rh(I)At



Scheme 27 ^{211}At labelling by formation of an At-metal bond

complex, the metal being bound to a *N*-heterocyclic carben, a ligand known to strongly stabilize low valency transition metal centre (Rajerison et al. 2014). Starting from the already formed complex with chloride as leaving group, the ^{211}At-labelled complex was formed with RCY > 80% within 15 min in acetonitrile and did not show decomposition for 15 h in human blood serum. Stability in animals were not reported to date.

Complexation of metallic astatine

A noticeable property of astatine is its dual behaviour: halogen and metal. Astatine's metallic character is reflected in typical properties shared with metals, i.e. the stability of At^+ in non-complexing aqueous medium unlike lighter halogens, but also the ground state reversal of AtO^+ upon hydration. In addition, its propensity to form complexes with anionic and neutral ligands has been well documented (Guérard et al. 2021).

First evidence of complex formation was reported in the late 1970's with halogenide ligands in electromobility experiments from which formation of AtX_2^- and $AtOX_2^-$ (X = Cl, Br or I) was assumed (Dreyer et al. 1979). The softer ligand Br^- formed stronger complexes than Cl^-. Follow up studies confirmed this behaviour with neutral ligands forming AtL_2^+ in the presence of thiourea and thioacetamide (Dreyer et al. 1985) or their

selenium analogues (Fischer et al. 1987). Selenoureas achieved higher stability in this series of monodentate ligands. A larger study comparing 18 polydentate linear ligands comprised of sulphur, oxygen and nitrogen donor atoms was also reported (Ludwig et al. 1991). Interestingly, while higher complex stability was expected for ligands containing sulphur atoms, presence of nitrogen atoms was essential to achieve high stability, while no complexes were formed with ligands containing only nitrogen as heteroatoms. Well known chelating agents for metal complexation were also investigated by Milesz et al., including EDTA (Milesz et al. 1988) and DTPA (Milesz et al. 1989). Depending on pH, positively charged, neutral and negatively charged species were observed, confirming the formation of AtL type complexes, the charge depending on the protonation of the ligand.

More recently, the metallic properties of At^+ and AtO^+ were revisited by using an alternative method based on the partition of At complexes in a biphasic system (Champion et al. 2009). Thiocyanate and a thiacalixarene were investigated as ligands, resulting in higher complex stability with At^+ than AtO^+. To strengthen these results, relativistic density functional theory (DFT) calculations were developed in follow up studies, whose results were in line with experimental data for various complexes formed with halogenides and thiocyanate or could predict experimental results (Champion et al. 2011; Guo et al. 2016). Contribution of molecular modelling is essential in understanding astatine properties and two recent studies highlighted the unexpected behaviour of the AtO^+ with ligands that do not form coordination complexes. For instance, it was concluded that the AtO^+ cation binds covalently to the sulphur or the carbon atom of the ligand via its oxygen atom (Bassal et al. 2020) or to the oxygen atom of ketone via its At atom (Burns et al. 2020).

To date, no efficient radiolabelling strategies were developed to produce radiopharmaceuticals by At complexation. The scarce reported examples lead to unstable complexes (Liu et al. 1998; Yordanov et al. 2000) whose nature are highly speculative considering the experimental conditions and in light of the recent theoretical investigations cited above. Current efforts put in At chemistry investigations could however renew the interest for this labelling approach.

^{211}At-labelled Prosthetic groups for proteins labelling

Prosthetic groups have played a crucial role in the advancement of radiopharmaceutical development for radiolabelled peptides and proteins. Several prosthetic groups have been introduced for radiolabelling of biomolecules in order to minimize the impact on biological activity. This is achieved either by first radiolabelling of the prosthetic group, followed by conjugation with the biomolecule under mild conditions (referred to as the 2-step approach), or by first conjugating the prosthetic group precursor to the biomolecule and then radiolabelling the conjugate (known as direct or late-stage radiolabelling). Both approaches aim to enhance radiolabelling efficiency and result in the formation of a stable biomolecule suitable for in vivo studies. These groups provide a broad range of options for radiolabelling, including the ability to label through lysine, tyrosine and cysteine residues. Additionally, they allow for site-specific labelling on *N*-terminal cysteine residues (Bibi et al. 2024).

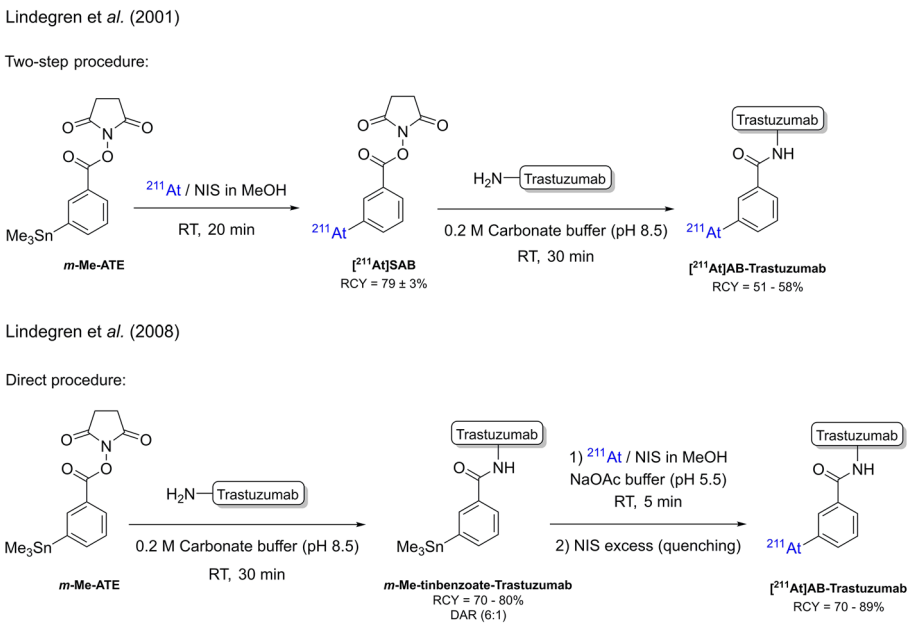
The development of prosthetic groups for astatination of biomolecules originates from similar precursor molecules developed for radioiodination (Petrov et al. 2022). The

necessity for prosthetic groups arises from the requirement for adequate stability for in vivo applications, as direct labelling on tyrosine residues is ineffective. Here we elaborate on three of the most advanced examples: SAB, SAGMB and the boron cluster B10 (closo-decaborate).

N-succinimidyl 3-[^{211}At]astatobenzoate ([^{211}At]SAB)

Radioimmunotherapy with anti-HER2 monoclonal antibodies (mAbs) such as trastuzumab is a promising strategy for treating HER2-positive breast, ovarian and gastric carcinoma patients. Akbani and colleagues (Akabani et al. 2006) have investigated the therapeutic potential of ^{211}At-trastuzumab for the treatment of HER2-positive cancers. Trastuzumab was labelled with using [^{211}At]SAB as described by Zalutsky and colleagues (Zalutsky et al. 2001). Labelling was performed in 30 min at room temperature in chloroform from *N*-succinimidyl 3-(tri-*n*-butylstannyl)benzoate with *tert*-butylhydroperoxide, and acetic acid to form the electrophilic At species. Purification of SAB was performed by solid-phase extraction followed by evaporation to dryness before conjugation to trastuzumab in borate buffer (pH 8.5–8.9) at room temperature for 15 min. The final ^{211}At-labelled trastuzumab was obtained after size-exclusion chromatography (SEC) with a RCP of > 98% (the RCY was not reported). The authors have demonstrated that under single cell conditions, ^{211}At-trastuzumab effectively killed HER-2 positive breast carcinoma cells with a relative biological effectiveness about 10 times higher than external beam radiotherapy, and requiring much lower mAb concentration compared with cold drug alone. This is highly important to overcome dose-limiting toxicities in patients with low or heterogeneous HER-2 expression, which have a poorer response rate to trastuzumab treatment. An important finding of this study was the demonstration that subpopulations of tumour cells were not killed by α-particle therapy, which was consistent with a lower target expression. They attributed this effect to competition of cold mAb at low specific activity (A_s), showing the relevance of this parameter. They have developed a pharmacokinetic model that considered the kinetics of receptor binding and internalization and the heterogeneity of receptor expression within a population of tumour cells, demonstrating the effects of competition between cold and radiolabelled mAb.

Palm and colleagues (Palm et al. 2007) have investigated the potential use of ^{211}At-labelled trastuzumab for the treatment of HER-2-positive, radioresistant SKOV-3 ovarian carcinoma xenografts. SAB was prepared as described before by Lindegren and colleagues (Lindegren et al. 2001). Here, ^{211}At in NaOH was added to a solution of *N*-succinimidyl 3-(tri-*n*-methylstannyl)benzoate (Me-ATE for methyl-activated tin ester) and *N*-iodosuccinimide (NIS) in methanol: 1% acetic acid, followed by incubation for 10 min at room temperature. Before antibody conjugation, unreacted astatine and NIS were reduced by the addition of sodium ascorbate. Trastuzumab was then added to the crude labelling mixture and allowed to react for 20 min. The ^{211}At-trastuzumab was finally isolated by SEC with a RCY of 37% and RCP > 95% (Scheme 27). The radioimmunoconjugate showed intact immunoreactivity (75–89% to SKOV-3 cells). It is noteworthy that this one-pot procedure yielded improved RCY due to the absence of a purification step before conjugation. This was made possible by using only 0.5 nmol of precursor compared to previously reported procedures using μmol amounts of precursor (Zalutsky et al. 2001). A dose–response study using a single injection of



Scheme 28 Two-step and one-step procedures developed by Lindegren for the ^{211}At-labelling of antibodies via the astatobenzoyl prosthetic group

^{211}At-trastuzumab with high A_s showed that increasing ^{211}At activity resulted in better therapeutic response. However, no additional benefit was found when using a fractionated treatment regimen. Instead, the authors hypothesized that unlabelled trastuzumab can be used to radiosensitize radioresistant tumour cells. Indeed, they found a proportional relationship between the amount of cold trastuzumab and reduction of tumour growth, which can be explained by antibody-dependent cellular cytotoxicity. The combination of 500 µg of trastuzumab and 400 kBq of ^{211}At-trastuzumab resulted in complete tumour eradication in SKOV-3 xenografts.

While beta particle-emitting radionuclides are best suited for the treatment of bulk diseases due to the relatively long tissue range, α particles are more attractive to be targeted to micrometastases or compartmentally spread disease due to a higher fraction of emitted energy absorbed by the tumour cells. Applications relevant to HER2 regarded to benefit from alpha particle therapy include ovarian carcinoma and breast cancer carcinomatous meningitis, which is characterized by the dissemination of malignant tumour cells into the subarachnoid space along the brain and spine. The therapeutic effect of ^{211}At-trastuzumab was demonstrated in a rat model of breast carcinoma carcinomatous meningitis following intrathecal administration (Boskovitz et al. 2009), resulting in significant prolongation in median survival and in some cases complete remission.

To provide a radioimmunoconjugate amenable for clinical applications, Lindegren and colleagues (Lindegren et al. 2008) have developed a late stage astatination procedure consisting of a direct radiochemical reaction of antibody/Me-ATE conjugates. The trastuzumab conjugates were prepared via reaction with the epsilon-lysine amines on the antibody and isolated by size exclusion chromatography to remove excess m-MeATE, resulting in the antibody with epsilon-lysyl-3-(trimethylstannyl)benzamide residues (Scheme 28).

The immunoconjugates obtained were then labelled with electrophilic astatine produced using NIS, followed by quenching of the remaining stannyl residues with excess NIS (Scheme 28). Compared with the 2-step radiolabelling approach, the direct astatination reaction proceeded almost instantaneously (15 *vs.* 60 min) and with improved RCY (60–80% vs. 30–60%). According to the authors, the reasons for this increased reactivity are twofold: the solvent-exposed epsilon-lysyl-3-(trimethylstannyl)benzamide residues of the immunoconjugates are more accessible; and the use of NIS as oxidizing agent to activate astatine results in the formation of the dihalide AtI or the trihalide anion AtI_2^- which act as good electrophiles in the destannylation reaction. In addition, the direct radioastatination method is less dependent on the concentration of the antibody conjugate, leading to higher A_s (1 GBq/mg) at a high-activity level with less radiolysis. The resulting directly labelled ^{211}At-AB-trastuzumab showed quality, stability and biodistribution comparable to ^{211}At-AB-trastuzumab obtained by the conventional 2-step procedure. However, a lower affinity (K_d) of directly astatinated trastuzumab (1.0 ± 0.06 nM *vs.* 0.44 ± 0.06 nM) was observed due to the presence of a high number of prosthetic groups per antibody (6:1), which may decrease the binding of the mAb to its antigen. Importantly, the trastuzumab-based immunoconjugates showed good shelf-life making it amenable for long distance shipping (Aneheim et al. 2015c).

To facilitate clinical translation, the synthesis of ^{211}At-labelled antibodies should be reproducible, reliable and have a short synthesis time. In this regard, automation of the synthesis of radiolabelled antibodies is very important. A radiosynthesis module is usually housed within a hotcell with all operations controlled by an external computer software. The system can perform the radiopharmaceutical synthesis, purification, and reformulation automatically, without manual intervention, with the final radiopharmaceutical product ready for use automatically dispensed into a presterilized vial. Aneheim and colleagues (Aneheim et al. 2014) have developed a system for the direct astatination of antibodies preconjugated with the tin precursor, which has the benefit of decreasing the number of synthesis steps and thus simplifying technical implementation (Fig. 2). The Me-ATE-Trastuzumab immunoconjugates were labelled at reasonnably high activity levels (up to 219 MBq) using the synthesis module resulting in ^{211}At-trastuzumab with good RCY (62%), high A_s (280 MBq/mg) and uncompromised immunoreactivity. Of critical importance to obtain a high RCY was the addition of NIS in methanol to the dry astatine residue. The automated system was further developed to also include dry distillation of astatine from the irradiated target material (Aneheim et al. 2015a). The choice of elution solvent proved to be critical for achieving a high RCY. When using chloroform as eluent, the time window to perform radiolabelling was longer than when using methanol. This new platform may allow hospitals with access to ^{211}At to perform all production steps to obtain ^{211}At-labelled radiopharmaceuticals for large scale clinical trials.

The efficacy of the directly astatinated trastuzumab, was evaluated in a clinically relevant mouse model of liver metastasis from a primary gastric cancer that expresses HER2 (Li et al. 2021). A systemic injection of ^{211}At-trastuzumab was able to achieve complete eradication of liver metastasis and prolonged mouse survival. The estimated dose in metastatic tumours was tenfold higher compared with the normal liver, suggesting specific target accumulation of the alpha particles.

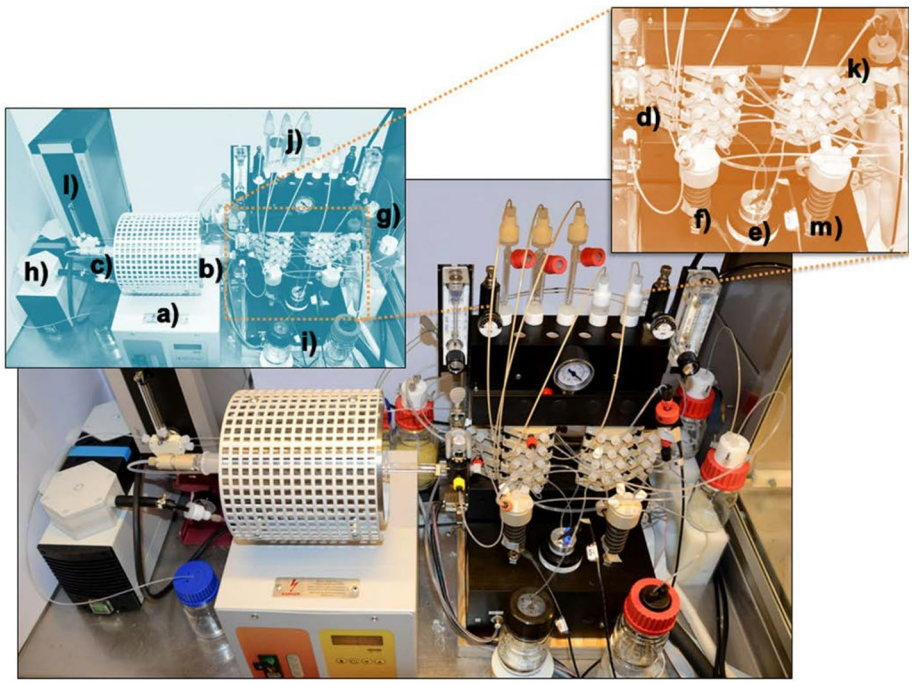


Fig. 2 Automated system for ^{211}At purification and synthesis. Reproduced from Aneheim et al. 2015a

Due to their excellent properties, including high in vivo affinity and specificity of binding, together with fast kinetics of elimination, single-domain antibody fragments (sdAbs; aka nanobodies or VHH) have gained great interest in targeted alpha therapy. In 2019, Dekempeneer et al. reported a potential therapeutic strategy that uses an HER2-targeting sdAb, referred to as 2Rs15d, labelled with ^{211}At, to selectively target HER2 + cancer cells (Dekempeneer et al. 2019). In this study a direct labelling procedure was followed, where *m*-MeATE was conjugated to ε-amino groups of lysine residues on a 2Rs15d sdAb and thereafter labelled with ^{211}At. The RCY for the one-step radiolabelling of 2Rs15d sdAb was higher when compared with the two-step radiolabelling approach (60–80 vs. 25–40%). Likewise, the A_m was also higher for the directly labelled conjugates (2.3–3.3 vs 0.9–1.3 MBq/nmol). Despite promising in vitro plasma stability, ^{211}At-2Rs15d revealed low in vivo stability with signs of deastatination, as seen by the high radioactivity accumulation in the lung and stomach.

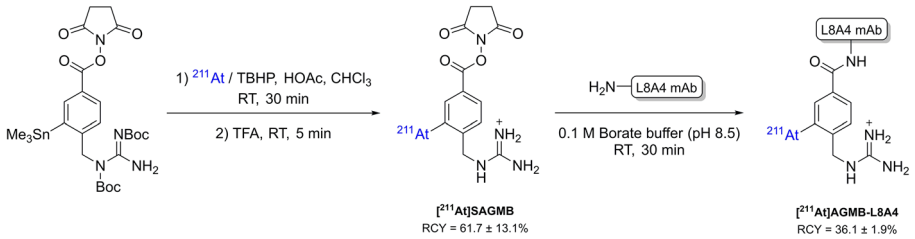
Clinical trials The clinical application of [^{211}At]SAB has been reported for the labelling of antibodies and antibody fragments for the treatment of several tumour types (reviewed in Albertsson et al. 2023). Two landmark phase one trials have investigated the efficacy of intracavitary radioimmunotherapy to treat solid tumours (NCT00003461 and NCT04461457). This local administration of the radiopharmaceuticals optimizes the dose delivered to tumour cells that are present at a defined anatomical cavity and limits the release of ^{211}At from its carrier molecule compared to a systemic injection, thus minimizing the issue related to stability. Zalutsky et al. have reported for the first time the evaluation of a ^{211}At-targeted therapy in cancer patients (Zalutsky et al. 2008). They have demonstrated that radioimmunotherapy with ^{211}At-ch81C6 mAb targeting tenascin C, an extracellular matrix protein overexpressed in high-grade gliomas, injected locally

in patients with malignant gliomas was well tolerated and associated with a promising antitumour benefit in patients. This was also the first clinical trial showing the benefit of highly potent and short-range alpha particles, which are less dependent on tumour oxygenation for achieving efficient cell killing compared with beta-emitting radioimmunotherapies, for the local treatment of residual diseases. Labelling of chimeric 81C6 was accomplished using a 2-step radiolabelling approach by first synthesizing [^{211}At]SAB and then conjugating it with the mAb. During the study, the authors were unable to scale up activity levels of ^{211}At-ch81C6 due to radiolysis-mediated effects lowering of SAB and mAb radiolabelling yields when high ^{211}At activity levels were used (Zalutsky et al. 2001). It was reported by the same group and others that the solvent-mediated radiolytic decomposition of the tin precursor and/or the [^{211}At]SAB at high radiation doses can occur, especially when the astatination reaction was performed in chloroform (Aneheim et al. 2019; Pozzi and Zalutsky 2005). Another possible explanation was the radiation-induced change of astatine species required for efficient radiolabelling reactions (Aneheim et al. 2019; Ghalei et al. 2022; Pozzi and Zalutsky 2007). As an alternative, other studies suggest the use of methanol as a solvent for [^{211}At]SAB radiosynthesis. The formation of reducing species during methanol radiolysis is also possible. Indeed, the formation of a reduced form of astatine, unsuited for electrophilic labelling reactions, was increased with the radiation dose. However, the formation of this astatine species could be mitigated using an oxidizing agent, such as NCS, to regenerate the reactive electrophilic astatine species during radiolabelling (Pozzi and Zalutsky 2017, 2007). In order to obtain clinical doses of ^{211}At-81C6, Vaidyanathan and colleagues have added NCS to methanol, to counteract radiolytic decomposition of the tin precursor and to avoid formation of reduced astatine, in a high activity level SAB synthesis. Starting with 2.74 GBq of ^{211}At, they were able to obtain a clinically relevant activity level ^{211}At-81C6 (1.0 GBq) (Vaidyanathan et al. 2020).

In a second clinical study, intraperitoneal administration of ^{211}At-labelled MX35-F(ab′)$_2$ was evaluated for the treatment of patients with recurrent ovarian cancer (Andersson et al. 2009). This antibody fragment targets a cell surface protein (NaPi2b), which is overexpressed in human epithelial ovarian cancers. This phase 1 clinical study was supported by promising preclinical data, where efficient cell killing in tumours with 0.5 mm in diameter was observed. Here, intraperitoneal infusion of ^{211}At-MX35-F(ab′)$_2$ demonstrated to be well tolerated and was efficient at eradicating peritoneal micrometastases. However, only limited ^{211}At-labelled mAb activity levels (150 MBq) were obtained, which limited the evaluation of the maximum tolerated dose.

N-succinimidyl-3-[^{211}At]astato-4-guanidinomethylbenzoate ([^{211}At]SAGMB)

[^{211}At]AGMB-L8A4 To develop a strategy for ^{211}At-labelling of highly internalizing monoclonal antibodies (mAbs) in general, and those targeting the epidermal growth factor receptor variant III (EGFRvIII) in particular, Vaidyanathan et al. investigated the potential of the guanidine-containing agent, [^{211}At]SAGMB, for labelling the anti-EGFRvIII mAb L8A4 (Vaidyanathan et al. 2003). Previous work by the same group had demonstrated that when L8A4 was labelled with *N*-succinimidyl 4-guanidinomethyl-3-[^{131}I]iodobenzoate ([^{131}I]SGMIB), intracellular retention of radioiodine was three-to-four-fold higher than that observed for the same mAb labelled via direct



Scheme 29 ^{211}At-labelling of an antibody using the [^{211}At]SAGMB prosthetic group agent

electrophilic iodination or that labelled with a less basic positively charged structure such as *N*-succinimidyl-3-[^{125}I]iodo-5-pyridinecarboxylate ([^{125}I]SIPC) (Vaidyanathan et al. 2002, 2001). [^{211}At]SAGMB in its Boc-protected form was prepared from a tin precursor in 62% RCY and, following deprotection by TFA, was coupled to the L8A4 mAb in 36% RCY (Scheme 29). The lipophilicity of [^{211}At]AGMBA (3-[^{211}At]astato-4-(guanidinomethyl)benzoic acid) was compared to that of its iodine analogue, [^{131}I]GMIBA, by means of a paired-label octanol-saline partition coefficient experiment (Vaidyanathan et al. 2003). At the physiologic pH, the partition coefficient of AGMBA was 0.03 ± 0.01 compared with 0.05 ± 0.01 for GMIBA. At lysosomal pH (pH 4.75), a partition coefficient of 0.13 ± 0.01 was measured for both AGMBA and GMIBA. This means that an increase in lipophilicity due to astatine for iodine substitution was not significant.

Paired-label internalization assays in the EGFRvIII-expressing cell line U87MGΔEGFR demonstrated that tumour cell retention of radioactivity for [^{211}At]AGMB-L8A4 was almost identical to that of [^{131}I]GMIB-L8A4 (22.7 ± 1.4% and 26.6 ± 1.7%, at 16 h, respectively) and three-to-four-fold higher when compared to ^{131}I-L8A4 directly labelled using Iodogen.

Paired-label biodistribution studies of labelled [^{211}At]AGMB-L8A4 and [^{131}I]GMIB-L8A4 in athymic mice bearing U87MGΔEGFR xenografts demonstrated identical tumour uptake of both ^{211}At- and ^{131}I-labelled L8A4 mAb over 24 h. Although higher levels of ^{211}At compared with ^{131}I were occasionally observed in tissues known to sequester free astatide, such as the stomach, lungs and spleen, these ^{211}At/^{131}I uptake ratios were significantly lower than those seen with other labelling methods, suggesting improved inertness towards in vivo dehalogenation.

[^{211}At]AGMB-2Rs15d sdAb In their search for an optimal method of labelling 2Rs15d sdAb with ^{211}At**,** Dekempeneer et al. investigated the potential of SAGMB (Dekempeneer et al. 2019). For this, Boc$_2$-SGMTB was labelled with ^{211}At and conjugated to the lysine residues of the 2Rs15d following the above-mentioned two-step labelling procedure. RCY ranged between 25 and 40% with a A_m between 0.9 and 1.3 MBq/nmol. The resulting radioconjugate, [^{211}At]AGMB-2Rs15d, was tested in vitro in HER2 + cells and in vivo in SKOV-3 xenografted mice and the data collected were compared to those obtained for [^{211}At]AB-2Rs15d and 2Rs15d labelled with *N*-[2-(maleimido)ethyl]-3-(trimethylstannyl)benzamide (MSB) prepared by direct labelling with ^{211}At. Because of the additional bioconjugation step required, both RCY (25–40%) and A_m (0.9–1.3 MBq/nmol) were lower for [^{211}At]AGMB-2Rs15d. RCP exceeded 95% after purification and was similar to the other astatinated

conjugates. Although [^{211}At]MSB labelled 2Rs15d revealed to be less stable, both [^{211}At]AGMB-2Rs15d and [^{211}At]AB-2Rs15d were found to be stable in PBS and in serum up to 24 h.

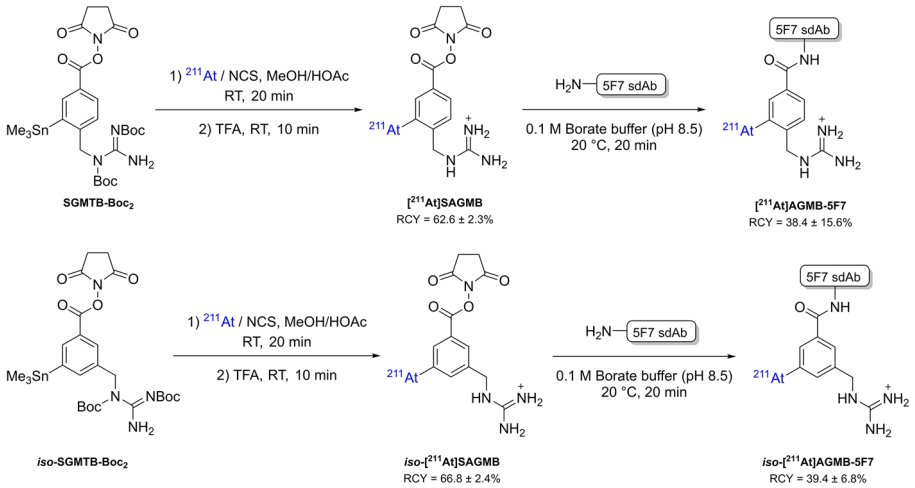
Specific targeting to SKOV-3 cells was observed in vitro for all radioconjugates with a similar degree of internalization over time. While the biodistribution pattern revealed comparable tumour uptake for all radioconjugates (higher than 8% ID/g at 1 h) [^{211}At]AGMB-2Rs15d showed only minimal uptake in normal tissues. Only in the kidneys, a higher uptake was measured after 1 h, but it decreased rapidly after 3 h. Alpha-camera imaging of tumours indicated a uniform distribution of radioactivity in tumour tissue at all time points for all three conjugates. The radioactivity in the kidneys was initially concentrated in the renal cortex, while after 3 h most radioactivity was measured in the medulla, confirming the fast washout into urine.

When compared to [^{211}At]AB-2Rs15d and to [^{211}At]MSB-labelled 2Rs15d, [^{211}At]AGMB-2Rs15d seemed to be the preferred radioconjugate to target HER2 + cancer cells due to its high tumour uptake, low background signals as a result of its higher stability, and fast renal excretion.

[^{211}At]AGMB-5F7 and iso-[^{211}At]AGMB-5F7 Several sdAbs with high affinity for HER2 have been generated and reported to target HER2-positive cancers (Keyaerts et al. 2016; Pruszynski et al. 2013; Vaneycken et al. 2011). Encouraging results were obtained when an anti-HER2 sdAb (5F7) was labelled with [^{131}I]SGMIB (Pruszynski et al. 2014). The advantage of using [^{131}I]SGMIB for labelling 5F7 sdAb conjugates has been described in previous publications that demonstrated increased intracellular retention and in vivo tumour uptake when compared with 5F7 sdAb labelled by direct iodination (Pruszynski et al. 2014, 2013) or with uncharged halobenzoyl reagents (Vaidyanathan et al. 2016). The potential utility of [^{211}At]SAGMB and its isomer *N*-succinimidyl 3-[^{211}At]astato-5-guanidinomethyl benzoate (*iso*-[^{211}At]SAGMB) wherein the guanidinomethyl group was moved from the *ortho* to the *meta* position for ^{211}At-labelling of the anti-HER2 single-domain antibody fragment 5F7 was later investigated by Choi and colleagues (Choi et al. 2018). Both [^{211}At]SAGMB and *iso*-[^{211}At]SAGMB, were synthesized by halodestannylation of the respective tin precursor and coupled to the anti-HER2 5F7 sdAb with similar RCYs (63% *vs* 67%, respectively) and coupling efficiencies (38% vs 39%, respectively). RCPs were higher than 98% (Scheme 30). The radioiodinated analogues [^{131}I]SGMIB and *iso*-[^{131}I]SGMIB were also synthesized and coupled to the anti-HER2 5F7 sdAb for comparison.

To determine the extent of intracellular trapping of radioactivity, in vitro paired-label internalization assays of [^{211}At]AGMB-5F7, *iso*-[^{211}At]AGMB-5F7, [^{131}I]GMIB-5F7 and *iso*-[^{131}I]GMIB-5F7, were performed on HER2-expressing BT474M1 cells. High intracellular trapping of radioactivity was observed, with little difference between corresponding ^{211}At- and ^{131}I-labelled 5F7 conjugates. Higher BT474M1 intracellular retention was observed from 1–6 h for the *iso*-conjugates (*iso*-[^{211}At]AGMB-5F7, 74.3 ± 2.8%, *vs.* [^{211}At]AGMB-5F7, 63.7 ± 0.4% at 2 h). However, unlike the behaviour of [^{131}I]GMIB-5F7 and [^{211}At]AGMB-5F7, intracellular radioactivity level from *iso*-[^{131}I]GMIB-5F7 (49.0 ± 3.6%) and *iso*-[^{211}At]AGMB-5F7 (48.4 ± 5.5%) was significantly lower at 24 h.

Two-paired label experiments were performed in SCID mice with subcutaneous BT474M1 breast carcinoma xenografts to directly compare the tissue distribution of



Scheme 30 Labelling of anti-HER2 single domain antibody 5F7 with ^{211}At using [^{211}At]SAGMB or iso-[^{211}At]SAGMB. Adapted from Choi et al. (2018)

[^{211}At]AGMB-5F7 and *iso*-[^{211}At]AGMB-5F7 to their ^{131}I-labelled counterparts. Tumour uptake of [^{211}At]AGMB-5F7 remained constant from 1 to 4 h post injection (15–16% ID/g) and then declined to 9.49 ± 1.22% ID/g at 21 h. Similar tumour uptake values were observed for co-administered [^{131}I]GMIB-5F7 except at 21 h when values were about 20% higher (11.8 ± 1.5% ID/g). Similar trends were observed in the second experiment where tumour uptake of *iso*-[^{211}At]AGMB-5F7 was compared to that of its radioiodinated counterpart. Nevertheless, tumour uptake of *iso*-[^{211}At]AGMB-5F7 was nearly 50% higher than that of [^{211}At]AGMB-5F7 at all time points, peaking at 23.4 ± 2.2% ID/g at 4 h. Also, tumour uptake of *iso*-[^{131}I]GMIB-5F7 was considerably higher than that of [^{131}I]GMIB-5F7 at all time points.

Thyroid and stomach uptake for both ^{211}At-labelled 5F7 conjugates was low, but significantly higher than that observed with their co-administered ^{131}I-labelled counterparts indicating some in vivo deastatination. However, the observed twofold lower thyroid and stomach activity levels for *iso*-[^{211}At]AGMB-5F7 compared to [^{211}At]AGMB-5F7 suggests a lower degree of deastatination in vivo for *iso*-[^{211}At]AGMB-5F7.

Apart from kidneys and lungs, tumour-to-normal tissue ratios for *iso*-[^{211}At]AGMB-5F7 were significantly higher than those for [^{211}At]AGMB-5F7 and greater than 10:1 by 2 h. Likewise, tumour-to-normal tissue ratios for *iso*-[^{131}I]GMIB-5F7 were much higher than those for [^{131}I]GMIB-5F7 in all tissues. Finally, tumour-to-normal tissue ratios were generally higher for the radioiodinated 5F7 conjugates compared with the corresponding ^{211}At-labelled 5F7 conjugates, which apparently reflects the higher in vivo stability of the radioiodinated constructs.

iso-[^{211}At]AGMB-VHH_1028 and [^{211}At]AGMB-NB7H6 VHH-1028, a single-domain antibody fragment that binds with high affinity to Domain IV of HER2 is an attractive tool for targeted α-particle therapy, particularly with ^{211}At. Feng et al. evaluated the therapeutic efficacy of VHH-1028 labelled with ^{211}At using *iso*-[^{211}At]SAGMB (*iso*-[^{211}At]AGMB-VHH_1028) and compared it to that of *iso*-[^{211}At]AGMB-5F7 that has previously demonstrated excellent uptake in BT474 cells and xenografts (Feng et al. 2023).

HER2-irrelevant *iso*-[^{211}At]AGMB-VHH_2001 was also used in this experiment as control. Conjugation of *iso*-[^{211}At]SAGMB to sdAbs proceeded in 52% RCY with no significant differences observed among 5F7, VHH_1028 and VHH_2001. In the same report, the cytotoxicity of HER2-specific iso-[^{211}At]AGMB-5F7 and irrelevant iso-[^{211}At]AGMB-VHH-2001 was compared on HER2-expressing BT474 breast carcinoma cells. Clonogenic survival of BT474 cells was reduced after exposure to *iso*-[^{211}At]AGMB-5F7 (D0 = 1.313 kBq/mL) while *iso*-[^{211}At]AGMB-VHH_2001 was ineffective.

The biodistribution of *iso*-[^{211}At]AGMB-VHH_1028 and HER2-irrelevant *iso*-[^{211}At]AGMB-VHH_2001 was carried out in athymic mice with subcutaneous BT474 xenografts and compared to previous data reported by Choi for *iso*-[^{211}At]SAGMB-5F7 in the same animal model (Choi et al. 2018). The tumour uptake of *iso*-[^{211}At]AGMB-VHH_1028 was 10.28% ID/g and 10.21% ID/g at 1 h and 4 h, respectively, decreasing to 4.00% ID/g at 21 h. Fast clearance of radioactivity from normal organs was found. The highest ^{211}At levels were seen in the kidneys, which decreased from 34.20% ID/g (1 h) to 4.46% ID/g (4 h). Thyroid activity levels ranged from 0.53 ± 0.25% at 4 h to 0.38 ± 0.17% at 21 h consistent with low dehalogenation in vivo. Tumour uptake of HER2-irrelevant *iso*-[^{211}At]AGMB-VHH_2001 was lower, 3.17%, 2.05% and 0.58% ID/g at 1, 4 and 21 h, respectively, values that were 3.2, 5.0 and 7.7 times lower than those found for *iso*-[^{211}At]AGMB]-VHH_1028. The biodistribution of co-administered *iso*-[^{131}I]GMIB-VHH_1028 and *iso*-[^{125}I]GMIB-5F7 was also performed in athymic mice hosting SKOV-3 xenografts. With few exceptions, the normal tissue levels for the two sdAbs were not significantly different. ^{131}I-to-^{125}I tumour uptake ratios were not significantly different from unity (1.07, 0.99 and 0.91, at 1, 4 and 24 h) corroborating similar tumour localizing capacities for both conjugates.

Dose-dependent tumour growth inhibition was observed with ^{211}At-labelled HER2-specific 5F7 and VHH_1028 but not with HER2-irrelevant VHH_2001. At a higher dose (3.0 MBq) complete tumour regression was observed in 3 of 4 mice treated with *iso*-[^{211}At]AGMB-5F7 and 8 of 11 mice treated with *iso*-[^{211}At]AGMB-VHH_1028. Prolongation in median survival was 495% and 414%, respectively.

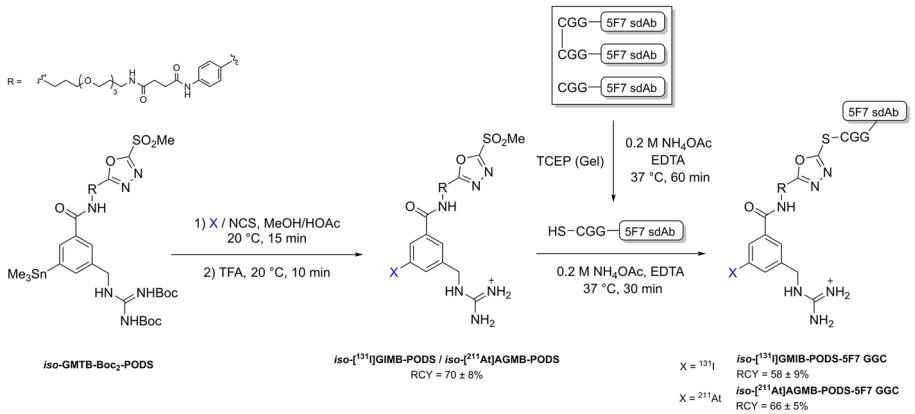
The therapeutic responses observed after a single dose of *iso*-[^{211}At]AGMB-VHH_1028 in the BT474 breast carcinoma model were quite encouraging. Remarkably, the significant increase in median survival time from 44 to 159 days, along with the observation of complete tumour regression in 4 out of 11 cases, underscores the efficacy of the treatment, even at the lowest dose level (1.0 MBq). In contrast, no significant increase in median survival time was observed with the HER2-irrelevant ^{211}At-sdAb control, highlighting the specificity and effectiveness of targeting HER2 for effective treatment outcomes.

Results obtained so far corroborate the potential advantages of TAT for cancer treatment—a single dose of *iso*-[^{211}At]AGMB-VHH1028 was considerably more effective than 4 weekly doses of the analogous beta-emitting *iso*-[^{131}I]GMIB-VHH_1028 conjugate in the same BT474 xenograft model (Feng et al. 2022a). Therefore, due to their significant therapeutic effects associated to minimal toxicity, both *iso*-[^{211}At]AGMB-5F7 and *iso*-[^{211}At]AGMB-VHH_1028 are worth investigating as attractive therapeutic options for patients who do not respond to conventional HER2-targeted therapies.

In a very similar comparative study was reported the evaluation of two versions of an anti-PSMA sdAb – without (NB7) or with His6-Tag (NB7H6) – radiohalogenated using [*I]SGMIB, [^{211}At]SAGMB or their isomeric analogues (Huynh et al. 2024). In vitro studies in PC3 PIP cells demonstrated that cellular uptake and intracellular trapping of [^{211}At]AGMB-NB7H6 were virtually identical to those observed for [^{131}I]GMIB-NB7H6. Moreover, in vivo studies in PSMA + PC3 PIP xenograft models revealed no significant differences in tumour uptake and kidney clearance between [^{211}At]AGMB-NB7H6 and its ^{131}I-labelled analogue at any time point. As in previous study, deastatination was higher than deiodination, but remained at a low level.

Site-specific radiolabelling strategies While ^{211}At-labelled sdAb conjugates, especially *iso*-[^{211}At]AGMB-5F7, showed some promise as candidates for HER2-targeted cancer therapy, there is one drawback associated with prosthetic agents like *iso*-[^{211}At]SAGMB. *Iso*-[^{211}At]SAGMB contains active esters that can react with multiple lysine residues within the sdAb sequence yielding a mixture of different radioconjugates. The heterogeneity and lack of reproducibility that are typical of random labelling, pose significant challenges to the effective use of radioimmunoconjugates, particularly at the higher radioactivity levels required for clinical application. Both the site of conjugation and the number of modifications per molecule can have a significant effect on the pharmacokinetic profile, affinity, and therapeutic efficacy. The modification at single location within a biomolecule via site-specific radiolabelling provides an attractive way to address these issues. The most used approach for site-specific conjugation uses the Michael addition reaction between a maleimide-bearing reagent and a cysteine sulfhydryl group on a protein (Feng et al. 2022b). Unfortunately, the succinimidyl thioether linkage that is formed can undergo retro-Michael additions under physiological conditions (Ravasco et al. 2019). Moreover, in the case of radioimmunoconjugates this thio-ether linkage is less than ideal for bioconjugation due to its instability to hydrolysis and propensity for exchange reactions with endogenous, thiol-bearing species (Adumeau et al. 2016).

A variety of alternative thiol-reactive prosthetic groups have been exploited to mitigate these drawbacks, including, more recently, a phenyloxadiazolyl methylsulfone (PODS) reagent developed for the site-specific cysteine labelling of intact mAbs with radiometals such as ^{89}Zr and ^{177}Lu (Sharma et al. 2021). This site-specific labelling strategy was shown to produce radioimmunoconjugates with markedly superior in vivo performance compared with analogues created using maleimide-based probes. These encouraging results led Feng et al. to explore the possibility of extending the PODS strategy to radiohalogenation of anti-HER2 5F7 sdAb. More specifically, PODS-bearing analogues of the residualizing *iso*-[^{131}I]SGMIB and *iso*-[^{211}At]SAGMB reagents were evaluated for thiol-selective conjugation to anti-HER2 5F7 sdAb carrying a C-terminus GGC tail (Feng et al. 2022b). Both radiohalogenated PODS-5F7GGC conjugates were synthesized in good RCYs, 58% and 64% for *iso*-[^{131}I]GMIB-PODS-5F7GGC and *iso*-[^{211}At]AGMB-PODS-5F7GGC (Scheme 31), respectively and retained high binding affinity on HER2-positive BT474 breast carcinoma cells. *Iso*-[^{211}At]AGMB-PODS-5F7GGC was significantly more stable in vitro than its maleimide analogue and exhibited excellent tumour uptake and better in vivo stability. Higher tumour-to-kidney activity ratios were found for both radiohalogenated



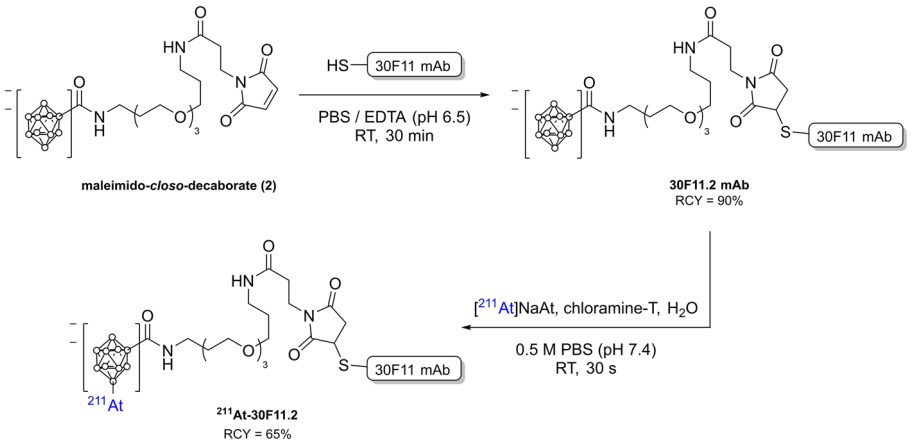
Scheme 31 Preparation of iso-[^{131}I]GMIB-PODS-5F7GGC and iso-[^{211}At]AGMB-PODS-5F7GGC (Feng et al. 2022b)

PODS-5F7GGC conjugates when compared to [^{177}Lu]Lu-DOTA-PODS-5F7GGC. Taken together all these findings suggest a potential role of *iso*-[^{211}At]AGMB-PODS-5F7GGC in the treatment of HER2-expressing malignancies.

Overall SAGMB and *iso*-SAGMB appear to be able to improve labelling stability, especially with small proteins. However, the need of a deprotection step that requires strongly acidic conditions makes incompatible its pre-conjugation to the carrier protein and subsequent one-pot labelling, limiting strongly the potential RCYs and A_s achievable.

Boron clusters

The use of boron clusters based prosthetic groups, mainly studied by the Wilbur group, have demonstrated a higher in vivo stability than astatoaryl compounds as covered in first part of this review. Several applications using this chemistry were focused on CD45, a receptor-type protein tyrosine phosphatase ubiquitously expressed in all nucleated hematopoietic cells. Previous reports indicated that beta-emitting radionuclides are not ideal candidates for killing the targeted hematopoietic cells owing to their long path length and low dose rates (Couturier et al. 2005; Mulford et al. 2005). Although specific targeting of hematopoietic cells may be achieved with the antibodies, most of the emitted energy is deposited outside of the targeted cells, reducing efficacy and causing nonspecific toxicity to neighbouring normal tissues. On the other hand, owing to their short path lengths of 40–90 μm in vivo, alpha-particles have the potential to deliver a lethal radiation dose to small metastatic cell clusters with less non-specific toxicity. Thus, a ^{213}Bi-labelled anti-CD45 mAb has been investigated as an alternative to total body gamma-irradiation in a nonmyeloablative conditioning regimen for hematopoietic cell transplantation (HCT) in a canine model (Bethge et al. 2004; Bethge and Sandmaier 2004; Sandmaier et al. 2021). In spite of the efficacy of the treatment, the very high cost and lack of availability of adequate quantities for clinical trials of actinium-225, the parent radionuclide of ^{213}Bi, have prevented translating ^{213}Bi-labelled mAbs into clinics. The successful studies with the ^{213}Bi-labelled mAbs targeting CD45 led to the investigation of the alpha-emitting radionuclide ^{211}At for the same clinical application. The longer half-life of ^{211}At ($t_{1/2}$ = 7.2 h), 9.5 times longer than that of ^{213}Bi ($t_{1/2}$ = 45.6 min), brings

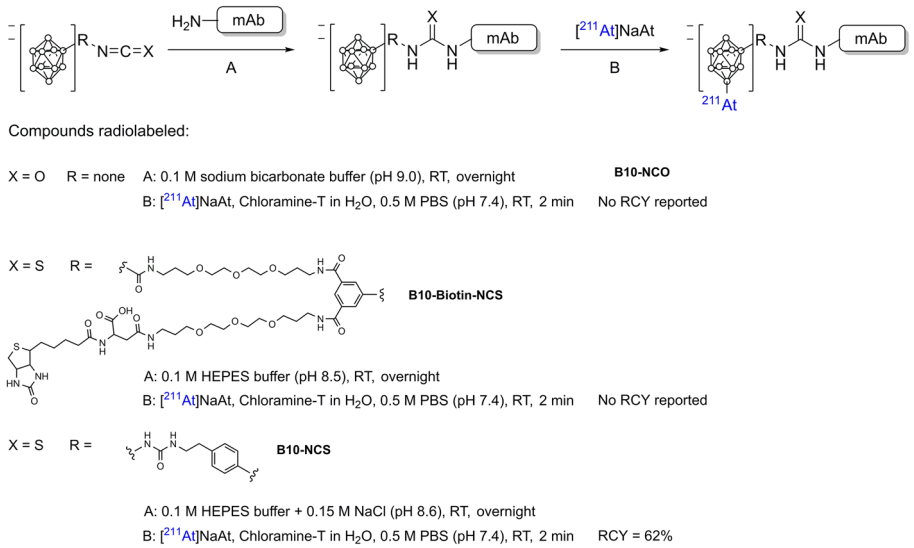


Scheme 32 Late-stage ^{211}At-labelling approach where sulfhydryl groups resulting from reduction of the disulphides on the mAb 30F11 are conjugated with a maleimido-closo-decaborate(2-) reagent (Wilbur et al. 2009b)

some important benefits such as a considerably lower expected amount of ^{211}At necessary to deliver a therapeutic dose. In order to target the CD45 antigen, studies were performed to compare the in vivo tissue distribution of a rat anti-murine CD45 monoclonal antibody (30F11) labelled with ^{211}At using the classical SAB prosthetic group or the conjugation of a maleimide-closo-decaborate(2-) reagent to 30F11 and the subsequent radiolabelling of the mAb-decaborate(2-) conjugate. Conjugation of maleimide-closo-decaborate(2-) was performed at pH 6.5 after reducing disulfide bridges of the mAb using DTT. At least one boron cage was conjugated per mAb, although the exact number could not be determined. Radiolabelling was performed in PBS (pH 7.4) in just 30 s using ^{211}At oxidized to its electrophilic form with chloramine-T, followed by addition of sodium metabisulfite to stop the reaction. (Scheme 32). Data from a biodistribution study in healthy mice demonstrated that using the closo-decaborate(2-) labelling approach resulted in higher concentrations of ^{211}At in target tissue (spleen) and higher tolerance to in vivo deastatination as seen by the low uptake in thyroid, and stomach (Wilbur et al. 2009a). This aspect, together with the simplicity of the labelling process and the potential benefit for scaling-up to clinically relevant activity levels of ^{211}At labelled antibodies, made the mAb-*closo*-decaborate(2-) conjugates promising candidates for further studies with ^{211}At labelled anti-CD45 antibodies.

In a follow-up study that compared ^{211}At-labelled anti-CD45 mAb (30F11 conjugated with a maleimido-*closo*-decaborate (2-), ^{211}At-B10-30F11) and ^{213}Bi-labelled anti-CD45 mAb (30F11 conjugated with isothiocyanatobenzyl-CHX-A"-DTPA), similar biodistribution patterns were observed with female BALB/c mice. No significant renal toxicity was observed in mice administered with either ^{213}Bi- or ^{211}At-labelled conjugates. However, ^{211}At-labelled 30F11 showed a more favourable therapeutic profile with less toxicity and superior myelosuppressive properties (Nakamae et al. 2009).

Radioimmunotherapy with ^{211}At-B10-30F11 combined with bone marrow transplantation has also shown to improve median survival in a disseminated murine acute myeloid leukaemia (AML) model in a dose-dependent manner (Orozco et al. 2013). AML is an aggressive malignancy with few options that induce prolonged remissions



Scheme 33 Approaches for late-stage ^{211}At-labelling of mAbs by which the lysine amines are conjugated with an isocyanato-closo-decaborate(2-) (B10-NCO) or isothiocyanatophenyl-closo-decaborate(2-) reagent (B10-Biotin-NCS and B10-NCS) (Wilbur et al. 2012)

in high-risk patients. Despite aggressive chemotherapy combined with hematopoietic stem cell transplantation (HSCT) many patients still relapse. Biodistribution studies in leukemic SJL/J mice showed excellent localization of ^{211}At-B10-30F11 to leukaemia sites, bone marrow and spleen, within 24 h with lower kidney and lung uptake. Outcomes of syngeneic HSCT studies were improved when using ^{211}At-B10-30F11 RIT, as seen by the prolonged median survival of leukemic mice. Data suggested that ^{211}At-anti-CD45 RIT in combination with HSCT might be a promising therapeutic option for AML.

Prior results obtained in mice have encouraged the above investigators to carry out dose-escalation studies in a dog model. The anti-canine CD45 mAb CA12.10C12 was used for targeting hematopoietic cells in this animal model. Dose-escalation of ^{211}At-labelled CA12.10C12 conjugated with maleimido-*closo*-decaborate (2-) resulted in an unacceptable kidney toxicity, as suggested by the high blood urea nitrogen values and creatinine concentrations in blood (Wilbur et al. 2012). This outcome was not anticipated, since no significant renal toxicity had been observed in mice in previous studies (Nakamae et al. 2009).

Initially it was believed that the renal toxicity might be caused by the nature of the mAb conjugate. Indeed, the reduction of disulphides followed by conjugation resulted in radiolabelled protein fragments that were sequestered in the kidneys. This was later confirmed by a biodistribution study in a dog model where the observed renal concentration of ^{123}I-labelled CA12.10C12 conjugated with maleimido-*closo*-decaborate (2-) through reduced disulphide bonds was 10 × higher than that obtained with ^{123}I-labelled CA12.10C12 conjugated to the DTPA-CHXA" chelate on amine side chains of lysine residues (Sandmaier et al. 2002; Wilbur et al. 2012). It was then anticipated that this significant difference in kidney concentrations could be due to the nature of the conjugates and an investigation of the conjugation reagents was

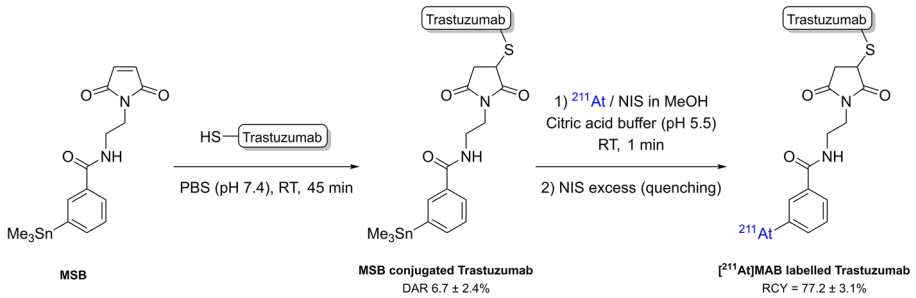
carried out (Wilbur et al. 2012) using the two labelling approaches: preparation of mAb conjugates by conjugation of a maleimido closo-decaborate (2-) after reduction of disulphides (Scheme 32) or by conjugation of an amine-reactive reagent with mAb (Scheme 33).

Indeed, evaluation of the CA12.10C12 conjugates formed by reaction with maleimido-*closo*-decaborate (2-), phenylisothiocyanato-closo-decaborate(2-) (Scheme 32), B10-NCO, and biotin containing phenylisothiocyanato-closo-decaborate (2-), B10-Biotin-NCS (Scheme 33) by nonreducing SDS-PAGE clearly indicated the presence of protein fragments when the mAb was labelled via disulphide reduction and conjugation of maleimido-closo-decaborate (2-), suggesting that conjugations with lysine amines were more advantageous (Wilbur et al. 2012). To overcome the low reactivity of the isocyanato-closo-decaborate (2-) reagent, B10-NCO and the complexity of the biotin containing phenylisothiocyanate reagent, B10-Biotin-NCS, a new easily synthesized amine-reactive reagent B10-NCS was explored. The purity of the CA12.10C12 mAb conjugate, obtained via this amine-reactive conjugation reagent was higher than that of the mAb radiolabeled with the maleimide approach as shown by SE-HPLC analysis. A dual-label biodistribution study with the CA12.10C12 mAb conjugate, B10-NCS labelled with ^{125}I- and ^{211}At also supported the finding that the kidney localization issue could be minimized by conjugation of an amine-reactive reagent (Wilbur et al. 2012).

The favourable biodistribution profiles obtained using B10-NCS led to further dose-escalation studies with the ^{211}At-labelled CA12.10C12 conjugate, (Chen et al. 2012). Consistent with other studies using anti-CD45 mAb (Matthews et al. 1992, 1991), including their own with ^{213}Bi-labelled anti-CD45 mAb (Sandmaier et al. 2002), the highest uptake was found in blood, lymph nodes, bone marrow, and spleen. Among non-target organs, the liver, gallbladder, and thyroid had the highest uptake, whereas no appreciable uptake was observed in the kidney.

Ongoing clinical trials with ^{211}*At-labelled mAbs using boron clusters* Five early-phase clinical trials in patients after allo-HCT are currently ongoing at the Fred Hutchinson Cancer Research Center (Seattle, USA) with the approved ^{211}At-labelled antibody constructs, anti-CD45 (^{211}At-BC8-B10) and anti-CD38 (^{211}At OKT10-B10).

(^{211}At-BC8-B10). The promising preclinical results obtained in a canine transplantation model (Chen et al. 2012; Nakamae et al. 2009; Orozco et al. 2013) together with the cGMP production of ^{211}At-labelled anti-CD45 antibodies (Li et al. 2018) have allowed the initiation of three clinical protocols with ^{211}At-BC8-B10 (NCT03128034, NCT03670966, and NCT04083183). As of October 24, 2017, the clinical phase I/II study NCT03128034**,** enrolling 75 patients with high-risk AML, Acute Lymphoblastic Leukemia (ALL) or Myelodysplastic Syndrome (MDS), is conducted to determine side-effects and the best dose of ^{211}At-BC8-B10. In 2021, preliminary results were presented for twenty patients, median age 60 years (AML = 14; MDS = 5; Mixed-phenotype acute leukemia (MPAL) = 1). From bone marrow evaluation on day 28 after allogenic HCT, 16 of 19 patients had a remission (12 MRD negative, 4 MRD positive). Eight patients died with relapse/progression of disease and 4 died from non-relapse mortality (NRM). With a median follow-up of 1.6 years, the 1-year overall and recurrence-free survival were 43% and 35%, respectively. The 1-year incidence of relapse and NRM were 40% and 25%,



Scheme 34 Late stage ^{211}At-labelling of Trastuzumab using MSB-conjugated precursor (Aneheim et al. 2016)

respectively (Sandmaier et al. 2021). This study is similar in size and in primary outcome to the **NCT03670966** phase I/II study that has started on July 10, 2019, differing only in population of patients, cell transplantation and conditioning regimen.

^{211}At OKT10-B10. The **NCT04083183** phase I/II trial,, intends to demonstrate that the addition of the anti-CD45 ^{211}At-BC8-B10 to conditioning may overcome graft rejection issues in non-malignant diseases treated with allogenic HCT, as already addressed in preclinical studies in a canine model (Frost et al. 2015; Nakaya et al. 2021).

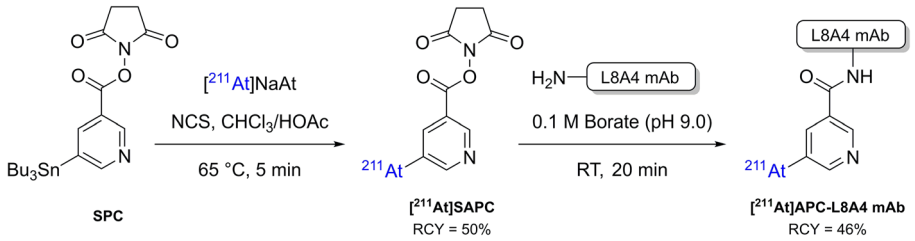
In 2019, O'Steen et al. demonstrated that ^{211}At-OKT10-B10 targeted to CD38, could eradicate multiple myeloma in minimal residual disease (MRD) mouse models (O'Steen et al. 2019). Following these successful studies in NRG mice bearing flank MM xenograft (OPM-2^{Luc} or NCI-H929 cells), two clinical trials with ^{211}At-OKT10-B10 (**NCT04466475** and **NCT04579523**) aiming to achieve eradication of multiple myeloma MRD were planned to be conducted at the Fred Hutchinson Cancer Research Center. The phase I trial **(NCT04466475**) planned to start on January 27, 2024 has been closed even before any participants were enrolled since NIH has terminated funding.

In the **NCT04579523** clinical trial, escalating doses of the anti-CD38 mAb ^{211}At-OKT10-B10 followed by Human leukocite antigen (HLA)-matched or haploidentical donor HCT for high-risk multiple myeloma will be evaluated in 30 patients assigned to one of the two arms, differing in cell transplant and conditioning issues. This phase I trial will investigate the side effects and best dose of ^{211}At-OKT10-B10 when given together with fludarabine, alone or in combination with cyclophosphamide and low-dose total body irradiation (TBI) before donor stem cell transplant, in treating high-risk newly diagnosed, recurrent or refractory multiple myelomas. However, this study has not yet started recruiting.

Other prosthetic groups

N-[2-(maleimido)ethyl]-3-(trimethylstannyl)benzamide (MSB) An alternative method has been used for site-selective modification of antibodies and sdAbs using the *N*-[2-(maleimido)ethyl]-3-(trimethylstannyl)benzamide (MSB) reagent. This reagent has been used for astatine labelling via disulphide bridges in the antibody structure or via cysteines introduced at specific positions in the sdAb sequence.

Aneheim and colleagues (Aneheim et al. 2016) attempted coupling of MSB with two different antibodies, trastuzumab and MX35. The resultant MSB-conjugated mAb were stable with a long shelf-life. The degree of antibody modification was similar for MSB



Scheme 35 ^{211}At-labelling of a mAb using SAPC prosthetic group (Reist et al. 1999)

(6 per antibody) and m-MeATE (7 per antibody) conjugation protocols. Both radioimmunoconjugates were obtained after one-step radiolabelling with astatine in high RCY (76%) and high A_s (>700 MBq/mg), which are often a prerequisite for efficient alpha therapy (Scheme 34). From biodistribution studies in mice, no significant differences in organ activity accumulation were found when comparing astatinated MSB-derived immunoconjugates with ATE-immunoconjugates of MX35 or trastuzumab. The MSB conjugation method represents a viable alternative to other tin-based derivatives for successful labelling of monoclonal antibodies with ^{211}At.

Site-specifically modified sdAbs can be obtained via conjugation of cysteines introduced at specific positions in the sdAb sequence. The HER2-targeting 2Rs15d sdAb was site-specifically conjugated with the MSB reagent onto the cysteine at the carboxyl-terminal end of the sdAb (Dekempeneer et al. 2019). The radioimmunoconjugate was obtained via one-step radiolabelling in high RCY (60–80%) and apparent molar activity (2.3–3.3 MBq/nmol), however [^{211}At]MAB-2Rs15d was less stable in vitro when compared with [^{211}At]AB/AGMB-2Rs15d, showing some degree of deastatination over time. When evaluated in vivo, high uptake levels of [^{211}At]MAB-2Rs15d were measured in lungs, stomach and spleen. The main advantage of this method is that homogeneous and better characterized radioconjugates are obtained for clinical evaluation.

N-succinimidyl 5-[^{211}At]astato-3-pyridinecarboxylate ([^{211}At]SAPC) Non-internalizing antibodies have been successfully radiolabelled using SAB, which reacts with the epsilon-amino groups of lysine residues. However, there is evidence that this may not be the adequate precursor for labelling internalizing antibodies. Since internalization of the antibody-receptor complex generally leads to rapid degradation of the antibody and loss of the radiolabel from the tumour cells, enhanced intracellular retention of activity the cell is required. Therefore, a method for labelling internalizing mAbs with ^{211}At has been developed (Reist et al. 1999), using *N*-succinimidyl 5-[^{211}At]astato-3-pyridinecarboxylate, [^{211}At]SAPC. [^{211}At]SAPC can be prepared by reaction of *N*-succinimidyl 5-(tri-*n*-butylstannyl)-3-pyridine-carboxylate (SPC) with ^{211}At using NCS as oxidant, with a RCY of approximately 50%. Conjugation of [^{211}At]SAPC with L8A4 mAb yielded the ^{211}At-L8A4 in about 50% RCY and with intact affinity for its target (Scheme 35).

The hypothesized mechanism of this enhanced cellular retention relies on the generation of positively charged astatopyridine-substituted catabolites which do not easily traverse biological membranes. Guanidine containing radioastatination agents, such as [^{211}At]SAGMB, were later developed for labelling of internalizing mAbs and mAb fragments (Vaidyanathan et al. 2003). These agents are hypothesized to become trapped within the tumour cells via the same mechanism as [^{211}At]SAPC (due to the presence of

a positively charged guanidine moiety), resulting in improved cellular retention of ^{211}At. Besides mAbs, peptides can also be labelled using this strategy. The synthetic somatostatin analogue, octreotide, was initially labelled with ^{211}At using either an indirect or direct labelling method (Vaidyanathan et al. 2000). In the indirect method, Boc-octreotide was radiolabelled using [^{211}At]SAB resulting in a 50–70% RCY. The direct method involved acylation of the Boc-protected peptide with *N*-succinimidyl 3-(tri-*n*-butylstannyl)benzoate, rendering the tin precursor peptide, followed by radiohalogenation with a similar overall RCY (50%). This one step procedure reduced the total synthesis and purification time to about 1 h and improved the amount of obtained product activity by a factor of 2. One alternative approach attempted labelling with ^{211}At by direct labelling of the peptide via electrophilic reaction on aromatic amino acid residues (Phe and Trp), which resulted in low peptide stability (Zhao et al. 2018). To overcome this, the peptide was indirectly radiolabelled involving two radiochemical steps. First [^{211}At]SAPC was prepared, and the organic solvent removed, and in a second step octreotide was radiolabelled under mild conditions. Astatination of octreotide by direct and indirect labelling method was achieved with about 60% and 30% RCY (non-decay corrected), respectively. The results showed that the indirect labelling method afforded higher stability in vitro. In addition, octreotide can be labelled with ^{211}At using [^{211}At]SAPC without sacrificing the SSTR2 binding ability of the peptide, and has the potential to improve the retention of astatine by the SSTR-positive tumour cells. However, high uptake in the lung, spleen, stomach and intestines was observed at earlier timepoints, which decreased slowly after 24 h post-injection, which was attributed to high SSTR2 expression in these tissues. In contrast to free ^{211}At, low accumulation of activity was observed in the thyroid at all timepoints, suggesting a low level of peptide deastatination.

The ability to synthesize another ^{211}At-labelled peptide, has been demonstrated. VP2 is a small molecule fusion peptide that can specifically bind to the vasoactive intestinal peptide receptor type 1 (VPAC1 receptor) which is overexpressed in several tumours (Moody et al. 2016). This peptide was radiolabelled in one from the peptide conjugated with SPC, yielding [^{211}At]APC-VP2 (Liu et al. 2020). The radiolabelled peptide demonstrated favourable tumour growth inhibition and improved survival when compared with [^{211}At]NaAt. Importantly, despite the lack of renal and hepatic toxicity, pathological evidence of gastric toxicity was associated with the accumulation of ^{211}At in the stomach (astatine-seeking organ). This highlights the potential instability of astatinated ligands and the need to develop novel strategies to enhance stability of ^{211}At-labelled radiopharmaceuticals.

Conclusions

After a long period from the late 1970's to the mid 2010's, during which electrophilic labelling of tin precursors was by far the main approach to access ^{211}At-radiopharmaceuticals, a renewed interest in developing improved reactions and procedures has been generated. Several new methods using electrophilic and nucleophilic astatine to produce ^{211}At-(hetero)aryl compounds were reported, facilitating access to a broader scope of ^{211}At-labelled radiopharmaceuticals. In addition, alternative structures to the At-C aryl bonds, that were long considered as the only option to reach acceptable stability, were proposed, which opened new perspectives to ensure higher in vivo stability. The

increased research activity related to the development of novel ^{211}At-based radionuclide therapies has led to a growing number of new small ^{211}At-molecules. However, most advanced carrier molecules that have reached the clinical trial stage are still monoclonal antibodies for which novel ^{211}At-prosthetic groups for bioconjugation have been heavily investigated.

Despite all the progress reported recently, challenges remain, in particular the scaling up of the radiolabelling from preclinical to clinical doses that could prove difficult due to radiolysis, and the automation of reported labelling procedures that has been somewhat neglected until now. This will certainly be one of the main aspects to be considered by the growing community involved in ^{211}At radiopharmaceutical research.

Abbreviations

ALL	Acute Lymphoblastic Leukemia
A_m	Molar activity
A_s	Specific activity
AP	[^{211}At]-astatophenylananine
DME	1,2-Dimethoxyethane
DFT	Density functional theory
IBCF	*iso*-Butylchloroformate
HLA	Human leukocite antigen
HPLC	High-performance liquid chromatography
mAb	Monoclonal antibody
MDS	Myelodysplastic Syndrome
MPAL	Mixed-phenotype acute leukemia
NCS	*N*-Chlorosuccinimide
NBS	*N*-Bromosuccinimide
NIS	*N*-Iodosuccinimide
NpG	Neopentyl glycol
PBS	Phosphate-buffered saline
PODS	Phenyloxadiazolyl methylsulfone
PSMA	Prostate-specific membrane antigen
RCP	Radiochemical purity
SAB	*N*-Succinimidyl-[^{211}At]astatobenzoate
SAGMB	*N*-Succinimidyl-3-[^{211}At]astato-4-guanidinomethylbenzoate
SAPC	*N*-Succinimidyl-5-[^{211}At]astato-3-pyridinecarboxylate
ROS	Reactive oxygen species
sdAb	Single domain antibody
SEAr	Aromatic electrophilic substitution
SEC	Size-exclusion chromatography
SIPC	*N*-Succinimidyl-5-[*I]iodo-3-pyridinecarboxylate
SPE	Solid phase extraction
TBI	Total body irradiation
TBA	Tributylamine
TEMPO	2,2,6,6-Tetramethyl-1-piperidinyloxy, free radical
TAT	Targeted alpha therapy
RCY	Radiochemical yield

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