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N3-substituted thymidine bioconjugates for cancer therapy and imaging

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Abstract

The compound class of 3-carboranyl thymidine analogues (3CTAs) are boron delivery agents for boron neutron capture therapy (BNCT), a binary treatment modality for cancer. Presumably, these compounds accumulate selectively in tumor cells via intracellular trapping, which is mediated by hTK1. Favorable *in vivo* biodistribution profiles of 3CTAs led to promising results in preclinical BNCT of rats with intracerebral brain tumors. This review presents an overview on the design, synthesis, and biological evaluation of first- and second-generation 3CTAs. Boronated nucleosides developed prior to 3CTAs for BNCT and non-boronated N3-substituted thymidine conjugates for other areas of cancer therapy and imaging are also described. In addition, basic features of carborane clusters, which are used as boron moieties in the design and synthesis of 3CTAs, and the biological and structural features of TK1-like enzymes, which are the molecular targets of 3CTAs, are discussed.

Boron neutron capture therapy (BNCT) is a binary cancer treatment modality that is based on the selective accumulation of ¹⁰B in tumor cells followed by irradiation with low-energy (thermal) neutrons. Neutron capture by ¹⁰B produces high linear energy transfer α -particles (⁴He²⁺) and ⁷Li³⁺ nuclei, both of which can selectively destroy tumor cells. Concurrently, nearby healthy cells are spared because of the limited path lengths of 5–9 μ m of these particles. Approximately 10⁹ ¹⁰B atoms per tumor cells and tumor-to-normal tissue ratios of >3 to 1 are required for successful BNCT. Natural boron consists of stable ¹⁰B- (~20%) and ¹¹B-isotopes (~80%). Only the former is suitable for BNCT [1,2].

Boronophenylalanine (**1**) and sodium borocaptate (**2**) are two boron delivery agents that are/were used clinically in ¹⁰B-enriched form in, for example, Japan, the USA, Europe and Argentina (Figure 1) [1,2]. The development of novel improved tumor selective boron

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delivery vehicles has been one of the most pressing tasks of BNCT research because of suboptimal clinical results obtained with BPA and BSH [1]. These novel boron delivery agents include amino acids, polyamines [3–5], DNA-binding agents [6,7], porphyrins and related structures [8–10], carbohydrates [11–14], monoclonal antibodies [15–17] and liposomes [18–20].

Endogenous nucleosides have also attracted considerable attention as potential boron delivery vehicles for BNCT [21]. These structures are known to accumulate selectively in rapidly proliferating tumor cells, as they are building blocks for DNA and RNA synthesis [22]. The same is true for artificial nucleoside derivatives, such as 3-[^{18}F] fluoro-3'-deoxythymidine ([^{18}F]FLT), which is widely used in PET imaging of cancer [23].

In recent years, the development of boronated nucleosides for BNCT has mainly focused on 3-carboranyl thymidine analogues (3CTAs) [21,24], which possibly localize selectively in tumor cells by a process described as **kinase-mediated trapping (KMT)** [25]. In this review, we will present an overview on the design, synthesis and biological evaluation of first- and second-generation 3CTAs. We will also describe the biological and structural features of TK1-like enzymes, which are the molecular targets of 3CTAs, as well as the structural and chemical properties of carborane clusters, which are used as boron moieties in the design and synthesis of 3CTAs. Another topic of this review will be boronated nucleosides that have been the focus of agent development efforts in BNCT prior to 3CTAs.

The general concept of using thymidine modified at the N3-position with a therapeutic or imaging entity in the context of KMT was initially developed and explored using the example of 3CTAs. However, in recent years numerous non-boronated N3-modified thymidine bioconjugates were successfully developed for areas of cancer therapy other than BNCT as well as for tumor imaging, thereby validating this concept [26–30]. Thus, these conjugates will also be discussed in this review.

Carboranes

Three highly hydrophobic *closo*-carborane clusters with the chemical formula $\text{C}_2\text{B}_{10}\text{H}_{12}$ have been used widely in the design and synthesis of 3CTAs. These are 1,2-*closo*-carborane (*ortho*), 1,7-*closo*-carborane (*meta*) and 1,12-*closo*-carborane (*para*) [31]. The former two cages can be transformed easily to anionic *nido*-carboranes by the action of fluoride anions or bases (Figure 2) [31]. Both *nido-o*-carborane and *nido-m*-carborane were also used in the synthesis of 3CTAs [21,24,32].

Closo- and *nido*-carborane cages have found diverse applications not only in the development of boron delivery agents for BNCT [1,24]. The hydrophobic character of the *closo*-carboranes and the hydrophilic character of the *nido*-carboranes combined with their rigid geometry, high physiological stability, and synthetic versatility have made both species also attractive entities for other areas of medicinal chemistry, such as their use as pharmacophores in conventional drug design and as prosthetic groups for radiohalogens in molecular imaging applications [33–35].

TK1-like enzymes

TK1-like enzymes include hTK1, vaccinia virus thymidine kinase and several bacterial thymidine kinases [36–42]. Cytosolic hTK1 is an essential deoxynucleoside kinase of the salvage pathway of DNA synthesis [43,44]. It is the primary molecular target of 3CTAs. This enzyme phosphorylates thymidine to thymidine monophosphate, which is followed by the conversion of thymidine mono-phosphate to thymidine diphosphate by TMPK. Phosphorylation of thymidine diphosphate by NDPK leads to thymidine triphosphate, which

is incorporated into DNA by DNA polymerase (Figure 3) [43,44]. There is a second mammalian thymidine kinase, designated TK2, which is localized in mitochondria. Based on amino acid sequences and substrate specificities, TK1 differs significantly from TK2 but also from other dNKs, such as dCK and dGK [44–48]. Human TK1 activity is cell-cycle dependent increasing in the G1/S phase. It declines rapidly in G2/M phase and is absent in resting cells. In contrast, TK2 activity is cell cycle independent [45–48].

Cellular hTK1 is found in a dimeric form with low affinity for thymidine ($K_m = 15 \mu\text{M}$) and a tetrameric form (Figure 4B) with high affinity for thymidine ($K_m = 0.7 \mu\text{M}$) [49]. Substrate binding is demonstrated at the example of thymidine tri-phosphate to a monomeric subunit of hTK1 [36], which represents a holo form of the enzyme (Figure 4A). Hydrogen bonds between $\text{N}_3\text{-H}$, $\text{C}_2=\text{O}$ and $\text{C}_4=\text{O}$ of the thymidine scaffold and carbonyl and amino groups of the peptide backbone primarily of the 'lasso loop' of hTK1 and other TK1-like enzymes are important for substrate binding [30–36]. As discussed by Welin *et al.* [36], the N_3 nitrogen of thymidine appears to be positioned towards the surface of the enzyme, which could possibly allow bulky substituents at this position, albeit accompanied with loss of hydrogen bonding. A small hydrophobic pocket accommodates the 5-methyl group of thymidine [36] and also slightly larger ethyl and halogen substituents, as has been confirmed by various structure–activity relationship (SAR) studies [48,50]. The 3-hydroxyl group of thymidine may also have some access to the surface of the enzyme [36], which explains some tolerance for small substituents at this position [44].

5-carboranyl- & 3'-aminocyanoborane-substituted pyrimidine nucleoside analogues for BNCT

Early design strategies for boronated nucleosides for BNCT focused on the C5-position of 2-deoxyuridine for attachment of boron moieties rather than on the N_3 -position of thymidine, as in the case of 3CTAs. These efforts were probably inspired by the knowledge that various 5-halo-2-deoxyridines are rapidly incorporated into DNA, and, thus, selectively accumulate in proliferating tumor cells [51,52]. Schinazi and Prusoff prepared the first boronated nucleoside analogue, 5-(dihydroxyboryl)-2-deoxyuridine (**3**; Figure 5) [53]. The compound did not appear to cause significant cytotoxicity in various mammalian cell lines. *In vitro* studies indicated that **3** was less effectively incorporated into DNA than 5-iodo- and 5-bromo-2-deoxyuridine [54].

Yamamoto *et al.* synthesized the first 5-substituted carboranyl nucleoside, 5-(1-*o*-carboranyl)-2-deoxyuridine (**4**) [55]. *In vitro* data indicated that **4** was overall more cytotoxic than its dihydroxyboryl counterpart **3**. Studies by Lunato *et al.* demonstrated that **4** was a substrate of TK2 rather than TK1 [56], which is not surprising considering the limited tolerance of TK1 with respect to 5-substitution. Phosphorylation of radiolabeled **4** *in vitro* was observed in, for example, human T-cell leukemia and peripheral blood mononuclear cells [57].

Spielvogel *et al.* synthesized and evaluated 3-aminocyanoborane-2,3-dideoxythymidine (**5**) [58]. Phosphorylation of thymidine analogues with minor modification at the 3-position (e.g., F and N_3) by TK1 is possible [44]. Compound **5** demonstrated significant cytotoxicity against various murine and human cancer cell lines, which was attributed to the inhibition of enzymes involved in both purine and pyrimidine nucleotide *de novo* synthesis [21,58].

3CTAs

First-generation 3CTAs & KMT

In 3CTAs, a carborane cage is linked through a spacer to the N3 position of thymidine, as represented by the examples of first-generation 3CTAs in Figure 6. In the case of 3CTAs **6–18**, the attached cluster is *o*-carborane. These compounds are effectively 5 - monophosphorylated by TK1. This presumably causes the selective accumulation of 3CTAs in tumor cells [21,24,59–62] since the obtained negative charge should prevent passive diffusion out of the tumor cells and nucleoside transporters apparently do not accept nucleotides as substrates [63]. This process has been described as KMT (Figure 3) [25]. Theoretically, intracellular entrapment of 3CTAs could be further enhanced by di- and tri-phosphorylation followed by incorporation of the 3CTA-triphosphate into DNA (Figure 3). However, it is unknown if 3CTAs undergo these biochemical conversions.

The original design concept for 3CTAs was developed by Lunato *et al.* [56]. It is based on observations made during the purification of various kinases, including thymidine kinases, by affinity chromatography [64–66]. In these studies, kinase substrates were covalently linked to a solid matrix. In the case of thymidine, linkage occurred presumably through the N3-position. It was discovered that for a successful purification of the kinases, linkers of specific lengths between solid matrix and nucleoside/nucleotide scaffold were of crucial importance to overcome steric hindrance. This simple concept was adapted for the design of 3CTAs. Here, the solid support was replaced with the bulky carborane cluster hoping that the resulting construct would remain susceptible to phosphorylation by kinases. Fortuitously, 3CTAs eventually proved to be selective substrates of TK1 [21,24,59–61] confirming this design concept.

Among the first-generation 3CTAs represented in Figure 6, those with a pentylene linker between thymidine scaffold and carborane cluster were the best substrates of hTK1. These agents were designated as N5 (**10**) and N5-2OH (**16**). Their apparent relative k_{cat}/K_m values (rk_{cat}/K_m s) were 26.7 and 35.8%, respectively, which is comparable to clinically used hTK1 substrates, such as AZT [24]. k_{cat}/K_m values are a measure of the apparent catalytic efficiency of the enzyme for its substrates. In this case, the k_{cat}/K_m values for the 3CTAs are expressed relative to that of endogenous thymidine, which is arbitrarily set to 100% [67]. On the other hand, these 3CTAs did not appear to be substrates of TK2 and dCK [24]. Those 3CTAs with an ethylene spacer (**7 & 13**) were as good substrates of hTK1 as **10** and **16**. However, they were apparently not as effective as the latter two agents in competing with endogenous thymidine at the substrate-binding site of hTK1 [59]. This was an important finding, as it is known that competition of endogenous nucleosides, such as thymidine, with nucleoside analogues used in imaging, such as [^{18}F]FLT, negatively affects the uptake of the latter in tumor tissue [23]. *In vitro* uptake, retention and cytotoxicity of **10** (N5) and **16** (N5-2OH) were evaluated in F98 glioma, MRA 27 melanoma, and TK1(+)/TK1(–) L929 cells [59]. Both compounds were moderately cytotoxic in all four cell lines with IC_{50} values ranging from 18–70 μM . The cellular uptake of N5-2OH was highest in MRA 27 with 169 $\mu\text{g B}/10^9$ cells after 24 h incubation with 22% retention following additional 12 h of incubation in compound-free medium. More importantly, uptake/retention of N5-2OH in L929 TK(+) and L929 TK(–) differed significantly (92 $\mu\text{g B}/10^9$ cells/29% vs 9.3 $\mu\text{g B}/10^9$ cells/0%) indicating that KMT may be an important factor in the cellular accumulation of this compound. In L929 TK(+) and L929 TK(–) tumor-bearing nude mice, an uptake ratio of approximately three in favor of the TK1(+) tumor was found for N5-2OH (22.8 vs 8.4 $\mu\text{g B/g}$ tumor tissue) [62]. Survival times of rats with intracranial RG2 glioma were increased by a factor of two by BNCT following prior intracranial (ic.) administration of N5-2OH compared with a control group of RG2 glioma bearing rats that were subjected to neutron radiation without prior injection of N5-2OH [62]. A group of rats without tumors that had

received N5-2OH by ic. administration without neutron irradiation was monitored for signs of toxicity [62]. There was no evidence for neurological deficit. The animals lost initially <10% of their weight, which was, however, regained after a short period of time. A pathologic examination of the rat brains did not reveal any significant tissue alterations.

A hypothetical model for the binding of 3CTAs to the active site of hTK1 was developed by Tjarks *et al.* [24,61,68]. It is demonstrated at the example of a docked pose of N5-triphosphate in the active site of a homology model of hTK1 (Figure 7) [68]. The use of the triphosphate form of N5 proved to be useful in stabilizing the docked pose of the thymidine portion of the molecule within the substrate binding site [68]. This protein structure presumably represents an apo form of the enzyme because it was derived from an apo form of *TmTK* [40,68]. TK1-like enzymes respond with dramatic conformational changes upon ATP and/or thymidine binding, which was discussed in detail by Lavie *et al.* [40]. This is especially obvious in the case of the lasso loop, which takes a far more open conformation in apo forms [40] as compared to holo forms (depicted in Figure 4). As represented in Figure 7, docking of N5 to the substrate-binding site of this apo form can be accommodated because the bulky carborane remains outside of the protein. Similar results have been observed for the docking of N5-2OH [24] and its isomeric forms **20** and **21** [61] into apo forms of TK1-like enzymes. On the other hand, docking of 3CTAs into the active site of holo forms of TK1-like enzymes was not possible [24]. This rudimentary hypothetical binding mode of 3CTAs to TK1-like enzymes seems plausible. However, the exact binding mode of these structures to this type of enzymes remains unknown.

Extended synthetic & biological studies on N5-2OH (16)

Byun *et al.* reported the synthesis of both (*R*)-epimeric- (**20**) and (*S*)-epimeric (**21**) forms of N5-2OH (**16**) with natural abundance of boron isotopes as well as racemic N5-2OH (**19**) in ¹⁰B-enriched form (Figure 8) [61]. In addition, structural isomers of racemic N5-2OH (**16**) containing either *m*-carborane (**22**) or *p*-carborane (**23**) were prepared. The relative hTK1 phosphorylation rates (relative PRs) of **16** and **20–23** ranged from 32–45%. PRs are used to estimate the capacity of, for example, 3CTAs to function as substrates for hTK1 in a screening setting. As in the case of the k_{cat}/K_m values, PRs are expressed relative to that of endogenous thymidine, which is arbitrary set to 100% [67].

Water-soluble amino acid ester prodrugs of 3CTAs

An approach focusing exclusively on improving the water solubilities of N5 and N5-2OH was recently reported by Hasabelnaby *et al.* [69]. A library of 3 -monosubstituted, 5 -mono-substituted, and 3 ,5 -disubstituted L-valine-, L-glutamate-, and glycine-ester prodrugs of N5 and N5-2OH was synthesized and representative structures (**24–27**) are shown in Figure 9. These prodrugs were specifically designed for ic. administration to treat high-grade brain tumors, such as glioblastoma multiforme by BNCT. The interstitial fluid (ISF) of the brain appears to have significantly lower protein (enzyme) concentrations than normal serum [70,71]. This could impede cleavage of ester-type prodrugs that would rely on esterase activity following ic. injection into brain ISF. Therefore, amino acid esters were chosen as promoieties for N5 and N5-2OH, as it is known that amino acid esters of nucleoside derivatives are susceptible to both chemical and enzymatic hydrolysis [72–75]. Overall, 5 -L-glutamate- and glycine-ester prodrugs of N5 and N5-2OH (**25–27**) had the best prodrug properties among all synthesized compounds. Their half-lives in bovine cerebrospinal fluid, a medium that presumably mimics the protein (enzyme) concentration of brain ISF, ranged from 0.96 to 2.11 h. The authors indicated that these half-lives should be suitable for ic. administration. In addition, aqueous solubilities of the prodrugs **25**, **26**, and **27** improved by factors ranging from approximately 40 to approximately 2360 compared with parental N5.

Carborane cluster halogenated & radiohalogenated 3CTAs

The synthesis and biological evaluation of cage-halogenated and radiohalogenated derivatives of N5 were recently described by Tiwari *et al.* (**28–34**; Figure 10) [68]. Radiolabeled compounds, such as **32** and **33**, have the potential to be used for tumor imaging and radiotherapy [35]. They may also serve in the real-time monitoring of compound concentrations in tumors during BNCT. Compounds **30/31** and **32/33** were synthesized by palladium-catalyzed halogen or isotope exchanges using either Na^{79/81}Br or Na¹²⁵I as the halogen source. The relative hTK1 PRs of compounds **28/29** and **30/31** were 29.6% and 36.9%, respectively, which was comparable with that of N5 in this specific study (38%). Thus, the addition of iodine or bromine atoms to the carborane cluster does not seem to have a significant effect on the phosphorylation of these 3CTAs by hTK1. *In vitro* uptake studies with N5, **28/29** and **30/31** in L929 TK1(+) and L929 TK1(–) cells resulted in preferential uptake of the compounds in the former cell line.

Second-generation 3CTAs

3CTAs with ethyleneoxide moieties

Major shortcomings of N5-2OH (**16**) and other first-generation 3CTAs that were recognized during biological studies included suboptimal competition with endogenous thymidine at the substrate-binding site of hTK1 [24] and lack of water-solubility [69]. In addition, the capacity of first-generation 3CTAs to function as substrates for hTK1 seemed to be in need of improvement. Thus, Johnsamuel *et al.* initially attempted to enhance the pharmacodynamic and physiochemical properties of 3CTAs by using hydrophilic and highly flexible ethyleneoxide moieties in their synthesis (Figure 11) [76]. Compounds with ethyleneoxide spacer between carborane and thymidine scaffold (**35–39**) demonstrated slightly better relative hTK1 PRs (37–42%) than 3CTAs **40–45** (17–26%), which contained ethyleneoxide substituents at the second carbon of the carborane cluster. Overall, however, compounds **35–39** did not appear to be superior to N5-2OH as substrates of hTK1.

3CTAs containing polyhydroxylated functions

Tjarks *et al.* designed and synthesized a small library of 3CTAs containing cyclic and acyclic polyhydroxylated functions in the spacer between a *p*-carborane cluster and thymidine (**46**) or attached to the second carbon of this cage (**47–49**; Figure 12) [77,78]. Compounds **46–49** demonstrated relative hTK1 PRs ranging from 44–67%. In addition the rK_{cat}/K_m value of **46** was 57.4%, which was significantly higher than that previously reported for N5-2OH (35.8%) [59]. Unfortunately, compound **46** was a far less potent inhibitor of thymidine phosphorylation by hTK1 than N5-2OH (92 vs 17 μ M).

3CTAs with triazole & tetrazole linkers

Lesnikowski *et al.* [79,80] synthesized a series of 3CTAs utilizing click chemistry [81] to incorporate a 1,2,3-triazole linker between carborane moiety and thymidine scaffold (**50 & 51**; Figure 13). The triazole group may participate in hydrogen bond formation between amino acids of the lasso loop and substrate thereby improving binding affinity. Compound **50** contains a metallacarborane moiety whereas **51** is substituted with *nido-o*-carborane. An evaluation of both compounds as potential substrates of hTK1 has not yet been published. Agarwal *et al.* recently synthesized a series of 3CTAs with tetrazolylmethyl- and tetrazolyl linker (**52–57**) based on the hypothesis that these hydrogen acceptor-rich structures may improve the binding affinity of 3CTAs to the active site of hTK1 [67]. Relative hTK1 phosphorylation rates of **52–57** ranged from 39.5–63.7%. A detailed comparative enzymatic evaluation indicated that compound **57** might be slightly superior to N5-2OH as a hTK1 substrate.

3CTAs containing amidinyl & guanidyl linkers

Agarwal *et al.* synthesized a series of 3CTAs with amidinyl and guanidyl groups in the spacer between the N3 position of thymidine and a *m*-carborane cluster (Figure 14) [67]. In this case, the hypothesis was that these groups might improve the binding affinity of the 3CTAs to the active site of hTK1 due to their hydrogen bond donating capacity. In addition, the highly basic properties of the amidinyl and guanidyl groups (**58–62**) should render these compounds water soluble under physiological aqueous conditions. Unfortunately, compounds **58–62** proved to be unstable under prolonged storage conditions and during enzyme assays, which prevented an accurate evaluation of their enzymatic and solubility properties. However, in the case of compound **61**, phosphorylation by hTK1 could be confirmed by MS.

3CTAs with amino & ammonium functions attached to the carborane cluster

In another attempt to improve the enzymatic properties and water solubility of 3CTAs, Byun *et al.* [32] synthesized a library of 12 analogues either with an amino group attached to *closo-m*-carborane (**63–67,73**; Figure 15) or an ammonium function attached to *nido-m*-carborane (**68–72,74**). Thus, the latter 3CTAs are zwitterionic structures. Both subseries appeared to be sufficiently lipophilic to enter into tumor cells via passive diffusion. In particular the compounds with ethylene linkers (**63 & 68**) had among the highest relative PRs observed for any 3CTAs (71.9% and 89.4%, respectively). In addition, compound **63** was approximately 10^3 times more soluble than N5-2OH in phosphate-buffered saline at pH 7.4 [HK Agarwal, Pers. Comm.]. Biodistribution studies of **69** in mice with subcutaneous L929 TK1(+) and L929 TK1(–) tumors resulted in intratumoral boron concentrations of 21.7 and 7.0 $\mu\text{g B/g tumor}$, respectively (3:1 ratio). These data were comparable with those obtained for N5-2OH in the same study. Interestingly, HPLC studies revealed two close peaks for **68** in chromatograms. A similar splitting phenomenon was observed for various signals in both ^1H - and ^{13}C -NMR spectra of **68**. On the other hand, such splitting patterns were not observed for any of the other 3CTAs of this library indicating steric hindrance between the bulky *nido-m*-carborane cluster and the thymidine scaffold resulting in the formation of atropoisomers. Overall, the compounds of this library appeared to be the most promising second generation 3CTAs.

Non-boronated N3-substituted thymidine analogues

N3-alkyl & haloalkyl thymidine analogues

Several thymidine analogues with small alkyl groups at the N3-position were evaluated with respect to their CNS depressant (**75 & 76**), anti-nociceptive (**75 & 76**) and antiviral activities (**77 & 78**) by Yamamoto, Kitade, and Grierson and their collaborators (Figure 16) [82–85]. In most cases, however, interest in the exploration of non-boronated N3-substituted thymidine analogues appeared to be triggered by discoveries related to 3CTA research. These studies primarily focused on the design, synthesis and biological evaluation of thymidine analogues with radiolabels at the N3-position for cancer therapy and imaging (Figures 16 & 17). Alauddin *et al.* reported the synthesis of a library of compounds with chloro-, bromo-, and iodo-substitutions at various positions of a N3-phenylalkyl side chain at thymidine (**79–81**) [86]. These agents may have been synthesized in preparation of a later synthesis of their radiohalogenated counterparts. Relative TK1 phosphorylation rates for some compounds of this series ranged from 2–6% [26]. Members of the research teams of Toyohara, Alauddin and Balzarini synthesized and evaluated several analogues with ^{18}F or ^{19}F attached to N3-alkyl side chains (**82–87**) [27,28,86–89,101]. Balzarini *et al.* observed that **83** was a relatively poor inhibitor of thymidine phosphorylation by TK1 [28]. Toyohara reported that **82–85** were effectively monophosphorylated by TK1 with rPRs ranging from 26–47% [27]. However, studies by the same group indicated that administration of **86**

(the ^{18}F counterpart of **83**) into LL/2 Lewis lung carcinoma bearing mice did not result in tumor selective accumulation of the compound [87]. In contrast, Alauddin *et al.* observed that **86** and **87** were selectively retained in H441G1 tumors implanted into mice [88]. However, a review by the same group discusses the possibility that **83** was not a substrate of TK1 [27]. Alauddin *et al.* also synthesized compounds **88–91** [90]. No biological data have been published for these compounds as yet. Smith *et al.* [29,102] employed click chemistry to link ^{18}F via a triazole moiety to the N3 position of thymidine (**92 & 93**). The corresponding ^{19}F derivatives were also synthesized. Only the ^{19}F derivative of **93** was moderately phosphorylated by TK1 [29].

Thymidine analogues conjugated at the N3-position with metal chelating functions

The synthesis and biological evaluation of several thymidine analogues conjugated at the N3 position to metal chelating complexes have been reported in recent years for potential applications in the radiotherapy and imaging of cancer [30,91–97]. Schmid *et al.* introduced 1,4,7,10-tetraazacyclododecane-1,4,7-tris(acetic acid) (DO3A) at the N3 position of thymidine to yield 3-(5-DO3A-pentyl)thymidine (D3T) (**94**; Figure 17) [91]. Compound **94** was radiolabeled with ^{68}Ga and ^{111}In to produce ^{68}Ga [D3T and ^{111}In]D3T. Cellular uptake of the ^{111}In -labeled compound in DoHH2 and HL60 cells was low and phosphorylation by TK1 was not observed. Celen *et al.* synthesized N3-monoaminomonoamidodithiol-substituted thymidine derivative labeled with $^{99\text{m}}\text{Tc}$ (**95**) [98]. The metal-free compound was a poor inhibitor of thymidine phosphorylation by TK1. However, the $^{99\text{m}}\text{Tc}$ -labeled compound demonstrated some tumor selectivity in fibrosarcoma-bearing mice.

Zubieta *et al.* synthesized and evaluated a series of N3- $\text{Re}(\text{CO})_3$ -thymidine conjugates (**96**; Figure 17) [93,94]. These compounds were generally poor inhibitors of thymidine phosphorylation by hTK1. Some derivatives of this library demonstrated significant cytotoxicity in A549 lung carcinoma cells *in vitro*. A substantial number of structurally different N3- $\text{Re}(\text{CO})_3$ – and N3- $^{99\text{m}}\text{Tc}(\text{CO})_3$ -thymidine conjugates were prepared by Schibli *et al.* [30,92,95–97]. A representative structure is compound **97**. Many of these compounds were good substrates of TK1. In the case of one of these agents, monophosphorylation by TK1 was confirmed by MS [92]. *In vitro* uptake in various cancer cell lines was generally low to moderate [30,97].

Thymidine conjugated at the N3-position with cancer chemotherapeutic drugs

Aspland *et al.* synthesized a series of thymidine derivatives N3-conjugated with paclitaxel or vinblastine as potential cancer therapeutics (**98–107**; Figure 18) [25]. Many of these conjugates were phosphorylated by TK1 with rPRs ranging from 13–68%. Several conjugates retained the microtubule-binding activities of unconjugated paclitaxel or vinblastine.

Conclusion

During the last 15 years, 3CTAs have become one of the most widely studied pre-clinical BNCT agents. First-generation 3CTAs, such as N5 and N5-2OH, exhibited good relative hTK1 PRs, selective *in vitro* uptake in TK1(+) versus TK1(–) cells, and favorable *in vivo* biodistribution profiles leading to selective accumulation in tumors. N5-2OH proved to be effective in preclinical BNCT studies with rats bearing intracerebral brain tumors. Techniques were developed to use the carborane cluster of N5 as a platform for radiohalogens. Disadvantages of first-generation 3CTAs are that they demonstrate poor water solubility and limited capacity to compete with endogenous thymidine at the substrate-binding site of hTK1. These shortcomings could be overcome partially with the

development of prodrug strategies and second-generation 3CTAs; the most promising, to date, appear to be those with amino substituents at the carborane cage. Research related to 3CTAs has sparked significant efforts in the development of thymidine conjugates with radiolabels in the N3 side chain. Promising biological data have also been reported in this new area of bioconjugate development for therapeutic and imaging applications. A remaining problem with second-generation 3CTAs and non-boronated radiolabeled N3-substituted thymidine analogues appears to be a very limited understanding of SARs, even within smaller sub-libraries of these compound classes. In addition, almost no information is available regarding the molecular interactions between N3-thymidine conjugates and their target, the hTK1 protein. In the case of some compounds conflicting data have been reported. These problems have to be addressed on the road to agents with improved capacities to act as substrates and inhibitors of hTK1.

Future perspective

3CTAs and other N3-substituted thymidine conjugates have significant potential in cancer therapy and imaging. However, an improved understanding of basic SARs has to be attained and structural biology and/or computational chemistry techniques have to be employed to gain insights into the molecular interactions between N3-thymidine conjugates and hTK1. The compound class might advance significantly towards clinical trials within the next 10 years provided these hurdles can be overcome and extended SAR information can be utilized to design novel derivatives with improved pharmacodynamic and pharmacokinetic properties.

Key Terms

Kinase-mediated trapping

Selective uptake of cancer therapeutic and imaging agents in tumor cells through phosphorylation by intracellular kinases

Lasso loop

Flexible region of human thymidine kinase 1 (hTK1) that covers the substrate binding site of the enzyme. It is comprised of the amino acids Leu166 to Lys 180

References

Papers of special note have been highlighted as:

of interest

of considerable interest

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Patents

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Executive summary

Background

- The development of novel improved tumor-selective boron delivery vehicles has been one of the most pressing tasks of boron neutron capture therapy (BNCT) research.
- 3-carboranyl thymidine analogues (3CTAs), which, possibly, localize selectively in tumor cells by a process described as kinase-mediated trapping (KMT), are one of the most widely studied preclinical BNCT agents.
- The general concept of using thymidine modified at the N3-position with a therapeutic or an imaging entity in the context of KMT has been validated successfully by recent studies on non-boronated N3-modified thymidine bioconjugates.

Carboranes

- Hydrophobic *closo*-carborane and hydrophilic *nido*-carborane clusters have been used widely in the design and synthesis of 3CTAs.
- *Closo*- and *nido*-carborane cages have found diverse applications not only in the development of 3CTAs.

TK1-like enzymes

- hTK1 is the primary molecular target of 3CTAs.
- hTK1 is only active in proliferating cells causing tumor-selective KMT of 3CTAs via 5'-monophosphorylation.

5-carboranyl- & 3-aminocyanoborane-substituted pyrimidine nucleoside analogues for BNCT

- These agents are the products of early design strategies for boronated nucleosides as boron delivery agents for BNCT.

First-generation 3CTAs

- Among the first-generation 3CTAs, N5-2OH was the best substrate of hTK1 leading to successful preclinical BNCT studies with rats bearing intracerebral brain tumors.
- Disadvantages of first-generation 3CTAs were poor water solubility, suboptimal competition with endogenous thymidine at the hTK1 binding site, as well as incomplete understanding of structure–activity relationships and molecular interactions between 3CTAs and hTK1.

Second-generation 3CTAs

- 3CTAs with various structural elements, including ethylene oxide, polyhydroxyl, triazolyl, tetrazolyl, amidinyl and guanidyl and amino moieties, were synthesized and evaluated for improved pharmacodynamic and pharmacokinetic properties.
- 3CTAs with amino and ammonium functions attached to the carborane cluster appeared to be the most promising second-generation 3CTAs.

Non-boronated N3-substituted thymidine analogues

- The development of non-boronated N3-substituted thymidine analogues for areas of cancer therapy other than BNCT as well as for tumor imaging is very promising but in an early stage.

Future perspective

- 3CTAs and non-boronated N3-substituted thymidine conjugates have great potential in cancer therapy and imaging provided currently encountered shortcomings can be overcome and missing information can be filled in.

Figure 1.
Boronophenylalanine (1) and sodium borocaptate (2).

Figure 2. Carborane structures

(A) *closo-o*-carborane, (B) *closo-m*-carborane, (C) *closo-m*-carborane, (D) *nido-o*-carborane, (E) *nido-m*-carborane.

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Figure 3. Kinase-mediated trapping of N5

NDPK: Nucleoside diphosphate kinase; TMPK: Thymidine monophosphate kinase.

Adapted with permission from The Royal Society of Chemistry [24].

Figure 4. hTK1

(A) Monomer subunit of hTK1 with thymidine triphosphate. (B) Tetramer structure of hTK1.

Data taken from [39] (PDB: 1W4R).

Figure 5. Boronated thymidine analogues synthesized and evaluated prior to the discovery of 3-carboranyl thymidine analogues

CDU: 5-(1- α -carboranyl)-2-deoxyuridine; DBDU: 5-dihydroboryl-2-deoxyuridine.

Figure 6.
First-generation 3-carboranyl thymidine analogues.

Figure 7. Docking pose of N5-triphosphate in a homology model of hTK1 (PDB: 1W4R), which was developed from *Tm*TK (PDB: 2QPO) [34].

Adapted with permission [63] © American Chemical Society (2012).

Figure 8. ^{10}B -enriched racemic N5-2OH (19), (*R*)-N5-2OH (20), (*S*)-N5-2OH (21), *m*-carboranyl racemic N5-2OH (22), and *p*-carboranyl racemic N5-2OH (23).

Figure 9.
Amino acid ester prodrugs of N5 and N5-2OH.

Figure 10.
Halogenated and radiohalogenated 3-carboranyl thymidine analogues.

Figure 11.
3-carboranyl thymidine analogues containing ethyleneoxide moieties.

Figure 12.
3-carboranyl thymidine analogues containing cyclic and acyclic polyhydroxylated functions.

Figure 13.
3-carboranyl thymidine analogues with triazole and tetrazole spacers.

Figure 14.
Amidinyl and guanidyl 3-carboranyl thymidine analogues.

Figure 15.
3-carboranyl thymidine analogues with amino and ammonium groups at the carborane cage.

Figure 16. Various non-halogenated, halogenated and ${}^{18}\text{F}$ -labeled N3-alkyl thymidine analogues.

Figure 17.
N3-substituted metal complex-thymidine conjugates.

Figure 18.
Paclitaxel or vinblastine conjugated with thymidine.