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Review Article

Boron-containing nucleosides as tools for boron-neutron capture therapy

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Abstract: Despite the significant progress in cancer cure, the development of new approaches to cancer therapy is still of great importance since many deadly tumors remain untreatable. Boron neutron capture therapy (BNCT), proposed more than eighty years ago, is still considered a potentially advantageous approach. Irradiation of cells containing ^{10}B isotopes with epithermal neutrons and the consequent decay of boron nuclei releases particles that deposit high energy along a very short path, inflicting heavy damage on the target cells but sparing the neighbouring tissue. Delivery and preferential accumulation of boron in cancer cells are the major obstacles that slow down the clinical use of BNCT. Since DNA damage caused by irradiation is the major reason for cell death, the incorporation of boron-containing nucleotides into the DNA of cancer cells may significantly increase the efficacy of BNCT. In this review, we discuss the current state of knowledge in the synthesis of boron-containing nucleosides and their application for BNCT with a special focus on their possible incorporation into genomic DNA.

Keywords: Boron neutron capture therapy, therapeutic nucleosides, carboranes, DNA damage, DNA repair, drug metabolism

Introduction

Although significant progress in cancer therapy was made over the past 20 years, human malignancies remain one of the most challenging health problems. According to the World Health Organization, cancer is the second leading cause of death and was responsible for an estimated 10 million deaths in 2020. Globally, about one in six deaths are due to cancer (https://www.who.int/news-room/fact-sheets/detail/cancer). Conventional cancer treatment is based mainly on surgical removal of tumors or targeting the most rapidly dividing cells by cytotoxic drugs and radiation. These approaches are associated with severe side toxicity, which is the very reason why most antineoplastic treatments must be used intermittently, allowing partial recovery of normal cells but also providing time for cancer cell regrowth and hence for the evolution of drug resistance and invasiveness. During the last decades, many breakthroughs in understanding the basic principles of cell proliferation and cell death have been made, illuminating the molecular mechanisms that underlie the origin and progression of malignant diseases [1]. This knowledge is now being translated into the next generation of safer and more efficient approaches to cancer therapy.

The need for the development of new treatments in oncology is demonstrated by the poor survival rate of patients in many types of cancer. A glaring example is given by high-grade gliomas, and especially glioblastoma multiforme, which are uniformly fatal and have no curative treatment. Patients with these tumors have a life expectancy of 12-15 months, even with the current standard therapy consisting of surgery and radiation therapy with the concomitant administration of temozolomide [2]. The

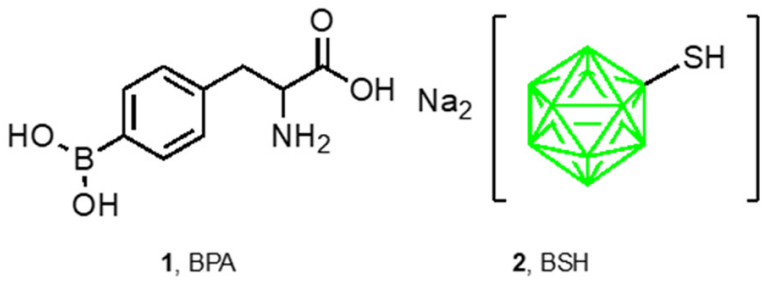

Figure 1. Chemical structures of boronophenylalanine (BPA) and sodium borocaptate (BSH).

failure of regular treatment stimulates work on new medical techniques. Boron neutron capture therapy (BNCT) is one of the most promising approaches to cure such cancers. The principle of BNCT was proposed by Gordon Locher in 1936 [3]. He hypothesized that if boron atoms could be selectively concentrated in tumor cells and then exposed to epithermal neutrons, a higher radiation dose could be delivered to the tumor relative to the adjacent normal tissue. Indeed, when a stable natural ^{10}B nucleus captures an epithermal neutron, it instantaneously decays to produce an α particle (a ^{4}He nucleus) and a recoiled ^{7}Li nucleus. The relevant feature of these particles is that they deposit high energy along a very short path (<10 μm), so their kinetic energy is efficiently spent within the diameter of a single cell. The resulting α particles have a high average linear energy transfer, which can induce complex DNA double-strand breaks [4] and consequent cell death, and the effectiveness of that depends on the intracellular localization of ^{10}B. If boron concentration is selectively higher in cancer cells, then normal cells adjacent to the cancer ones are mostly spared from neutron irradiation [4]. Consequently, the effectiveness and specificity of BNCT largely depend on two key factors: a reliable source of epithermal neutrons and precise delivery of ^{10}B into tumor cells. All clinical studies and use of BNCT to date has been limited to nuclear reactors as the neutron source, with many ensuing technical and regulatory limitations. However, the recent progress in compact accelerator neutron sources holds promise to eliminate this obstacle [5]. The other barrier is the very narrow range of compounds capable of selectively delivering boron isotopes to tumor cells, especially in the brain. The only agents currently used are boronated phenylalanine derivatives, such as boronophenylalanine (1, BPA), and sodium borocaptate (2, BSH; **Figure 1**) [6]. They are actively transported into the cells, but lack cell type selectivity, and are randomly distributed within a cell. Consequently, a rather high amount of boron (ca. 10^9 ^{10}B atoms per cell) is required for neutron absorption-induced cytotoxicity [6].

Active cell division is a defining feature of cancer cells, distinguishing them from the vast majority of mostly post-mitotic cells in the human body, and especially in the brain tissue. The ability of cancer cells to incorporate nucleotide analogues into replicating DNA underlies the mechanism of action of many anticancer chemotherapeutic drugs. Thus, boronated nucleotides (BNs) or their precursors seem to be perfect agents for selective delivery of ^{10}B into cancer cells where they would be stably integrated into DNA. Such precise targeting might significantly reduce the amount of boron required to kill a cell. Moreover, since DNA damage induced by ionizing radiation is the main reason for the death of actively dividing cancer cells, directing boron-containing molecules to DNA itself would enhance the damage in the targeted cells and alleviate it in the adjacent cells, which is difficult to achieve with other boron carriers. In this review, we address the chemistry and biology of BNs, with a special focus on their possible incorporation into genomic DNA.

Chemical routes to boron-containing nucleotides

Generally, BNs can be divided into nucleosides carrying one boron atom, similar to BPA, and nucleosides carrying a boron cluster, similar to BSH. Due to their structural features and unique chemical properties, nucleosides and other molecules carrying boron clusters are considered popular synthetic targets for potential BNCT candidates. Carborane clusters and other polyhedral boranes feature delocalized electron-deficient bonding. Given the electronic nature of boron and hydrogen, such clusters form three-center two-electron (3c-2e) bonds with concomitant formation of trigonal faces and hyperconjugation.

Exact structures and related molecular formulas of boron clusters were described in the

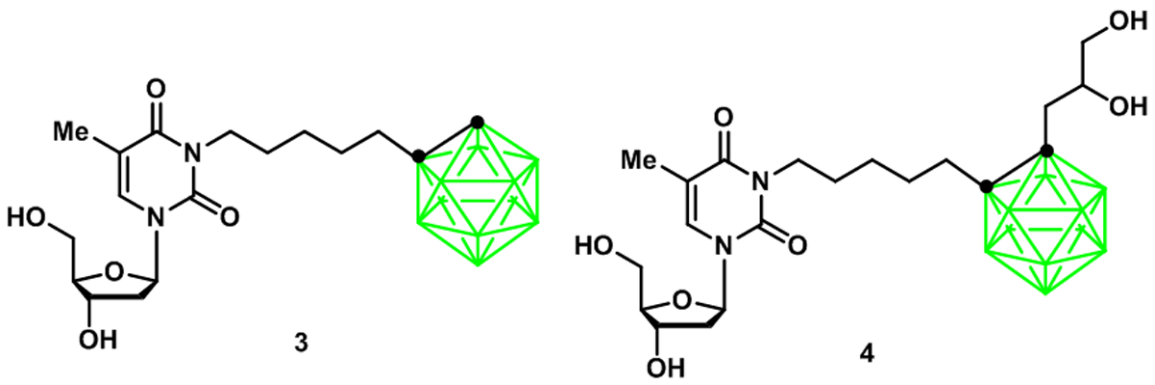

Figure 2. Structure of *N*3-carboranylated dThd derivatives 3-[5-{1-*o*-carboran-1-yl}pentan-1-yl]-2'-deoxyribothymidine 3 and 3-[5-{2-(2,3-dihydroxyprop-1-yl)-*o*-carboran-1-yl}pentan-1-yl]-2'-deoxyribothymidine (4, N5-2OH).

early 1970s by Wade and Mingos [7]. The most abundant type of boron cluster in medicinal chemistry are icosahedral dicarbadodecarboranes. Their sum formula is $C_2B_{10}H_{12}$ in which CH units replace two BH vertices. The B-B and C-B bonds form 12 vertices and measure 1.8 Å in length. Carboranes are sometimes considered inorganic benzenes due to their highly delocalized electronic structure and their hydrophobic properties [8]. However, regarding their size, carboranes are larger, resembling a planar benzene ring rotated around its C_2-axis. In terms of size and volume, carboranes could also be compared to adamantane.

One of the most important chemical features of carboranes is their reactivity at boron and carbon without degradation of the carborane cage. Boron clusters provide a broad range of properties valuable for drug design. First, they convey a unique non-covalent interaction ability, including ionic interactions, σ-hole bonding and dihydrogen bond formation. Therefore, regarding their binding to biological targets, boron clusters differ from ordinary organic molecules, which makes them bioorthogonal and less prone to metabolism. Given that boron clusters can be hydrophilic, amphiphilic or lipophilic and their unique reactivity, which allows for fine-tuning of their chemical properties, carboranes may be used to adjust drug bioavailability and pharmacokinetics. Furthermore, they possess a rigid spherical or ellipsoidal geometry that facilitates computational modelling and design. As a major advantage over single boron carrying compounds in BNCT, boron clusters provide a high content of boron. The chemistry of carboranes is well explored and comprises manifold methods granting access to different substitution patterns that are important in drug design and thus to the exploration of new chemical space [9-11].

Boron cluster-bearing nucleosides were explored as an approach to improve the boron uptake in tumors, mainly glioma cells [12, 13]. A broad variety of different BNs were synthesized and their applicability for BNCT was evaluated. The focus was placed on boronated dThd and dUrd derivatives, since they are substrates for human thymidine kinase 1 (TK1, see below for a detailed discussion). Two known BNs that are known to be phosphorylated by TK1 are shown in **Figure 2**.

Synthetic approaches to obtain thymidine derivative 3, were first reported by Tjarks' group [14], starting from tosylate 5, which was boronated in the presence of bis(acetonitrile)decaborane complex, $B_{10}H_{12}(CH_3CN)_2$, which itself was obtained after refluxing decaborane in acetonitrile for 4 h. The boronation proceeded smoothly and yielded 62% of the carboranyl derivative 6. Subsequently, *N*3 alkylation of dThd in presence of potassium carbonate provided the boronated dThd 3 with a 49% yield.

In order to circumvent the high lipophilicity of 3 concomitant with solubility issues under physiological conditions, a dThd derivative N5-2OH (4) was synthesized [15] that bears a dihydroxy propyl moiety on the carborane cluster. In comparison to its analogue 3, N5-2OH showed higher affinities for TK1 and thymidine kinase 2 (TK2). The synthesis of 4 originally included 9-10 steps with rather unsatisfying overall yields [15]. A more elegant synthesis of 4 yielded the desired thymidine analogue in 4 steps

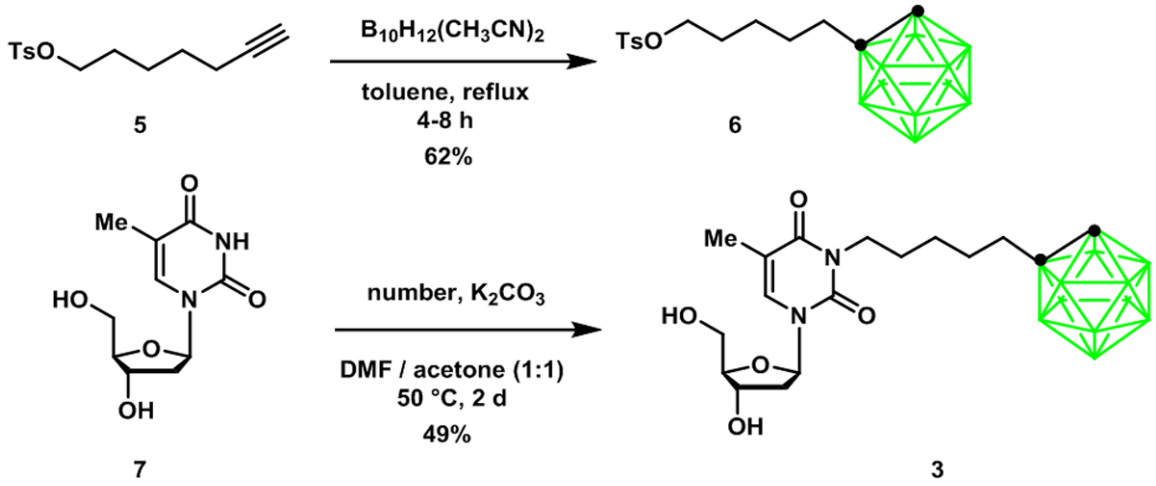

Figure 3. Synthesis of 3-[5-{1-*o*-carboran-1-yl}pentan-1-yl]-2'-deoxyribothymidine 3.

with an 8% overall yield [16]. The synthesis commences with mono allylation of *o*-carborane with *n*-butyllithium and allyl bromide yielding 70% of the allyl-*o*-carborane 8. Subsequent alkylation with homobifunctional 1,5-pentanediol di-*p*-tosylate provided access to connect the *N*3 position of thymidine with the carborane cluster 9 and consequently provided 10.

To prevent disubstitution during the second step with 1,5-pentanediol di-*p*-tosylate, temperatures between 5-10°C were found optimal, whereas higher temperatures resulted in the formation of the olefinic isomer of 10, which is difficult to separate from the desired terminal olefine by silica column chromatography. However, oxidation in presence of osmium tetroxide and 4-methylmorpholine-*N*-oxide yielded only the desired dihydroxide 4 in 40% yield, while the olefine isomer did not react under these conditions, possibly due to steric hindrance originating from the bulky carborane cluster. Additionally, syntheses of the ^{10}B-enriched form of 4 and its stereoisomers were presented, allowing detailed biological studies of their processing by TK1. Furthermore, access to the ^{10}B-enriched N5-2OH provided a basis for preclinical neutron irradiation studies of the survival of rats bearing intracranially implanted F98 glioma cells. The results underlined the suitability of 4 as a candidate for BNCT [16]. These findings were also supported in further studies employing other rodent tumor models [17, 18]. Nevertheless, some problems and limitations remain, including solubility problems under physiological conditions due to the hydrophobic carboranyl core and the absence of ionizable groups.

Further attention was paid to the synthesis and evaluation of metallacarboranes. In contrast to singly boronated or carborane-containing BNs, metallacarborane conjugates possess up to twenty times more boron than boronic acid derivates and twice as much boron as ordinary carboranes. Consequently, metallacarboranes appear as outstanding boron carriers considering their high inherent boron content. 4-*O*-bisethyleneoxy-8-{(1,2-dicarba-closo-undecaborane)-3,3'-cobalt(1)(1',2'-dicarba-closo-undecaborane)}deoxyribothymidine 15 was successfully obtained employing a base-activated nucleophilic substitution of 8-dioxane-[(1,2-dicarba-closo-undecaborane)-3,3'-cobalt(1)(1',2'-dicarba-closo-undecaborane)] zwitterion (12; [3,3'-Co(8-$C_4H_8O_2$-1,2-$C_2B_9H_{10}$)(1',2'-$C_2B_9H_{11}$)]) with protected 2'-deoxyribothymidine 13 as a key step in the synthesis (**Figure 5**) [19]. Separation of the *N*-isomer and *O*-isomer was necessary before deprotection of the hydroxyl moieties in the deoxyribose unit. Furthermore, 15 was incorporated into an oligonucleotide used as a primer for PCR amplification by Taq DNA polymerase [19]. Additional investigations revealed selective accumulation in rapidly multiplying neoplastic cells and entrapment within the cells after phosphorylation [18, 20]. Further metallacarborane-conjugated nucleosides were reported, featuring cobalt and iron as central metal atoms, and Ade and Gua as conju-

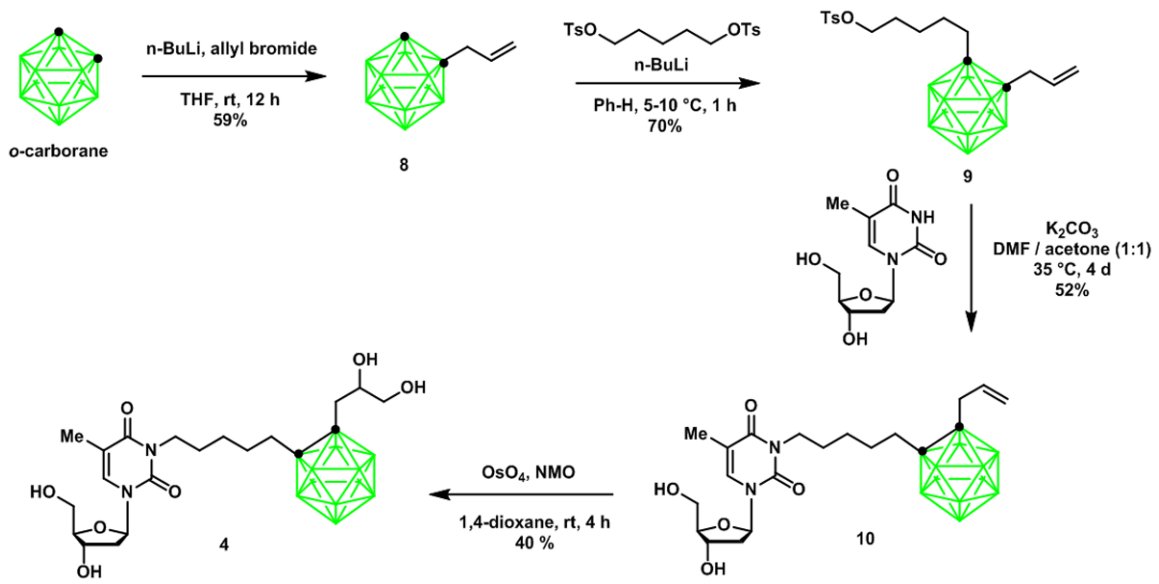

Figure 4. Synthetic route to N5-2OH (4).

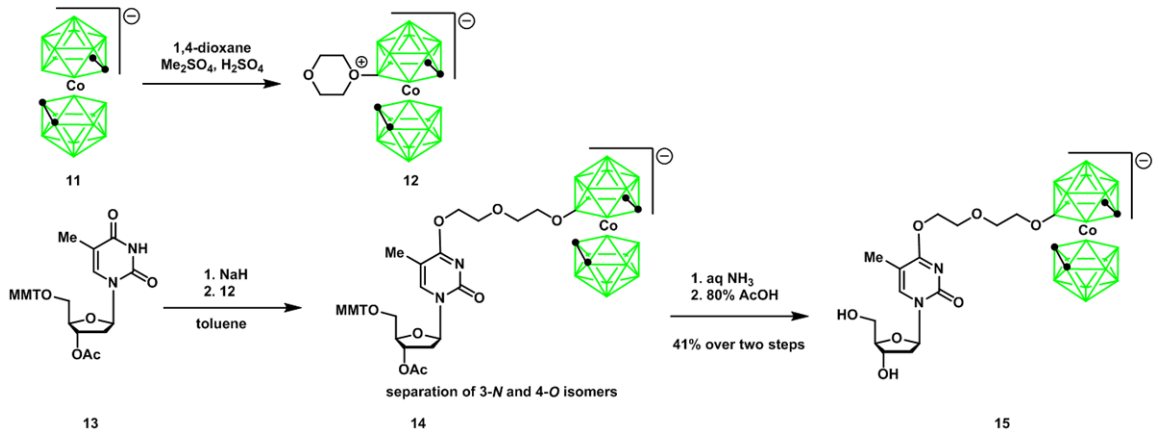

Figure 5. Synthesis of 4-*O*-bisethyleneoxy-8-{(1,2-dicarba-closo-undecaborane)-3,3'-cobalt(1)(1',2'-dicarba-closo-undecaborane)}deoxyribothymidine (15).

gated nucleobases, and various linker modifications [21-23].

Although carborane- and metallacarborane-containing BNs have excellent boron capacity, their solubility issues in physiological media render such compounds difficult to administer. Parallel to synthetic modifications of carborane nucleosides to improve their hydrophilicity, single boron carrying nucleosides have been synthesized with boron modifications in the nucleobase as well as in the backbone (**Figure 6**).

Multiple cyanoborane nucleosides were investigated and characterized with respect to their cytotoxic, anti-neoplastic, anti-inflammatory, anti-osteoporotic and hypolipidemic activities *in vitro* and *in vivo* [24-26]. Remarkable results in tumor selectivity were demonstrated for cyanoboro dCyd 16 in tumor-bearing mice, rendering 16 a suitable candidate for BNCT. Other approaches focused on the introduction of boron at the 5' position of nucleosides. Vasseur's group described a successful synthesis of a borononucleoside isostere of dTMP, which was furthermore shown to undergo coupling with diols including ribonucleosides and their monophosphates through a boronate linkage [27]. The TMP analogue 19 was synthesized starting from 5'-*O*-dimethoxytritylthymidine 21

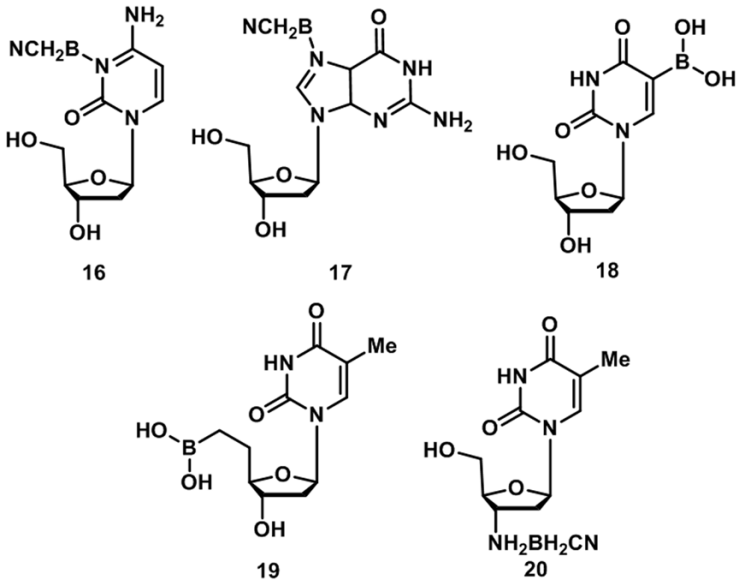

Figure 6. A representative selection of single boron bearing nucleosides.

in 7 steps with an overall yield of 31% (**Figure 7**). Following a three-step procedure, 21 was converted into the 3'-*O*-TBDMS-5'-aldehyde derivative 22. A modified Seyferth-Gilbert homologation of the aldehyde taking advantage of the Bestmann-Ohira reagent smoothly yielded alkyne 23 with a 76% yield. After quantitative reduction, alkene 24 underwent hydroboration in the presence of diisopinocampheylborane and gave the boronic acid TMP analogue 19 after removal of 3'-O-TBDMS protection.

Briefly considering the underlying chemistry of the past decades, which built the foundation of boronated nucleosides, it becomes apparent that long step syntheses and complex procedures so far dominated the synthetic approaches towards new BNs, especially for the preparation of a few single boronated nucleosides. Although successful procedures were reported, little progress was made to explore the chemical space of potentially new BNs for BNCT. Novel approaches in drug discovery like *in silico* screenings and combinatorial chemistry remain seldom used in BNCT research. However, a few reports took advantage of click chemistry for rapid access to new BNs [23, 28]. This, in combination with more accurate computer simulations, might open new ways towards accelerated screening of BNs for BNCT applications.

Cellular fate of boron-containing nucleotides

Uncharged normal nucleosides are efficiently transported through the cellular membrane [29, 30], accumulated in the cells and incorporated into DNA. Nucleosides delivered into the cell exist in two kinetic pools. A high-mobility pool that includes mononucleosides is turned over in metabolically active cells within minutes to hours. Pharmacologically, the elimination half-life for the high-mobility pool varies from hours for non-phosphorylated nucleosides to days for those that can be phosphorylated and thus trapped in the cell. A low-mobility pool is represented by dNMPs incorporated into genomic DNA and is essentially irreversible unless subject to excision by DNA repair mechanisms. Such pools may be hard to detect by regular pharmacokinetic measurements due to their small size compared with the total dose but they can have disproportionally large effects if used for BNCT. However, the processing of the boron-containing nucleosides by the cellular enzymes is unpredictable and potentially may prevent the formation of boron-containing dNTPs or their incorporation into DNA. Partially this problem may be solved by direct delivery of the boron-containing dNTPs into the cells. Recently developed drug delivery methods including liposomes and nanoparticles can be used not only transporting the boron-containing dNTPs into the cells but also to target them directly to cancer cells [31]. However, efficient incorporation of the boron-containing dNTPs into the cells still may be a problem. Below, we summarize the current knowledge on the cellular fate of BNs and attempt to make inferences about BNs from the mechanisms operating on other therapeutic nucleotides.

Uptake and metabolism

Therapeutic nucleosides and nucleobases are usually transported into human cells through several solute carrier proteins, most of which belong to two major families: Na^+-coupled

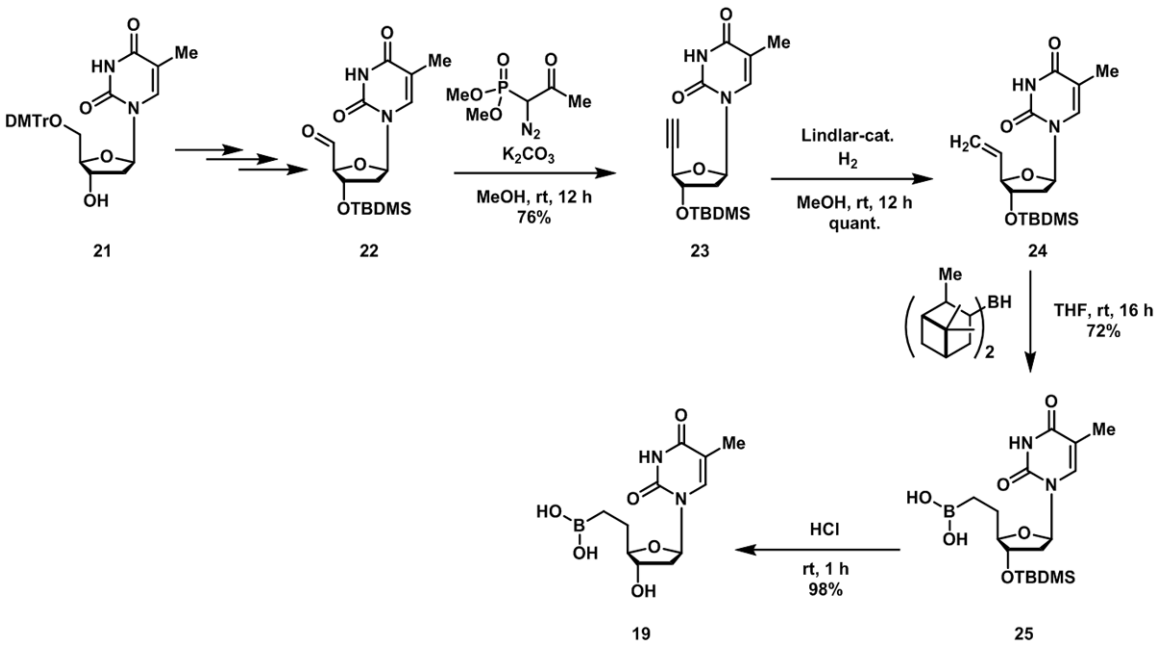

Figure 7. Synthesis of dTMP isostere 19.

concentrative nucleotide transporters (CNT1-CNT3) and equilibrative nucleoside transporters (ENT1-ENT3) [30, 32], (**Figure 8**). Not much is known about the transport of BNs into the cell. It was shown that an *N*3-carboranylated dThd derivative N5-2OH is taken up by cells through the broad-specificity equilibrative nucleoside transporter 1 (ENT1) [33], whereas 2'-*O*-(*o*-carboran-1-ylmethyl)uridine accumulates in cells in a nucleoside transport-independent manner [34]. Some little-studied possibilities of cellular entry for polynuclear inorganic clusters, including carboranes, are receptor-independent endocytosys and direct adsorption on membranes causing their leakiness [35].

Several BNs inhibiting nucleotide biosynthesis pathways have been reported [36, 37]. However, it should be reiterated that the logic of efficient BNs use in BNCT is different from therapeutic nucleoside antimetabolites: rather than inhibit nucleotide biosynthesis, BNs must be efficiently processed and converted to dNTPs that would be further incorporated into genomic DNA retaining boron atoms to the low-mobility pool. After the cell uptake, therapeutic nucleosides possessing a 5'-hydroxyl group are generally phosphorylated by the enzymes of nucleotide salvage pathways, such as cytidine kinase (DCK) or nuclear TK1, and further converted to NTPs or dNTPs by several nucleoside monophosphate kinases and nucleoside diphosphate kinases as well as metabolic enzymes such as pyruvate kinase and creatine kinase [38, 39], (**Figure 8**). BNs with a blocked 5'-hydroxy group or nucleotides with a modified 5'-phosphate, such as 5'-*O*-[(triphenylphosphine-boryl)carbonyl]-3'-acetylthymidine and 5'-(diethylphosphite-cyanoborane)-3'-acetylthymidine may serve as efficient inhibitors of nucleotide synthesis pathways, DNA and RNA polymerases [36] but are not drawn into the NTP/dNTP pool. Interestingly, the dTMP isostere 19 can be phosphorylated by thymidylate kinase (TMPK), albeit slowly, and acts as an inhibitor for TMPK and several other human NMP kinases [40]. However, the B-O-P linkage is unstable in an aqueous environment, and the dNTP analogue produced by TMPK cannot be processed further.

For BNs with a free 5'-hydroxyl, at least the first bioactivation step does not seem to be a problem: efficient phosphorylation by TK1 (but not by mitochondrial TK2) was reported for dThd with a carboranyl moiety linked to C3' [41] or N3 [33, 42-46]. Among these, several compounds resistant to dephosphorylation by cellular 5'-deoxynucleotidases and hydrolysis by

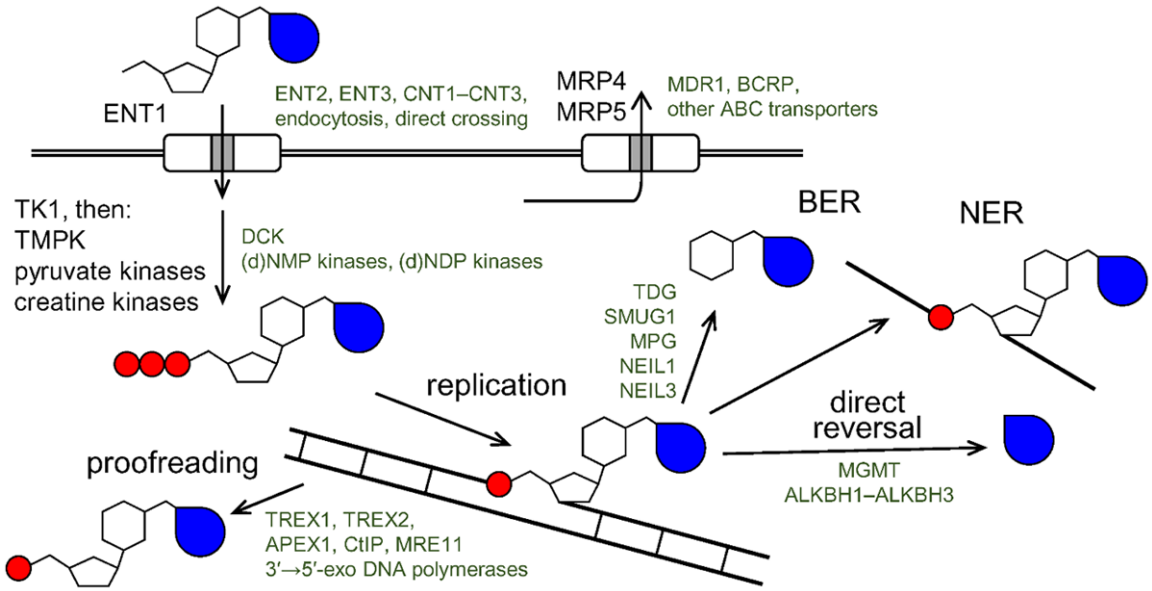

Figure 8. Likely cellular fates of boronated nucleotides. Enzymes and processes acting on other therapeutic nucleotides but not yet confirmed for BNs are listed in green. Red circles schematically depict phosphate groups, and blue drops, boron-carrying modifications.

thymidine phosphorylase were identified [15]. N3-carboranylated dThd derivatives, while acting as competitive inhibitors of TK1, had only moderate cytotoxicity in cell cultures (tens to hundreds μM IC_{50}), which did not depend on the cells' TK status [15, 43]. Further steps, however, may present a challenge. For example, N5-2OH is efficiently monophosphorylated but its monophosphate is not a substrate for human UMP-CMP kinase or TMP kinase, and the diphosphate is a poor substrate for nucleoside diphosphate kinase, so the nucleoside is not converted to di- and triphosphates in the cell [33]. On the other hand, 5'-(α-P-borano) dNDPs/NDPs are robustly phosphorylated by pyruvate kinase and creatine kinase [47]. 5'-α-P-borano derivatives of acyclic adenine nucleoside analogs (e.g., dA analogs adefovir and tenofovir) are also phosphorylated by adenylate kinases, although ~1000-fold less efficiently compared to natural dAMP/AMP [48].

N5-2OH and its monophosphate are actively excreted from cells by multidrug resistance-associated organic anion transporters MRP4 and MRP5 [33]. It is possible that other ATP-binding cassette (ABC) family transporters, such as MDR1 or BCRP that participate in the efflux of therapeutic nucleotides, are also involved [29, 30].

Incorporation into DNA

Incorporation of boronated dNTPs by DNA polymerases strongly depends on the site of conjugation of the boron-carrying moiety and follows general rules determining the dNTP substrate properties for template-directed synthesis. Substitutions at C5 are generally well tolerated: dUTP containing a carborane or boronic acid tethered through a linker at C5 is efficiently incorporated by several commercial thermophilic DNA polymerases used in molecular cloning and PCR (Klenow fragment, Taq, KOD, Vent and Pwo) [49-51]. These polymerases belong to Families A and B, which also include human replicative DNA polymerases α, δ, and ε [52], so there's a good chance that C5-substituted dNTP boron carriers can make their way into DNA in human cells (**Figure 8**). On the other hand, *N*3-carboranylated dTTP, in which the Watson-Crick face is disturbed, is not a substrate for the Klenow fragment [33]. *R* diastereomers of 5'-(α-P-borano)dNTPs/NTPs are good substrates for T7 DNA polymerase, HIV reverse transcriptase and hepatitis C RNA-dependent RNA polymerase while the *S* diastereomers are poorly incorporated and in some cases act as competitive inhibitors [53-56].

From a kinetic point of view, modified base pairs appear in DNA in two steps: a DNA poly-

merase must first incorporate a modified dNMP (or a normal dNMP opposite a modified one) and then extend from such a non-canonical structure [57]. In human cells, replication pauses due to DNA or dNTP damage are usually resolved through a polymerase switch mechanism, in which specialized DNA polymerases catalyze insertion or extension steps [58]. Bulky carborane clusters close to 3'-terminus were found to reduce the efficiency of primer extension by single DNA polymerases but not to stop it completely [59]. Thus, incorporation of boronated dNMPs with a free 3'-hydroxyl will likely be followed by extension, fixing boron in the low mobility pool.

The available data on the bypass of boronated nucleotides in the template are still contradictory. For C5-substituted dUrd, both efficient use of such templates [49, 50] and complete block of DNA synthesis [51] were reported. 5'-(α-P-borano)phosphates appear to be easily bypassed [60]. It should be noted, however, that these studies used PCR as a measure of the ability of polymerases to bypass the boronated template, which is generally not a reliable way to address the blocking and coding properties of modified nucleosides.

There are only a few examples where the incorporation of boronated nucleosides into cellular DNA was demonstrated by mass spectrometry [61-63]. However, none of these data have been either reproduced or disproved so far. The ability to deliver ^{10}B directly into genomic DNA remains a critical issue for the ultimate success of BNs in BNCT and warrants special studies.

Proofreading

Proofreading activity of DNA synthesis enzymes may significantly affect the incorporation of boron-containing nucleotides into DNA. The concept of exonucleolytic proofreading as a general mechanism of error correction in replication appeared in the 1980s and matured over the next two decades [57]. Many DNA polymerases have 3'→5' exonuclease activity, which is more specific for unpaired 3'-terminal nucleotides and serves to remove misincorporated nucleotides. In some cases, the proofreading activity belongs not to the DNA polymerase itself but to a separate protein (**Figure 8**). In human cells, proofreading can be performed both by replicative DNA polymerases δ and ε [64] and by separate exonucleases such as TREX1 and TREX2 [65], apurinic/apyrimidinic exo/endonuclease APEX1 [66, 67] or CtIP and MRE11 DNA repair exonucleases from the non-homologous end-joining pathway [68]. In addition, proofreading exonucleases also remove many nucleoside inhibitors that work through incorporation into DNA [68-73].

5'-(α-P-borano)dNMPs incorporated into DNA are poor substrates for exonuclease III, a bacterial homolog of human APEX1 [54, 60], and for other DNA-degrading enzymes such as phosphodiesterases from snake venom and bovine spleen, S1 and P1 nucleases [74]. C5-carboranylated dUrd was reported to be resistant to phosphodiesterases [59]. From the limited data available at present, it seems that proofreading would not be a major problem for DNA-targeting BNs.

DNA repair

DNA repair is a major factor limiting the efficiency of drugs whose action is based on their incorporation into DNA or the formation of adducts with DNA [75, 76]. BNs should not be an exception and are likely to be subject to removal by multiple DNA repair systems. However, no reports concerning the repair of boron-containing DNA is available at present. Considering the chemistry of known BNs, several well-studied DNA repair pathways might be involved in their removal (**Figure 8**).

Carboranyl moieties are of considerable size, comparable to "bulky adducts" in DNA such as benz[*a*]pyrenes, aflatoxins, etc. Such adducts are usually removed by a DNA repair pathway known as nucleotide excision repair (NER). In human cells, NER operates in two modes: global genome repair (GGR) and transcription-coupled repair (TCR), which rely on different mechanisms for the lesion search [77-79]. In GGR, the presence of damage in any part of the genome is sensed by specialized protein complexes such as XPC/RAD23B/CETN2 or DDB1/DDB2 based on the distortion of DNA duplex by a bulky adduct. In TCR, the sensor is an RNA polymerase molecule stalled at the lesion, and TCR predominantly repairs lesions in the template strands of transcribed genes. In both cases, the damage sensor serves as a platform to assemble the repair machinery involving a

dozen or so protein subunits. This complex makes two nicks in DNA at both sides of the lesion, excises a 20-30-nt single-stranded oligonucleotide, and replaces it with a newly synthesized undamaged strand [77-79].

Modified nucleobases may also be substrates for the base excision repair (BER) pathway. BER is initiated by DNA glycosylases, the enzymes that hydrolyze the *N*-glycosidic bond of damaged nucleotides and remove the base producing an apurinic/apyrimidinic (AP) site [80, 81]. The repair is continued with AP site nicking, end polishing at the nick, insertion of the normal dNMP by a DNA polymerase and the final ligation step [82]. Considering the major sites of the introduction of boron-containing groups into nucleobases (**Figures 2**-**6**), several DNA glycosylases could target such modifications. In human cells, DNA glycosylases TDG and SMUG1 can excise pyrimidine bases with bulky C5 substitutions, although the size limit for the exocyclic moiety has not been established [83-87]. *N*7-substituted purines are excised by alkylpurine-DNA glycosylase (MPG) [88, 89]. They are also prone to imidazole ring opening, and in the ring-open form may be excised by NEIL1 and NEIL3 DNA glycosylases, even with substitutions as large as an aflatoxin adduct [90, 91].

Finally, some adducts at ring nitrogens and exocyclic nitrogens and oxygens are substrates for a subset of direct reversal pathways. Adducts at O^6 of guanine and O^4 of thymine, including quite bulky ones, can be removed by O^6-methylguanine methyltransferase (MGMT) via a direct transfer of the whole alkylating moiety to a Cys residue in the enzyme's active site [92, 93]. In fact, dealkylation of a free guanine base modified at O^6 by a boron carrier was demonstrated in living cells, although in this case, it was a BODIPY fluorescent dye-labeled probe rather than a therapeutic compound [94]. Adducts at adenine exocyclic N6, N1 of purines and N3 of pyrimidines are repaired by oxidative dealkylation by AlkB family dioxygenases (ALKBH1-ALKBH3 in human cells) [95, 96], though the adduct size requirements have not been studied so far.

Conclusions

Future directions of research needed to improve BNCT. Today, the instrumental advances in neutron generation finally make BNCT a feasible therapeutic option. The main biological problem remaining in the BNCT's way into clinical use is the lack of selective ways to deliver boron to cancer cells and concentrate it in the critical cellular compartments. With random intracellular distribution, it has been estimated ~10^9 ^{10}B atoms are required to kill a cell at reasonably achievable neutron fluxes [97]. Specifically targeting DNA as the most sensitive cellular compartment holds promise to reduce this amount significantly, especially for tumors affecting nearly completely postmitotic organs and tissues, such as cerebral gliomas. However, several hurdles remain to be cleared to achieve this goal.

Although the chemistry of boron incorporation into different components of DNA is developed, there are currently no methods for screening multiple boron-containing nucleosides for their incorporation into DNA in living cells. Consequently, at this stage, making and testing individual boron-containing nucleosides is risky since it is difficult to predict which of them would be recognized by the cellular DNA biosynthesis enzymes and incorporated into DNA. The solution to this problem may be the employment of combinatorial chemistry, which allows the synthesis of a library of boron-containing nucleosides that can be used to treat cultured human cells to allow the cells themselves to identify which boron-containing nucleosides can be processed and incorporated into DNA. The nature of the boron-containing nucleotides incorporated into DNA can be identified by liquid chromatography/mass spectrometry (LC/MS) and synthesized at a large scale for further investigation.

The knowledge of BNs interactions with cellular uptake, metabolism, replication and repair systems is still unacceptably poor for potential drugs. In particular, more research is required to understand the stability of the incorporated boron-containing nucleotides in DNA because modified nucleotides are likely to be efficiently removed from DNA by the DNA repair systems. To increase BNCT efficacy, further studies are needed to find out optimal timing for tumor irradiation after treatment to hit the balance between the incorporation of boron-containing nucleotides into DNA and their removal. Additionally, sensitization of tumor cells through inhibition of DNA repair might aid the success

of BNCT, even with traditional boron carriers. This strategy, using poly(ADP-ribose) polymerase inhibitors to enhance the cytotoxicity of DNA-damaging drugs, is now successfully employed with ovarian and breast cancer and is under clinical trials in combination with radiation therapy. Only the ability to concentrate boron in the genomic DNA in a controlled way can unleash the full potential of BNCT in cancer cure.

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Disclosure of conflict of interest

None.

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