# Diamidines for human African trypanosomiasis

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*REVIEW*

# Diamidines for human African trypanosomiasis

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*Aromatic diamidines are potent trypanocides. Pentamidine, a diamidine, has been used for more than 60 years to treat human African trypanosomiasis (HAT); however, the drug must be administered parenterally and is active against first-stage HAT only, prior to the parasites causing neurological deterioration through invasion of the CNS. A major research effort to design novel diamidines has led to the development of orally active prodrugs and, remarkably, a new generation of compounds that can penetrate the CNS. In this review, progress in the development of diamidines for the treatment of HAT is discussed.*

***Keywords*** Blood-brain barrier, clinical trial, diamidine, drug metabolism, drug transport, furamidine, human African trypanosomiasis, pentamidine, sleeping sickness

## Introduction

Human African trypanosomiasis (HAT) is caused by parasitic protozoa of the *Trypanosoma brucei* subgroup [1]. The disease is endemic in sub-Saharan Africa, where its distribution is determined by the habitat range of the tsetse fly vector, which transmits the parasite. HAT has two defined stages: the first stage involves trypanosomes in the hemolymphatic system; and the second (or late) stage begins once parasites enter the CNS, where their presence initiates a deterioration in neurological function, including disruptions to sleep/wake patterns, resulting in this stage of the disease being referred to as 'sleeping sickness'.

Antigenic variation has precluded the development of vaccines against HAT [2], resulting in chemotherapy being the only option for disease intervention at the level of infected individuals [3]. The concept of integrated control, diminishing transmission by tsetse flies and reducing the infected population (ie, the primary reservoir for infection) by active surveillance and drug treatment, is attractive [1]; however, HAT, which afflicts the world's poorest populations exclusively, is a largely neglected disease, and many challenges, such as lack of funding, the general poor health of patients and their inaccessibility to healthcare, constrain progress toward the development of a successful therapeutic agent.

Current drugs for treating HAT are generally unsatisfactory because of varying degrees of toxicity, a need for parenteral administration, prohibitive cost and distribution difficulties, all of which negatively impact use [3]. For first-stage disease, prior to CNS involvement, two drugs are used: pentamidine (Figure 1) for *Trypanosoma brucei gambiense* infection; and suramin, largely

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current diamidines regarding mechanism(s) of action, distribution to the brain and accumulation in tissue can be used to design improved compounds for evaluation in animal models of efficacy and safety.

Enhanced awareness of HAT has attracted several research groups to begin developing new drugs and, in addition to the diamidines, several new classes of compounds, such as the nitroheterocycle fexinidazole, are entering preclinical and clinical development for second-stage disease through the DNDi program [62]. The clinical trials to test the efficacy and safety of pafuramidine in HAT coincided with a sustained and significant reduction in the incidence of the disease, which can be attributed partially to the trials themselves. The CPDD continues to evaluate diamidines in preclinical studies, and anticipates selecting a clinical candidate for second-stage disease in the near future, with DB-829 being the most likely candidate. It is anticipated that this renewed interest in HAT, coupled with the experiences gained from the first large-scale trials for an NCE for HAT, eventually will lead to a sustainable pipeline of medications to control the disease, or even fulfill the WHO mission of eliminating HAT [63].

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Furthermore, the accumulation of DB-829 in both the liver and kidneys was less than that of furamidine (≥ 50% and 20%, respectively). Taken together, these data suggest that parenteral DB-829 is promising as a treatment for second-stage HAT. A study of the safety of an acetate salt of DB-829 (CPD-0802), administered intravenously to uninfected vervet monkeys, is underway [Murilla GA, Thuita JK: unpublished data]. If successful, it is expected to trigger entry of CPD-0802 into clinical development.

## The clinical development of pafuramidine

The advantages of pafuramidine, an orally available analog of pentamidine, stimulated preclinical and phase I clinical trials. Pafuramidine was advanced to clinical development for the treatment of first-stage HAT by the CPDD and Immtech. Pafuramidine underwent preclinical microbiology, pharmacology and toxicokinetic evaluation, and demonstrated good efficacy with an acceptable safety profile [51-54]. Reversible liver toxicity in rodents and monkeys was observed, which was anticipated based on the known toxicity of pentamidine [55].

Phase I clinical trials of safety and pharmacokinetics in healthy volunteers indicated that pafuramidine was absorbed and converted to the active metabolite furamidine, yielding plasma concentrations appropriate for the treatment of HAT. The compound was well-tolerated in these trials [56].

Phase II clinical trials of pafuramidine (100 mg bid for 5 days) were initiated in 2001 by the Pharmaceutical Medicine Unit of the Swiss Tropical Institute and Immtech, and marked the first time an NCE had been studied in controlled trials for HAT. The trials were conducted in the Democratic Republic of Congo (DRC) and Angola in patients with first-stage *T b gambiense* HAT. Pafuramidine demonstrated promising results in terms of drug safety. Increased treatment duration of 10 days was needed for acceptable efficacy and resulted in a cure rate of 93% at 3 months post-treatment [57].

A phase III clinical trial of pafuramidine was conducted between August 2005 and March 2007 in the DRC, Angola and Southern Sudan, and enrolled 273 patients, including pregnant and lactating women and adolescents [58]. Patients were randomized in an open-label design to treatment with pafuramidine (100 mg po, bid for 10 days) or pentamidine (4 mg/kg im, qd for 7 days) and were monitored for 2 years. Both drugs were well tolerated, liver toxicity during treatment was significantly less in the pafuramidine group than in the pentamidine group. The primary endpoint of 12-month post-treatment efficacy for pafuramidine was 89% compared with 95% for pentamidine ($p = 0.067$) [59]. The 24-month post-treatment efficacy was 84 and 89% ($p = 0.212$), respectively.

In October 2007, a phase I clinical trial was initiated to expand the safety data needed for pafuramidine regulatory registration for trypanosomiasis and pneumocystis pneumonia [59]. The development program was suspended when ~ 25% of patients in the trial developed elevated transaminases ~ 5 days after the last dose; all patients returned to baseline transaminase levels without sequelae. When acute renal insufficiency was observed in five patients ~ 8 weeks after dosing, the pafuramidine program was discontinued [59]. The five patients were treated with corticosteroids and other supportive care, and blood urea nitrogen and creatinine levels improved significantly in the following months. The affected patients continue to be monitored by specialists.

The phase III clinical trial for HAT was in a follow-up period at the time of the suspension of the phase I trial. Three patients with glomerulonephritis or nephropathy post-pafuramidine treatment were subsequently identified; two of these cases retrospectively may be considered possibly related to pafuramidine [59]. These three patients recovered without sequelae following treatment with corticosteroids. The cause for these unforeseen adverse drug reactions is unclear and is being investigated. No new drug-related adverse events were observed in any other pafuramidine trial. Whether the adverse events are a class effect or specific to pafuramidine is not yet clear, but it is of significance that DB-829 accumulates in host tissue significantly less than furamidine, including in the liver and especially kidneys, as discussed in the previous section.

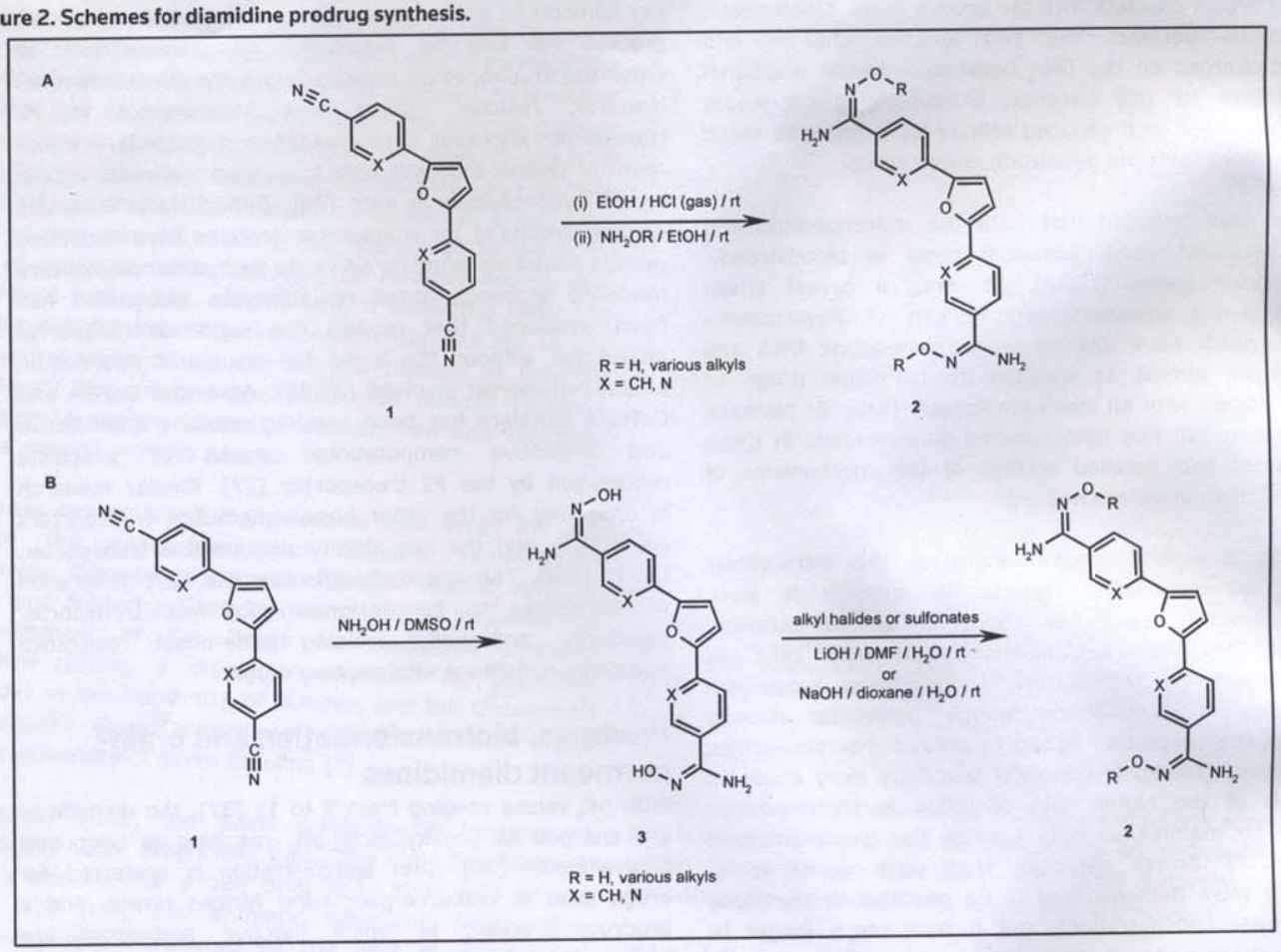
The subsequent discontinuation of the pafuramidine program, following the adverse effects identified in the phase I and III clinical trials, represented a major setback in drug development for HAT. However, many positive outcomes resulted from this project. For example, the program led to many infrastructure improvements and investigator training at the clinical sites. In addition, more than 250,000 patients were screened, and 416 patients were treated in the phase II and III clinical trials. The rate of participation in the 24-month follow-up visits for the phase III trial was 97% [59]. Patients who were actively screened for study participation, but who were ineligible because of the presence of second-stage HAT, also received appropriate treatment and follow-up.

It is important to note that the incidence of HAT has demonstrated a significant and sustained reduction over the time frame of the pafuramidine clinical trials. This decrease in prevalence suggests that the trials, along with other initiatives such as the WHO-sponsored surveillance, drug distribution campaigns and enhanced engagement of various non-governmental organizations (ie, MSF, Malteser International and Merlin), has contributed to the decrease in the prevalence of HAT during the past decade [60,61].

## Conclusion

The extraordinary efficacy of diamidines against trypanosomal infection indicates that this class of compounds continues to advance drug development campaigns for HAT. New scientific understanding of

**Figure 2. Schemes for diamidine prodrug synthesis.**




(**A**) The synthesis of furamidine prodrugs is illustrated. The original procedure for producing the amidoxime and the *O*-methylamidoxime of furamidine, involving a Pinner approach, is outlined [64]. In principal, the imidate ester intermediate can be allowed to react with hydroxylamine or any *O*-alkylhydroxylamine to form the corresponding *O*-alkylamidoxime. This approach is limited by the well-established drawbacks of the Pinner method, primarily the necessity for rigorous exclusion of water to prevent amide formation and the requirement for long reaction times because of very low solubility of the bis-nitriles. (**B**) The synthesis of *O*-alkyloxime prodrugs of furamidine is shown. *O*-Alkylation of the bis-amidoximes (compound **3**) can be readily achieved by base-mediated alkylation with alkyl halides or sulfonates at room temperature [40]. In each case, alkylation conditions should be optimized to minimize multi-alkylation (*N*-alkylation).

Pafuramidine has demonstrated efficacy in models of first-stage infection in mice [46] and vervet monkeys [47], as well as in clinical trials (discussed in the following section). The aza analogs DB-844 and DB-868 have exhibited efficacy in models of first- and second-stage infection in mice [46]. However, DB-844 was associated with dose-limiting gastrointestinal toxicity in uninfected vervet monkeys [Murilla GA, Thuita JK: unpublished data], and further research with this compound has been discontinued. An efficacy study of DB-868 in a vervet monkey model of second-stage HAT is underway.

The discovery that prodrugs of some diamidines treat disease in the CNS has offered hope for new drugs for second-stage HAT. The mechanism(s) by which the diamidines enter the CNS and elicit anti-trypanosomal activity is not fully elucidated. Furamidine [48] and the aza analogs DB-820 and DB-829 [Wang MZ: unpublished data] have been demonstrated to be substrates of the human facilitative organic cation transporter 1 (OCT1) in CHO cells stably transfected with OCT1. However, because expression of OCT1 and Oct1 in the blood-brain barrier (BBB) of humans and mice, respectively, is reportedly low [49,50], different transporters may facilitate the entry of certain diamidines (ie, DB-820 and DB-829) into the brain to treat second-stage disease in the mouse model.

A preliminary pharmacokinetic study in rats administered a single dose (3 mg/kg iv) of furamidine or DB-829 demonstrated biphasic plasma concentration-time profiles for both diamidines [Paine MF, Jones JP: unpublished data]. The systemic exposure (ie, AUC) of DB-829 was twice that of furamidine, a reflection of a lower systemic clearance for DB-829, and may imply that DB-829 has a higher likelihood of entering the brain. The steady-state volume of distribution was similar between the two compounds. Although the elimination half-life of DB-829 was twice that of furamidine, the distribution half-life of DB-829 was 3-fold that of furamidine, indicating that DB-829 distributed to tissues more slowly than furamidine.

van der Waals contacts with the groove walls. Electrostatic interactions between the two amidine charges and anionic charges on the DNA backbone provide additional stabilization for the complex. GC-containing sequences bind diamidines with reduced affinity as a result of steric interferences from the guanosine amino group.

Studies have revealed that both the mitochondrion and the kinetoplast serve essential roles in bloodstream-form trypanosomes [16,17]. However, a caveat arises in considering dyskinetoplastic strains of *Trypanosoma evansi*, which have lost condensed kineoplast DNA and yet remain almost as sensitive to diamidine drugs as trypanosomes with an intact kinetoplast [Brun R: personal communication]. Key mitochondrial genes persist in these organisms, and detailed studies of the mechanisms of action of diamidines are needed.

Although questions remain regarding the intracellular targets of diamidines, trypanocidal activity is slow, taking several days after exposure to kill parasites *in vitro*. Diamidines are dications that enter cells via plasma membrane transporters. Trypanosomes accumulate diamidines in high concentrations (millimolar overall, although the proportion bound to cellular macromolecules is unknown) [18,19]. Trypanocidal specificity likely arises as a result of the higher rate of influx in trypanosomes relative to mammalian cells against the transmembrane gradient. *T brucei* parasites that were pentamidine-resistant were demonstrated to be sensitive to the same intracellular concentrations, but it took much longer to achieve these concentrations than in wild-type organisms [19], suggesting that resistance was associated with a change in the mechanism of uptake. Disabling mutations in the transporters responsible for diamidine uptake in trypanosomes would be expected to lead to diamidine resistance.

Pentamidine is used to treat > 90% of all first-stage HAT cases and also has been used as a prophylactic agent on a population scale [20]. Despite long-term, widespread use, clinical resistance has been reported rarely. The lack of resistance and persistence of efficacy over time may relate to the uptake of pentamidine through multiple membrane transporters. The main trypanosome transporter for diamidines is the P2 aminopurine transporter [21-24]. The recognition motif for this transporter is shared between adenine-, adenosine-, amidine- and melamine-containing compounds [25-27]. While loss of this transporter underlies resistance to some diamidines [28-30], it leads to only minor loss of sensitivity to pentamidine [31]. High-level resistance to pentamidine (and melaminophenyl arsenicals) requires the loss of at least one other transporter, the so-called high affinity pentamidine transporter (HAPT), in addition to P2 loss [32,33].

An understanding of the kinetic parameters for each transporter would allow the prediction of their exact contribution to the net transmembrane flux for each diamidine (ie, drug accumulation) [33,34], representing a key component of the anti-trypanosome drug development process. For example, furamidine was demonstrated to enter the trypanosome principally via the P2 transporter. However, residual uptake was measureable in P2 transporter knockout cells, revealing a secondary minor route of uptake that was able to sustain sufficient uptake to kill trypanosomes in mice [29]. Difficulties in obtaining crystal structures for transporter proteins have restricted protein-based modeling for SARs. As such, a complementary modeling approach based on substrate recognition has been developed that models the substrate-transporter interaction without the need for structural information of the transporter involved [35,36]. Advanced CoMFA and CoMSIA software has been used to create a quantitative and predictive computational model of substrate recognition by the P2 transporter [27]. Similar research is underway for the other known diamidine transporters (ie, HAPT1 and the low affinity pentamidine transporter, LAPT1 [34]). This approach will allow the next generation of diamidines to be designed with multi-transporter selectivity, potentially avoiding early-onset resistance and cross-resistance with existing drugs.

## Prodrugs, biotransformation and brain-permeant diamidines

With $pK_a$ values ranging from 9 to 11 [37], the diamidines are charged at physiological pH, resulting in poor oral bioavailability [38]. Oral administration is preferred for drugs used in resource-poor rural African clinics, and a prodrug strategy, in which inactive precursors are biotransformed to active metabolites [39], was devised to investigate whether diamidine derivatives could be administered orally.

Pafuramidine and the aza analogs DB-844 and DB-868 (both from the University of North Carolina/Georgia State University; Figure 1) were designed as prodrugs of the diamidines furamidine, DB-820 (University of North Carolina/Georgia State University; Figure 1) and DB-829 (CPD-0801; University of North Carolina/Georgia State University; Figure 1), respectively [40]. These prodrugs involved masking the positive charge of the amidine functional group with *O*-alkyl moieties (Figure 2), resulting in increased lipophilicity and intestinal permeability [41-43].

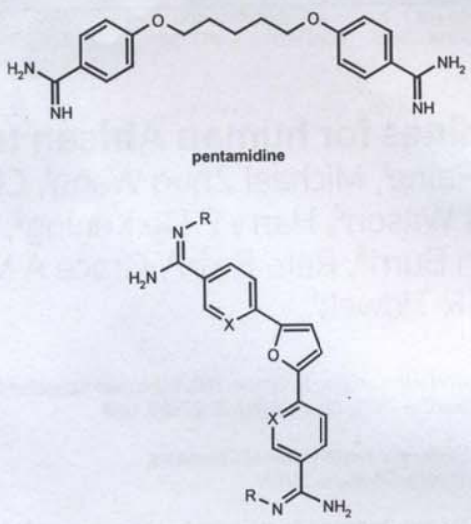
Biotransformation of prodrugs to the active diamidines requires a series of enzymatic reactions. The biotransformation of pafuramidine to furamidine involves oxidative catalysis by cytochrome P450 enzymes and reductive catalysis by the cytochrome $b_5$/NADH-cytochrome $b_5$ reductase system. Four intermediate metabolites (M1 to M4) precede the formation of furamidine [44,45]. Prodrug biotransformation occurs primarily in the liver, and the elimination of furamidine occurs via biliary excretion [43]. Tissue distribution data from rats administered a single oral dose (10 mg/kg) of pafuramidine indicated that furamidine was retained extensively in liver, as exemplified by a liver to plasma concentration ratio of 1300:1 [43].

for *Trypanosoma brucei rhodesiense* infection. For second-stage disease, melarsoprol (a melaminophenyl arsenical) was, until recently, the first-line drug of choice against gambiense disease and the only treatment for rhodesiense disease. Eflornithine has replaced melarsoprol as first-line therapy for second-stage gambiense disease in many foci (ie, geographically confined regions), but is of insufficient efficacy to treat the rhodesiense form of HAT. In 2009, a combination therapy, comprised of eflornithine at reduced dose plus nifurtimox, was added to the WHO list of essential medicines [4]. The reduced dosing with improved tolerability, coupled with a perceived reduction in the risk for selecting drug resistance, has led to the recommendation of the combination therapy as first-line treatment for second-stage gambiense disease [Simarro P: personal communication]; however, new and improved drugs are still urgently needed.

Progress has occurred in developing new drugs for HAT during the past few years. Several not-for-profit initiatives followed a major campaign against HAT by WHO and Médecins Sans Frontières (MSF), including the establishment of the Drugs for Neglected Diseases initiative (DNDi), a drug discovery unit for neglected diseases at the University of Dundee, and the Consortium for Parasitic Drug Development (CPDD), which is based at the University of North Carolina [3].

Sir James Black, the Nobel prize-winning inventor of β-blockers, noted insightfully that, "the most fruitful basis for the discovery of a new drug is to start with an old drug". For HAT, the aromatic diamidine pentamidine (Figure 1) was introduced as the best of a relatively small number of compounds of this class in the 1930s. Pentamidine, however, has several limitations: the drug is inactive against second-stage disease; it must be administered by intramuscular injection; and it can cause significant side effects, including hepatic toxicity and hyper-/hypoglycemia. Despite these limitations, pentamidine has been successful; however, the success of this drug discouraged further research into diamidines until the use of pentamidine against AIDS-associated pneumocystis pneumonia rekindled interest in this chemical class in the 1980s [5]. Numerous diamidines with improved pharmacological benefits compared with pentamidine have been identified, including orally available *N*-methoxy prodrugs. One of these diamidines is furamidine (DB-75; Immtech Pharmaceuticals Inc; Figure 1), which in its prodrug form pafuramidine (Figure 1) became the first HAT drug to enter a clinical trial pathway that conformed to contemporary standards in drug development; previous trials for HAT involved low patient numbers and relatively *ad hoc* protocols during patient follow up. Unfortunately, renal toxicity emerged several weeks after the last treatment with pafuramidine in a cohort of volunteers in an extended phase I, safety trial, halting the development of the drug. The recent discovery of CNS-permeable diamidines offers hope for the development of new drugs to potentially treat both first- and second-stage HAT.

**Figure 1. Structures of aromatic diamidines for human African trypanosomiasis.**




In this review, the understanding of how diamidines enter and kill trypanosomes, how the compounds are distributed within the body and how they offer great potential as new drugs for use in the treatment of HAT is discussed.

## Diamidine modes of action and mechanisms of resistance

A definitive intracellular target for diamidines, that results in anti-trypanosomal activity when inhibited, remains elusive [3]. However, diamidines bind with high affinity to DNA, and much research has focused on this interaction as a potential mechanism of action for these compounds. An extraordinary complex of intercatenated circular DNA that comprises the mitochondrial genome, known as the kinetoplast (which defines the Kinetoplastid taxon to which trypanosomes belong) [6,7], has been proposed as a possible target for diamidines. Fluorescent diamidines bind to the kinetoplast within seconds of exposure of the parasite to these compounds [8], and the kinetoplast disintegrates in diamidine-treated trypanosomes. Kinetoplast DNA comprises both minicircles and maxicircles. The minicircles contain repeated AT-base pair sequences that are in phase (ie, on the same side of the DNA double helix), producing a bent helical conformation [9-11], which can be disrupted by diamidines [11]. Minicircles encode the small guide RNA molecules that act as templates for the remarkable process of RNA editing of precursor transcripts encoded by the maxicircles [12,13]. Aromatic diamidines generally bind to the DNA minor groove in AT sequences of 4 to 5 base pairs [14,15]. The amidines hydrogen-bond to the thymidine keto or adenosine N3 groups at the floor of the minor groove, and the aromatic systems (or aliphatic in the case of pentamidine) make

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