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SYNTHESIS AND BIOLOGICAL EVALUATION OF A WATER SOLUBLE PHOSPHATE PRODRUG OF 3-AMINOPYRIDINE-2-CARBOXALDEHYDE THIOSEMICARBAZONE (3-AP)

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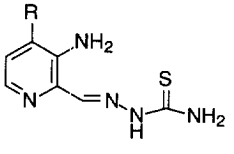
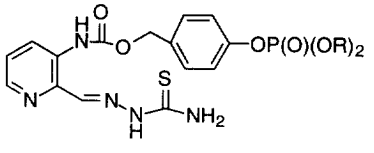
Abstract: With the aim of improving its biological and pharmaceutical profiles, two water soluble phosphate prodrugs of 3-AP, **3a** and **3b** were prepared. The detailed synthesis and the preliminary evaluation of these prodrugs are described.

Introduction: The reductive conversion of ribonucleotides to deoxyribonucleotides by the enzyme ribonucleotide reductase (RR) is a crucial, rate-limiting step in the pathway leading to the biosynthesis of DNA.[1] Since deoxyribonucleotides are present in extremely low levels in mammalian cells, an excellent correlation exists between tumor growth rate and the specific activity of ribonucleotide reductase.[2] Therefore, development of a strong inhibitor of RR , which is essential for cellular replication, would be a useful addition to the existing therapeutic agents against cancer. To date, gemcitabine[3] and hydroxyurea (HU)[4] are the only clinically approved ribonucleotide reductase inhibitors, the former acting at the nucleotide binding site (often referred to as M_1 subunit) and the latter at the metal binding site of the enzyme (often referred to as M_2 subunit). In 1992, Sartorelli and his coworkers at Yale reported on the synthesis and biological evaluation of 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP) and 3-aminopyridine-4-methyl-2-carboxaldehyde thiosemicarbazone (3-AMP), two new lead compounds in the HCT (a-N-heterocyclic carboxyaldehyde thiosemicarbazone) series.[5]



1 R = H (3-AP)
2 R = Me (3-AMP)



3-AP prodrug 3a R = H
3-AP prodrug 3b R = Na

Figure 1: Structures of 3-AP, 3-AMP and 3-AP prodrugs

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According to these authors, both 3-AP (**1**) and 3-AMP (**2**) were about 1000-fold more potent than hydroxyurea in L-1210 leukemia cell lines.[5] Prompted by this encouraging result, we conducted an in-house in vivo evaluationof these two agents in Madison-109 solid tumor model. The result obtained from this comparative in vivo efficacy study demonstrated that 3-AP (**1**) was superior to 3-AMP (**2**).[6] Very recently, Sartorelli and his coworkers found an excellent synergistic effect between 3-AP (**1**) and another DNA damaging drug, etoposide.[7a] On the basis of these observations, 3-AP (**1**) was chosen as the final lead compound within HCT series (see Figure 1 for structures)

Despite of its promising antitumor effect, the decision to develope 3-AP (**1**) as an anti-cancer agent still generates some serious concerns primarily due to its less favorable biological and pharmaceutical profiles such as poor water solubility and lower therapeutic index (in M-109 tumor model). With the aim to maximize the therapeutic utility of 3-AP and to circumvent the deficiencies mentioned above, we designed a series of phosphate-bearing 3-AP prodrugs including prodrugs **3a/3b** shown in Figure 1. The phosphate group attached to the benzyl moiety in **3a** or **3b** should significantly improve the water-solubility of 3-AP. The same phosphate moiety may also enhance the bioavailability of the drug.[7b]

The bioactivation pathway for prodrugs **3a/3b** is envisioned to occur through the cleavage of the phosphorus-oxygen bond, leading to the intermediate **4**, as shown in Figure 2. This would be followed by a cascade of fragmentation, with the loss of quinone methide **5** and CO2, providing the parent drug 3-AP (**1**). It is worthwhile to mention that the quinone methide **5** can cause damage to DNA and thereby contribute to inhibition of cellular replication.[8] Therefore, this quinone methide may act in an additive or synergistic manner to produce complimentary inhibition with 3-AP (**1**).[8]

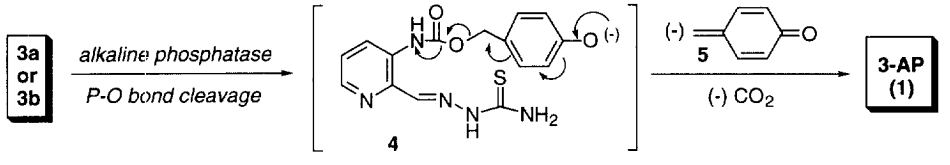
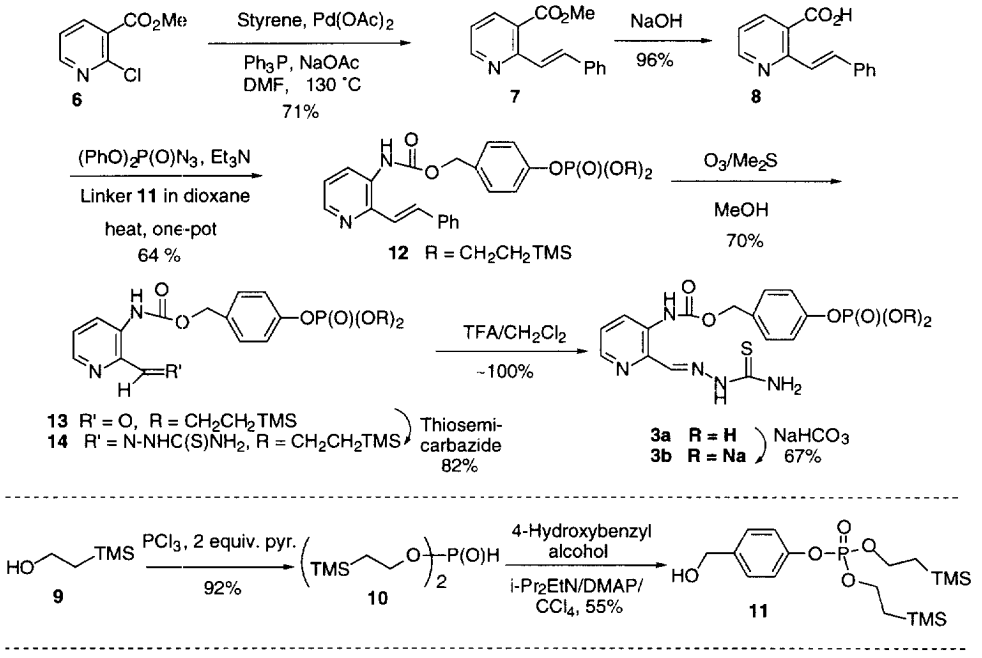


Figure 2: Prodrug activation pathway

Chemical Synthesis: The synthetic route employed for the preparation of **3a/3b** is shown in Scheme 1. The key step in this route was the use of a Curtius rearrangement[9] for the installation of the C-3 carbamate-containing phosphate linker (from **8** to **12**). As outlined in Scheme 1, the 2-styryl-3-nicotinic acid **8** was prepared from 2-chloro-3-nicotinic acid methylester **6** via a two-step sequence consisting of a Heck vinylation

reaction[10] and a NaOH promoted ester hydrolysis. The phosphate linker **11** was obtained in a regioselective manner from oxidative coupling of the phosphite **10** and 4-hydroxybenzyl alcohol in carbon tetrachloride.[11] The bis-Teocphosphite **10** was prepared in turn from 2-trimethylsilylethanol and PCl_3 according to McCombie et al..[12] Curtius rearrangement[9] on acid **8** provided the corresponding isocyanate, which was trapped with linker **11** to afford the desired carbamate **12**[13] in 64% yield. Treatment of a methanol solution of **12** with excess ozone yielded the aldehyde **13**[14] (70%), which was coupled with thiosemicarbazide to afford the corresponding thiosemicarbazone **14**[15] in 82% yield. Deprotection of the Teoc in **14** with trifluoroacetic acid (TFA)[16] furnished the desired 3-AP prodrug free acid **3a** (100%). The disodium salt **3b**[17] was obtained upon further treatment with sodium bicarbonate solution in 67% yield.

Scheme 1 **Synthesis of 3-AP Phosphate Prodrugs 3a/3b**



Evaluation of 3-AP Phosphate Prodrug 3b

Water solubility and thermal stability. The water solubility of 3-AP prodrug **3b** (disodium salt) was estimated to be 46 mg/mL, a more than 460-fold increase than the parent 3-AP, whose water-solubility was less than 0.1 mg/mL. The stability of prodrug **3b** was measured at three temperature (5 °C, 25 °C and 40 °C). The data

obtained for three month thermal stability of **3b** (in powder form) showed that it was stable at all three temperature tested.

*Bioconversion of **3b** to 3-AP catalyzed by alkaline phosphatase or by rat liver S-9 preparation.*[18] The bioactivation of prodrug **3b** was studied using 4.65 x 10^{-5} unit phosphatase enzyme solution (Type VII-SA, from Bovine Intestinal Mocose, Sigma). Upon incubation with phosphatase, **3b** was converted cleanly to the parent 3-AP. Under these experimental conditions, the half-life ($T_{1/2}$) of bioactivation of **3b** was found to be 56 min. In a separate experiment, the bioconversion of **3b** was also studied in vitro in the presence of the S-9 fraction of rat liver. A 1 uM solution of **3b** in cultire media was incubated with approximately 2.5 mg of liver S-9 fraction. The prodrug concentration decreased very rapidly and was below detection limits after incubation for 100 min at 37 °C. Approximately 80% (molar ratio) of the expected concentration of 3-AP was generated in this process and no other metabolites were found. The rate of conversion of **3b** was 20 nmol/min/mg rat liver tissue. When incubated in culture media alone at 37 °C for 2 h, **3b** was stable and no 3-AP was detected by HPLC assay. This result indicated that the release of 3-AP from **3b** was an enzymatic event.

*In vivo pharmacokinetics study of prodrug **3b** in dog.* [19] Prodrug **3b** was given to two dogs (~10 kg, 1M/1F) via the iv route and the other two dogs (~10 kg, 1M/1F) via oral dosing.The prodrug was given at the dose of 7.26 mg/kg (eqivalent to 3mg/kg of 3-AP). The drug was given 1 h before feeding. Blood samples for analysis of 3-AP levels were collected at numerous time-points following the dose.

Analysis of the blood samples collected from this experiment showed that when the drug was given by the iv route, the AUC value of 3-AP generated from **3b** was essentially the same as that achieved from dosing with 3-AP (74.04 mg/min/L from **3b** or 71.59 mg/min/L from 3-AP). The clearance rate for **3b** was also very similar to that of 3-AP (2.628 L/h/kg from **3b** or 2.437 L/h/kg from 3-AP). These observations clearly indicated that prodrug **3b** was converted rapidly and cleanly to 3-AP in dogs. When the dosing was administered orally, prodrug **3b** achieved an average oral bioavailability of 91%.

Antitumor activity in M-109 murine lung carcinoma.[20] Two experiments comparing the in vivo efficacy of prodrug **3b** and 3-AP (**1**) were performed using M-109 solid tumor model.[16] Balb/c mice were used for these experiments. Ten mice were used for each group. Injection of 3-AP (**1**) (4.5 mg/kg/injection), prodrug **3b** (10 mg/kg/injection), or vehicle controls (10% DMSO/H_2O for 3-AP or PBS solution for **3b**) were given intraperitoneally to the mice twice on Day 3 through Day 7. The size of tumors were measured by a caliper on days 7, 10, 13, 17. Both 3-AP and prodrug **3b** were capable of reducing tumor volume by 30 - 44% and 46 - 57%, respectively. Evidently, prodrug **3b** was slightly more efficacious on a mole-to-mole basis than 3-AP. Unfortunately however, no improvement on therapeutic index (TI) was achieved with **3b**.[21]

In summary, the first phosphate-containing 3-AP prodrug **3b** indeed exhibits improved pharmaceutical profiles over the parent drug (e.g., water-solubility and oral bioavailability). Further efforts aimed at improving biological profiles (e.g., in vivo efficacy and TI) are being pursued. The results from these investigations will be published in the near future.

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7. (a) Personal communication with Professor A. C. Sartorelli, Yale University. (b) c.f. etoposide phosphate prodrug (BMY-40481): Saulnier, A.; Langley, D. R.; Kadow, J. F.; Senter, P. D.; Knipe, J. O.; Tun, M. M.; Vyas, D. M.; Doyle, T. W. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 2567.
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13. ^{1}H NMR of **12** ($CDCl_3$, 300 MHz): δ 8.41 (dd, J = 1.2, 5.1 Hz, 1H), 7.74 (d, J = 15.9 Hz, 1H), 7.57 (d, J = 8.1 Hz, 2H), 7.1-7.45 (m, 10H), 6.77 (br, 1H), 5.20 (s, 2H), 4.20-4.32 (m, 4H), 1.05-1.20 (m, 4H), 0.03 (s, 18H). ^{13}C NMR of **12** ($CDCl_3$, 300 MHz): δ 153.78, 149.43 (d, J = 29 Hz), 146.05, 145.07, 136.65, 135.14, 131.50, 130.61, 129.98, 129.53, 128.56, 128.39, 128.28, 127.38, 127.27, 127.18, 125.09, 122.40, 120.97, 120.24 (d, J = 6.6 Hz), 67.29 (d, J = 26 Hz), 19.55 (d, J = 24 Hz), -1.61. HRMS(FAB) calcd. for $C_{31}H_{44}O_6N_2Si_2P$: 626.2478, found: 627.2475.

14. ^{1}H NMR of **13** ($CDCl_3$, 300 MHz): δ 10.48 (br, 1H), 10.07 (s, 1H), 8.83 (d, J = 8.7 Hz, 1H), 8.44 (dd, J = 0.9, 4.2 Hz, 1H), 7.49 (dd, J = 4.5, 8.7 Hz, 1H), 7.40 (Abq, J = 8.4 Hz, 2H), 7.23 (Abq, J = 8.1 Hz, 2H), 5.19 (s, 2H), 4.18-4. 28 (m, 4H), 1.02-1.20 (m, 4H), 0.02 (s, 18H). ^{13}C NMR of **13** ($CDCl_3$, 75 MHz): δ 197.18, 153.31, 150.98 (d, J = 29 Hz) 143.77, 138.55, 136.82, 132.09, 129.96, 128.71, 126.32, 120.20, 120.14, 67.09, (d, J = 25 Hz), 66.73, 19.52 (d, J = 23 Hz), -1.56. HRMS(FAB) calcd. for $C_{24}H_{37}O_7N_2Si_2P$: 554.2032, found: 554.2033.

15. ^{1}H NMR of **14** ($CDCl_3$, 300 MHz): δ 8.25-8.32 (m, 2H), 8.19 (s, 1H), 7.46 (Abq, J = 8.7 Hz, 2H), 7.41 (d, J = 4.8 Hz, 1H), 7.23 (Abq, J = 8.1 Hz, 2H), 5.21 (s, 2H), 4.20-4.40 (m, 4H), 1.02-1.20 (m, 4H), 0.03 (s, 18H). ^{13}C NMR of **14** ($CDCl_3$, 75 MHz): δ 155.48, 151.94 (d, J = 27 Hz), 134.83, 130.97, 130.70, 126.07, 123.47, 121.27 (d, J = 19 Hz), 68.80 (d, J = 27 Hz), 67.58, 20.34 (d, J = 22 Hz), -1.44.

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17. ^{1}H NMR of **3b** (CD_3OD, 300 MHz): δ 8.30-8.42 (m, 2H), 8.19 (s, 1H), 7.39 (dd, J = 4.5, 8.4 Hz, 1H), 7.28 (dd, J = 9.3, 10.5 Hz, 4H), 5.12 (s, 2H). ^{13}C NMR of **3b** (CD_3OD, 75 MHz): δ 180.90, 156.92, 155.70, 145.59, 144.31, 142.07, 136.51, 130.99, 130.28, 130.27, 129.67, 125.78, 121.17 (d, J = 20 Hz), 68.56.

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21. When **3b** was dosed at 12.5 mg/kg/inj., severe animal death was observed.