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The combination of the novel glycolysis inhibitor 3-BrOP and rapamycin is effective against neuroblastoma

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Summary

Children with high-risk and recurrent neuroblastoma have poor survival rates, and novel therapies are needed. Many cancer cells have been found to preferentially employ the glycolytic pathway for energy generation, even in the presence of oxygen. 3-BrOP is a novel inhibitor of glycolysis, and has demonstrated efficacy against a wide range of tumor types. To determine whether human neuroblastoma cells are susceptible to glycolysis inhibition, we evaluated the role of 3-BrOP in neuroblastoma model systems. Neuroblastoma tumor cell lines demonstrated high rates of lactate accumulation and low rates of oxygen consumption, suggesting a potential susceptibility to inhibitors of glycolysis. In all ten human tested neuroblastoma tumor cell lines, 3-BrOP induced cell death via apoptosis in a dose and time dependent manner. Furthermore, 3-BrOP-induced depletion of ATP levels correlated with decreased neuroblastoma cell viability. In a mouse neuroblastoma xenograft model, glycolysis inhibition with 3-BrOP demonstrated significantly reduced final tumor weight. In neuroblastoma tumor cells, treatment with 3-BrOP induced mTOR activation, and the combination of 3-BrOP and mTOR inhibition with rapamycin demonstrated synergistic efficacy. Based on these results, neuroblastoma tumor cells are sensitive to treatment with inhibitors of glycolysis, and the demonstrated synergy with rapamycin suggests that the combination of glycolysis and mTOR inhibitors represents a novel therapeutic approach for neuroblastoma that warrants further investigation.

Keywords

Neuroblastoma; 3-BrOP; Glycolysis; Rapamycin

Introduction

Neuroblastoma is the most common extra-cranial solid tumor in children and accounts for approximately 10% of new pediatric malignancies each year. Approximately half of children diagnosed with neuroblastoma present with high-risk disease [1]. Despite intensive chemotherapy and autologous stem cell transplantation, long-term survival continues to be less than 50% for these patients [2–4]. Similarly, children with relapsed neuroblastoma have a less than 50% response rate to salvage regimens and have very poor rates of survival [5, 6].

Novel treatment strategies are needed to improve outcomes and limit toxicity in these young patients.

A common property of cancer cells is an increased use of glycolysis for energy production, even in the presence of oxygen (termed the Warburg Effect) [7]. Features of the Warburg effect include high glucose uptake and utilization, excessive production of lactic acid, and decreased oxygen consumption. The recent increased use of ^{18}F -fluoro-deoxyglucose positron-emission tomography (FDG-PET) imaging for patients with a variety of cancers has demonstrated that most human cancers utilize significantly elevated levels of glucose [8, 9]. Furthermore, increased glucose uptake has been shown to correlate with increased invasiveness of tumors and poor outcomes [10–13]. This biochemical difference between normal and malignant cells represents an opportunity to exploit therapeutic strategies aimed at inhibiting glycolysis, thereby affecting the survival of malignant cells while sparing normal cells.

Several glycolysis inhibitors have been investigated for their anti-cancer effects, including lonidamine, 3-bromopyruvate (3-BrPA), and 2-deoxy-D-glucose (2-DG) [14]. Lonidamine and 3-BrPA are inhibitors of hexokinase, the first and rate-limiting enzyme in the intracellular pathway for glucose metabolism. Lonidamine has been employed in combination with other agents in clinical trials for patients with ovarian [15], breast [16], and brain [17] cancers, while 3-BrPA has been shown to be effective against a variety of tumor types in preclinical models, including pancreatic [18], breast [19], and colon [20, 21] cancers, in addition to cancer cells from mesothelioma [22] and hepatocellular carcinoma [23–25]. 3BrPA has also been shown to decrease ATP levels and induce cell death in leukemia and lymphoma tumor cells [26]. 2-DG, by contrast, competes with glucose for transmembrane transport and is converted to 2-deoxyglucose-phosphate, which cannot be further metabolized within the cancer cell, resulting in decreased cellular ATP production and cell death [27–31]. 2-DG has produced encouraging results in a variety of preclinical models [32–35] and is currently being investigated in a variety of early phase clinical trials [36, 37].

3-BrOP (3-bromo-2-oxopropionate-1-propyl ester) is a cell-permeable ester of 3-BrPA that is hydrolyzed by intracellular esterases to generate 3-BrPA. 3-BrOP has shown efficacy against leukemia and lymphoma cell lines [38, 39], in addition to demonstrated efficacy in pre-clinical models of hepatocellular carcinoma, glioblastoma multiforme, and pancreatic cancer (P.H., personal communication). Furthermore, the combination of inhibition of the mTOR pathway with inhibitors of glycolysis has been shown to be synergistically effective against lung cancer and leukemia cells [38–40]. Therefore, we hypothesized that 3-BrOP would inhibit glycolysis and be effective against neuroblastoma tumor cells and that the combination of inhibition of glycolysis with 3-BrOP and of mTOR with rapamycin would be synergistically effective against neuroblastoma.

Materials and methods

Cells and cell culture conditions

The neuroblastoma cell lines SK-N-SH, SH-EP, SH-SY5Y, NGP, CHP-134, SK-N-AS, SMS-KCNR, NBL-S, LA1-55N and IMR-32 used in this study have been previously described [41–46] and were generously provided by Susan Cohn (University of Chicago Children's Hospital, Chicago, IL) and John Maris (Children's Hospital of Philadelphia, Philadelphia, PA).

Neuroblastoma cell lines and HEK-293 cells (ATCC, used within 10 passages) were grown at 37°C in 5% CO₂ in RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (USB, Minneapolis, MN), non-essential amino acids, penicillin (10,000 units/mL) and streptomycin (10 mg/mL) in 0.9% NaCl (Sigma, St. Louis, MO). All experiments were performed with cells under exponential growth conditions.

For experiments under hypoxic conditions, cells were placed in a sealed modular incubator chamber (Billups-Rothenberg, Del Mar, CA) flushed with 5% CO₂ and 95% N₂. Final oxygen content in the hypoxia chamber was ~1% after achieving air equilibrium.

Therapeutic agents

3-bromo-2-oxopropionate-1-propyl ester (3-BrOP) was generously provided by Peng Huang (M.D. Anderson Cancer Center, Houston, TX). A stock solution of 1M was generated in 1-propanol, stored at 4°C, and diluted in fresh media immediately before use. Rapamycin (Sigma) was dissolved in DMSO to create a 10 mM stock solution stored at –20°C. Rapamycin was diluted in fresh media immediately before use.

Cell viability assays

Approximately 5000 neuroblastoma or HEK-293 cells were plated in individual wells of 96-well plates and allowed to adhere overnight. Cells were exposed to a range of 3-BrOP concentrations for up to 72 h. For combination studies, cells were exposed to 3-BrOP and rapamycin simultaneously at multiple concentrations over a 72 h time period. After experimental treatment, 10 µL of AlamarBlue reagent (Invitrogen) was added to each well and plates were incubated at 37°C for at least 2 h. Plates were read on a Spectromax Gemini EM spectrophotometer (Molecular Devices, Sunnyvale, CA) at 544 nm and 590 nm. Experiments were performed in triplicate for each treatment condition. IC₅₀ values were derived using best-fit trendlines and values calculated using the relevant curve-fit equations. The combination index (CI) was calculated using CalcuSyn software (Biosoft, Ferguson, MO, USA). CI values of less than 1, equal to 1, and greater than 1 indicate synergistic, additive, and antagonistic effects, respectively.

Cell morphology

Neuroblastoma cells were treated in 10 cm tissue culture dishes with 12 µM 3-BrOP for 12–72 h. Cells were photographed at 200× magnification with a Nikon Digital Sight DS-L1 camera attached to a Nikon Eclipse TS 100 microscope.

Immunoblot analysis

Whole cell lysates (50 µg) were separated by SDS-PAGE and proteins were transferred to polyvinylidene difluoride membranes (PVDF, Millipore Corporation) using standard techniques. Membranes were incubated with primary antibodies against hexokinase II (SantaCruz Biotechnology), caspase-9, caspase-8, caspase-3, Poly (ADP-ribose) polymerase 1 (PARP1), phospho-S6 Ribosomal Protein, and beta-actin (Cell Signaling). Membranes were washed and incubated with appropriate HRP-conjugated secondary antibodies (Cell Signaling) and then developed with the ECL Western Blotting Analysis System (ECLplus, Amersham International) following the manufacturer's instructions.

ATP assays

Cells were treated with 3-BrOP for 12–48 h at a range of concentrations. ATP concentrations were measured with the ATPlite kit according to manufacturer's instructions (PerkinElmer, Boston, MA).

Determination of glycolytic activity

To determine cellular lactate production, cells in exponential growth phase were washed and incubated with fresh medium for 48 h. Aliquots of the culture medium were removed for the analysis of lactate content using an Accutrend lactate analyzer with a linear range of standard lactate concentrations according to the manufacturer's instructions (Roche, Mannheim, Germany). To determine cellular oxygen consumption, 10⁶ neuroblastoma cells were resuspended in 1 mL fresh warm medium pre-equilibrated with 21% oxygen and placed in a sealed respiration chamber equipped with a thermostat control, a micro stirring device, and a Clark-type oxygen electrode disc (Oxytherm, Hansatech Instrument, and Cambridge, United Kingdom). The oxygen content in the starting medium was normalized to an O₂ concentration of 220 µM in air-saturated medium at 37°C. Oxygen concentrations were continuously recorded over 7.5 min. The oxygen content in the cell suspension was continuously monitored and oxygen consumption rate was determined as described previously [47].

Mouse experiments

Six to eight week-old NOD/SCID/IL2R-gamma mice (stock # 005557, Jackson laboratories, Bar Harbor, ME) were inoculated subcutaneously into the right flank with 10⁷ SK-N-SH neuroblastoma tumor cells as previously described [45]. Once tumors were palpable (~300 mm³), mice were randomly assigned to treatment with 20 mg/kg of 3-BrOP or with vehicle, once daily by tail vein injection. All mice remained healthy during treatment, with no signs of neurologic effects and no weight loss. Animals were sacrificed 14 days after the start of treatment and final tumor weights were measured. Protocols were approved by the Institutional Animal Care and Use Committee at the University of Texas M. D. Anderson Cancer Center.

Results

Neuroblastoma cells demonstrate decreased oxygen consumption and increased lactate production

To evaluate the dependence of neuroblastoma cells on glycolysis, we measured both oxygen consumption as a measure of oxidative respiration and lactate production as a measure of glycolysis in two neuroblastoma cell lines and in the non-malignant HEK-293 cells. We found that neuroblastoma cell lines consume oxygen at a significantly lower rate when compared to HEK-293 cells ($P<0.001$) (Fig. 1a). Under these conditions, the HEK-293 cells used over 65% of the oxygen in the media, while both neuroblastoma lines used $<25\%$ of the oxygen available, demonstrating lower rates of oxidative respiration.

To determine if these low oxidative respiration rates correlated with upregulation of glycolysis, we measured lactate levels in neuroblastoma and HEK-293 cell lines. Lactate levels were approximately 67% higher in both neuroblastoma cell lines when compared to HEK-293 cells ($p<0.01$), suggesting a higher rate of glycolysis (Fig. 1b).

Inhibition of neuroblastoma cell growth with 3-BrOP

To determine whether inhibition of glycolysis would be effective against neuroblastoma tumor cells, SH-SY5Y neuroblastoma cells were treated with the novel glycolysis inhibitor 3-BrOP for 12 h. Neuroblastoma cells demonstrated rounding and detachment from the surface, suggesting cell death (Fig. 2a). We then explored the effects of 3-BrOP on cell viability in a panel of ten neuroblastoma cell lines and HEK-293 cells. As shown in Fig. 2b, all neuroblastoma cell lines had IC₅₀ values in the range of 4–18 μM . In contrast, HEK-293 cells did not reach an IC₅₀ value when treated up to 25 μM 3-BrOP, suggesting a lack of dependence upon glycolysis for energy production (Fig. 2b and data not shown).

To evaluate this decreased cell viability after exposure to 3-BrOP, neuroblastoma cells were treated with 3-BrOP and viable cell counts were obtained after 12, 24, and 48 h of exposure using trypan blue exclusion. We found a decrease in the number of viable neuroblastoma cells after exposure to 3-BrOP that occurred in both a time and concentration dependent manner (Fig. 2c).

To further investigate the mechanism by which neuroblastoma cell numbers are reduced by 3-BrOP, we explored the ability of 3-BrOP to induce apoptosis. Neuroblastoma cells were treated with 3-BrOP and evaluated for cleavage of caspase-9, caspase-3 and PARP1 at 12, 24 and 48 h, respectively (Fig. 2d). Sequential cleavage of caspase-9, caspase-3, and PARP1 after 3-BrOP exposure suggests that the reduced neuroblastoma cell numbers are due at least in part to the induction of apoptosis. No evidence of caspase-8 cleavage was seen at any time point (data not shown).

Glycolysis inhibition reduces intracellular ATP concentration

Treatment of malignant cells with glycolysis inhibitors, including 2-DG, 3-BrPA, and 3-BrOP, has been shown to result in lower cellular ATP levels prior to cell death [27–30, 38]. In order to determine whether 3-BrOP treatment resulted in lower neuroblastoma tumor cell

ATP levels, NGP neuroblastoma and HEK-293 cells were treated with 3-BrOP and ATP levels and cell viability were measured over 48 h. NGP cells demonstrated a time and concentration dependent decrease in ATP levels that correlated with loss of cell viability (Fig. 3a). In contrast, HEK-293 cells demonstrated no decreased ATP levels or reduced cell viability, even after 48 h of exposure to 12 μ M 3-BrOP (Fig. 3b)

We next tested whether neuroblastoma cells would be more sensitive to glycolysis inhibition under hypoxic conditions. Neuroblastoma cells were treated with 3-BrOP at varying concentrations either at 21% O₂ (normoxia) or at ~1% O₂ (hypoxia). We found a small increase in neuroblastoma tumor cell sensitivity to 3-BrOP under hypoxic conditions, compared to normoxic conditions, across a wide range of doses (Fig. 3c).

3-BrOP inhibits neuroblastoma xenograft tumor growth

To determine whether 3-BrOP is effective against neuroblastoma xenograft tumors *in vivo*, immunocompromised mice were injected subcutaneously with neuroblastoma cells. Once tumors were palpable, mice were treated daily with either 3-BrOP or vehicle alone. At the conclusion of the experiment, tumors from control mice were grossly hemorrhagic, with poorly defined margins and friable tumor tissue. Neuroblastoma xenograft tumors from mice treated with 3-BrOP were firm and well-circumscribed. Treatment with 3-BrOP resulted in significant inhibition of tumor growth, with an average final tumor weight of 0.8+/-0.6 grams in the 3-BrOP treated mice, versus 3.6+/-0.6 grams in control mice, representing a 78% decrease in tumor mass in the 3-BrOP-treated mice ($P<0.001$) (Fig. 4).

mTOR inhibition synergizes with glycolysis inhibition

In order to further explore the role of the mTOR pathway in the cellular response of neuroblastoma tumor cells to 3-BrOP, we evaluated the phosphorylation of S6 protein before and after treatment of neuroblastoma cells with 3-BrOP. We found a dramatic increase in phosphorylated S6 protein within 6 h of exposure to 3-BrOP (Fig. 5a). We therefore evaluated the efficacy of the combination of 3-BrOP with rapamycin against neuroblastoma cells. Neuroblastoma tumor cells were treated with 3-BrOP, rapamycin, or both over 72 h using a range of concentrations, and synergistic loss of viability was observed for multiple dose combinations. For example, cell viability was noted to be 86% of control at a dose of 6 μ M 3-BrOP ($P=0.013$), while viability was 54% of control with exposure to 100 nM rapamycin ($P=0.007$) (Fig. 5b). When the two agents were combined at these doses, cell viability was decreased to 13% of control ($P=0.0002$), in contrast to the expected additive 46% viability (Fig. 5b, dashed line). The combination index for these doses is 0.3, making 3-BrOP and rapamycin a synergistic combination against neuroblastoma cells *in vitro*.

Discussion

New treatment strategies are clearly needed for children with high-risk and relapsed neuroblastoma. We have demonstrated that the novel glycolysis inhibitor 3-BrOP is effective against neuroblastoma tumor cells *in vitro* and *in vivo* and that the combination of glycolysis inhibition with 3-BrOP and mTOR inhibition with rapamycin demonstrates synergistic efficacy against neuroblastoma cells.

Many cancer cells have been shown to utilize the glycolytic pathway for energy generation in both normoxic and hypoxic conditions [9]. We have shown that neuroblastoma cells have lower rates of oxidative respiration and an upregulation of the glycolytic pathway, consistent with the Warburg effect in these cell lines. Preliminary studies over 30 years ago demonstrated that growth of neuroblastoma tumor cells could be reduced by inhibition of glycolysis with DL-glyceraldehyde [48], and recent studies have demonstrated that overexpression of the N-myc protein correlates with increased expression levels of glycolytic pathway proteins, although in a separate study *N-myc* gene amplification did not correlate with neuroblastoma tumor cell sensitivity to glycolysis inhibitors [49, 50]. However, the role of glycolysis in the pathogenesis of neuroblastoma is poorly understood, and the efficacy of novel glycolysis inhibitors in neuroblastoma model systems is unknown.

3-BrOP (3-bromo-2-oxopropionate-1-propyl ester) is a cell-permeable ester of 3-BrPA that is hydrolyzed by intracellular esterases to generate 3-BrPA. 3-BrOP is a novel inhibitor of glycolysis with efficacy in a variety of preclinical models [38, 39]. This study represents the first demonstration of the efficacy of 3-BrOP in a pediatric cancer model system and of the efficacy of hexokinase inhibition in neuroblastoma tumor cells. We have shown that 3-BrOP is effective against neuroblastoma tumor cells via induction of apoptosis, with IC50 values of 4–18 μ M. For comparison, leukemia and lymphoma cell lines had IC50 values of approximately 20 μ M after exposure to 3-BrOP [38], demonstrating that neuroblastoma cell lines are even more sensitive to glycolysis inhibition. The mechanisms of the observed variability in neuroblastoma cell line sensitivity to 3-BrOP are not clear. Responses to 3-BrOP do not appear to correlate with any particular biologic feature of the tumor cells, such as *N-myc* gene amplification or chromosome 1p deletion. Prior studies have demonstrated the importance of hexokinase II in the regulation of glycolysis, and many tumor types have increased hexokinase II expression [51, 52]. We confirmed the neuroblastoma cell lines express detectable levels of hexokinase II by Western blot (Supplemental Figure 1), but there was no correlation between hexokinase II expression and sensitivity to 3-BrOP ($p=0.8$, data not shown).

As glycolysis inhibition creates cellular stress, compensatory mechanisms, such as the mammalian target of rapamycin (mTOR) pathway, may lead to 3-BrOP resistance. These pathways represent additional targets for effective combination therapies. Inhibition of glycolysis has been shown to induce S6 phosphorylation through the mTOR pathway in the heart [53]. In lung cancer cells, mTOR inhibition with CCI-779 was synergistic with inhibition of glycolysis with 2-DG through down-regulation of HIF-1 [40], and in leukemia and lymphoma cell lines, the combination of 3-BrOP and rapamycin was found to have synergistic efficacy [38, 39]. We have also shown that the combination of 3-BrOP and rapamycin is effective against neuroblastoma tumor cells, further confirming the role of the mTOR pathway in the cellular response to 3-BrOP. Inhibitors of mTOR have been shown to be effective against neuroblastoma tumor cells [54], suggesting that the combination of 3-BrOP and rapamycin may be particularly effective in the treatment of children with neuroblastoma.

In summary, we have demonstrated that neuroblastoma tumor cells exhibit accumulation of lactate and low oxygen consumption, even in the presence of oxygen. Furthermore, we have

demonstrated that the novel glycolysis inhibitor 3-BrOP is effective against neuroblastoma tumor cells *in vitro* and *in vivo*, and that exposure of neuroblastoma tumor cells to 3-BrOP leads to dramatically decreased ATP concentrations followed by caspase-mediated apoptosis under both normoxic and hypoxic conditions. Importantly, we have also demonstrated significant anti-tumor efficacy in a xenograft mouse model without overt toxicity. Finally, we have demonstrated the synergistic effects of glycolysis inhibition and mTOR inhibition in neuroblastoma tumor cells. The evidence for the importance of the glycolytic pathway in neuroblastoma tumor cell growth and for the demonstrated efficacy of the combination of 3-BrOP and rapamycin in these studies provides strong biological and clinical rationale for further investigation of the role of inhibition of glycolysis in the treatment of neuroblastoma and for further testing of this combination for the treatment of children with neuroblastoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

FDG-PET	¹⁸ fluorodeoxyglucose positron-emission tomography
ATP	adenosine triphosphate
3-BrPA	3-bromopyruvate
2-DG	2-deoxy-D-glucose
3-BrOP	3-bromo-2-oxopropionate-1-propyl ester
mTOR	mammalian target of rapamycin
PARP1	Poly (ADP-ribose) polymerase 1

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Fig. 1.

Oxygen consumption and lactate production in neuroblastoma tumor cells. **(a)** Oxygen consumption of IMR-32 and NGP neuroblastoma cells and HEK-293 cells was determined as detailed in the methods. HEK-293 cells consumed a total of $130 \mu\text{M}$ of O_2 over 7.5 min, while the IMR-32 and NGP cells consumed only $56 \mu\text{M}$ and $55 \mu\text{M}$ O_2 , respectively ($P < 0.001$ vs. HEK-293). **(b)** Lactate concentration was measured after 48 h in culture for HEK-293, IMR-32, and NGP cells. Both neuroblastoma cell lines accumulated $>67\%$ more lactate than the non-malignant HEK-293 cells ($P < 0.01$)

Fig. 2.

Effects of 3-BrOP on neuroblastoma tumor cells. **(a)** Photomicrographs of SH-SY5Y cells treated with 12 μM 3-BrOP for 12 h. The left photograph shows untreated neuroblastoma cells, while the right photograph shows cells treated with 3-BrOP. **(b)** Neuroblastoma cell line sensitivity to 3-BrOP. A panel of neuroblastoma tumor cell lines were treated with 3-BrOP and IC50 values were calculated for each cell line after 72 h of exposure to 3-BrOP. Results represent the mean ± SEM from triplicate samples. **(c)** IMR-32 and CHP-134 cells were treated with 3-BrOP at doses of 6 and 10 μM over 48 h. Cell counts were done at 12, 24, and 48 h of 3-BrOP exposure. Results represent the mean ± SEM from triplicate samples. **(d)** IMR-32 cells were treated with 12 μM 3-BrOP over 48 h. Increased caspase-9 cleavage was observed by 12 h, increased caspase-3 cleavage by 24 h and increased PARP1 cleavage by 24 h of exposure to 3-BrOP

Fig. 3. 3-BrOP reduces ATP levels in neuroblastoma cells. (a) NGP neuroblastoma cells and (b) HEK-293 cells were treated with 3, 6, and 12 μM 3-BrOP for up to 48 h and cell viability and ATP levels were measured. (c) SK-N-SH and CHP-134 cells were incubated with increasing 3-BrOP concentrations in both normoxic (21% O_2) and hypoxic (1% O_2) conditions for 48 h. Results represent the mean $\pm$ SEM. All results are expressed as percentages of untreated cells

Fig. 4. 3-BrOP inhibits neuroblastoma tumor growth *in vivo*. SK-N-SH cells were injected subcutaneously into immunocompromised mice. When tumors were palpable, mice were randomized to receive 3-BrOP or vehicle control by daily tail vein injections. All mice were euthanized after 14 days and final tumor weights were obtained

Fig. 5. 3-BrOP induces S6 phosphorylation and is synergistic with rapamycin. **(a)** IMR-32 and NGP cells were treated with 12 μ M 3-BrOP over 24 h. Cell lysates were collected and were analyzed by immunoblot for phosphorylated S6 protein. **(b)** IMR-32 neuroblastoma cells were incubated with increasing concentrations of 3-BrOP, rapamycin, or the combination for 72 h. Data is shown for the combination of 3-BrOP at 6 μ M and rapamycin at 100 nM (CI= 0.3). Cell viability is expressed as a percentage of untreated control cells. The dashed line indicates the expected additive dose effect of the combination of 3-BrOP and rapamycin