# A *Drosophila* Model To Identify Polyamine−Drug Conjugates That Target the Polyamine Transporter in an Intact Epithelium

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*Received September 24, 2007*

Polyamine transport is elevated in many tumor types, suggesting that toxic polyamine−drug conjugates could be targeted to cancer cells via the polyamine transporter (PAT). We have previously reported the use of Chinese hamster ovary (CHO) cells and its PAT-deficient mutant cell line, CHO-MG, to screen anthracene−polyamine conjugates for their PAT-selective targeting ability. We report here a novel *Drosophila*-based model for screening anthracene−polyamine conjugates in a developing and intact epithelium (*Drosophila* imaginal discs), wherein cell−cell adhesion properties are maintained. Data from the *Drosophila* assay are consistent with previous results in CHO cells, indicating that the *Drosophila* epithelium has a PAT with vertebrate-like characteristics. This assay will be of use to medicinal chemists interested in screening drugs that use PAT for cellular entry, and it offers the possibility of genetic dissection of the polyamine transport process, including identification of a *Drosophila* PAT.

## Introduction

The native polyamines (putrescine **1**, spermidine **2**, and spermine **3**) are essential growth factors that exist mainly as polycations at physiological pH (Figure 1).[1,2] These charged growth factors can be internally biosynthesized and also imported from exogenous sources.[1] Many tumor types have been shown to contain elevated polyamine levels and an activated polyamine transporter (PAT[a]) for importing exogenous polyamines.[1,3] Thus, the PAT represents a potential target for anticancer strategies. Great strides have been made in the development of new polyamine synthetic methods and understanding the structure−bioactivity relationships of polyamine derivatives.[4–36] However, the identification of the genes and proteins involved in mammalian polyamine transport remains an important challenge for researchers in this field.[4,37] Indeed, there are only a handful of reports, that describe the PAT phenomenon beyond a descriptive account. The insightful X-ray crystal structures for the putrescine and spermidine transporters in *E. coli*[38] and the sequence information in yeast[39] and *Leishmania*[40] are all that are known from the structural perspective. In terms of transport itself, Poulin's recent model[41] is the most inclusive because it also incorporates the role of proteoglycans in polyamine uptake, which was recently described by Belting et al.[42] Even without structural information of the mammalian PAT itself, a number of studies have begun to decipher which polyamine architectures are accommodated by the PAT and imported into eukaryotic cells via this transport process.[4–36]

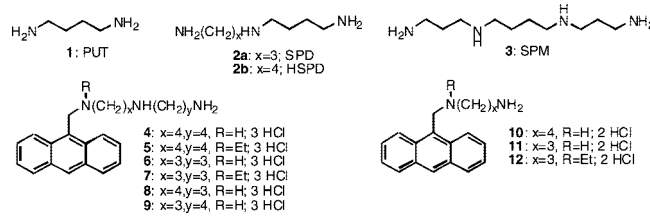


**Figure 1.** Native polyamines (putrescine **1**, spermidine **2a**, and spermine **3**), homospermidine **2b**, and the anthracene−polyamine conjugate library members **4**–**12**.

A key tool has been the use of comparative cytotoxicity and transport measurements in Chinese hamster ovary (CHO) cells and its mutant CHO cell line, CHO-MG.[4,14,43] These have been used to screen new polyamine conjugates for their PAT-selective targeting ability. First, the respective cytotoxicities of the polyamine−drug ($IC_{50}$) were determined in PAT-inactive cells (CHO-MG) and in PAT-active (CHO) cells. Conjugates that had high $IC_{50}$ ratios [($IC_{50}$ value in CHO-MG cells)/($IC_{50}$ value in CHO cells) ≫ 1] were deemed to be PAT-selective. In contrast, conjugates that do not utilize the PAT for cellular entry have $IC_{50}$ ratios near 1. After the synthesis and screening of numerous anthracene−polyamine conjugates (Figure 1) in CHO and CHO-MG cells, homospermidine **2b** was identified as an optimal polyamine vector for the transport of anthracene (into CHO cells) via the PAT (i.e., compound **4**).[4,7] Our synthetic "library" of anthracene−polyamine conjugates (**4**–**12**) provided a series of structurally related molecules each with different PAT recognition and use. Armed with this library, we were interested in developing new assays to screen PAT-selective agents and to identify a PAT in a multicellular eukaryote. To this end, we report here a novel *Drosophila*-based model for screening PAT-selective agents in a developing and intact epithelium.

The development of a *Drosophila*-based model for screening PAT-selective agents has many advantages. More than 100 years of genetic analysis in *Drosophila* have resulted in an unparalleled set of genetic tools that can be used to dissect important

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[a] Abbreviations: PAT, polyamine transporter; CHO, Chinese hamster ovary cells; CHO-MG, Chinese hamster ovary cells polyamine transport deficient mutant; L1210, mouse leukemia cells.

10.1021/jm701198s CCC: $40.75 
Published on Web 12/29/2007

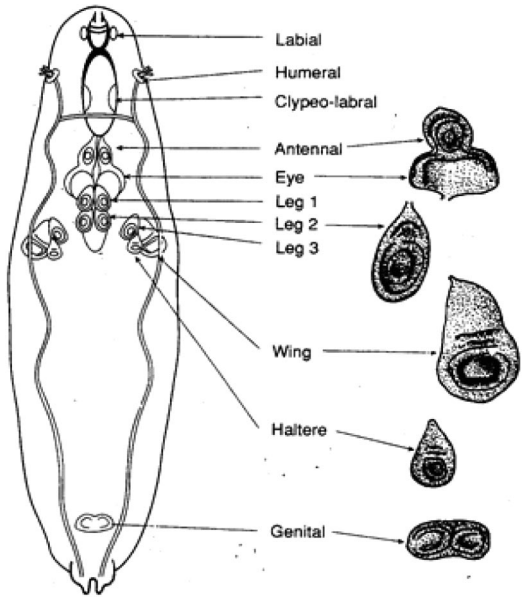


**Figure 2.** Location and morphology of imaginal discs in a *Drosophila* late larva from a ventral view. Reproduced, with permission, from "The Development of Drosophila melanogaster" (Fristrom, D.; Fristrom, J. W. The Metamorphic Development of the Adult Epidermis. In *The Development of Drosophila melanogaster*; Bate, M., Martinez-Arias, A., Eds.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1993; Vol. II).[49] Copyright 1993 Cold Spring Harbor Laboratory Press.

developmental and physiological processes. *Drosophila* has a relatively small genome size (one-tenth of humans) and limited genetic redundancy compared to mammals. Limited genetic redundancy has been key in the understanding of the functions of genes that are present in multiple copies in mammals. This has permitted the elucidation of many of the central signaling pathways that regulate metazoan development and homeostasis, including the Ras, Hedgehog, Wnt, and Notch pathways.[44–47] In the course of these and other studies it became apparent that the remarkable conservation of biochemical signaling pathways between *Drosophila* and mammals makes *Drosophila* an excellent model in which to study the principles underlying human development and pathology. Indeed, *Drosophila* models have been established for a variety of human pathologies including Huntington, Alzheimer's, and Parkinson's disease, in addition to the study of genes that play direct roles in human malignancy.[48] Thus, development of a *Drosophila* PAT screen would allow for genetic dissection of the PAT process and is likely to improve our understanding of important structure−function relationships between the PAT and biomolecules targeting the PAT in a higher eukaryote. Structure−function studies in *Drosophila* could, in turn, lead to identification and improved understanding of the human PAT.

We have chosen to study polyamine transport in *Drosophila* imaginal discs. Imaginal discs are the precursors of the adult epithelium and structures such as the legs, wings, and eyes (Figure 2).[49] Imaginal discs form as invaginations of the embryonic epithelium and grow by cell division during the larval period.[50] During the larval period, imaginal discs exist as a folded, single-cell thick epithelium consisting of approximately 30000 cells. At the onset of metamorphosis, a transient pulse of the steroid hormone 20-hydroxyecdysone (ecdysone) induces rapid tissue morphogenesis and cellular differentiation leading to the formation of adult structures.[49] We focus here on imaginal discs that give rise to the adult legs because the genetics and cell biology of the development of these organs are particularly well understood.[51] Most importantly, leg imaginal discs can be cultured in vitro where they mimic in vivo development and unfold into a rudimentary leg in the presence of ecdysone.[52] Thus, we have a rare opportunity to study PAT function in an intact and developing epithelium within which all cell−cell adhesion properties are maintained. Moreover, the developmental endpoint is easily detected as a phenotypic change, an everted leg.

In this report, we describe the development of the *Drosophila* leg imaginal disc assay and compare the screening results of the polyamine library in the *Drosophila* assay with previous L1210, CHO, and CHOMG screens. Indeed, data from the *Drosophila* assay are consistent with previous results in L1210 and CHO cells, indicating that the *Drosophila* epithelium has a PAT with vertebrate-like characteristics and that this assay will be of use to medicinal chemists interested in screening drugs that use PAT for cellular entry.

## Results and Discussion

**Synthesis.** The synthesis of the anthracene−polyamine conjugates (**4**–**12**) has been described in detail and involved standard reductive amination and N-alkylation techniques.[4,7,53,54]

**Cell Culture Studies with Anthracene−Polyamine Conjugates.** L1210 cells (murine leukemia) were chosen as one cytotoxicity model in order to compare the cytotoxicity of the new conjugates with those published by other authors. In addition, Chinese hamster ovary (CHO) cells were chosen along with a mutant cell line (CHO-MG) in order to evaluate how the synthetic conjugates gain access to cells.[4,7] The CHO-MG cell line is polyamine-transport-deficient and was isolated after selection for growth resistance to methylglyoxalbis(guanylhydrazone), MGBG, ($CH_3C[{=}N{-}NHC({=}NH)NH_2]CH[{=}N{-}NHC({=}NH)NH_2]$) using a single-step selection after mutagenesis with ethylmethanesulfonate.[43a] For the purposes of this study, the CHO-MG cell line represents cells with no PAT activity and provided a model for alternative modes of entry or action that are independent of PAT. These alternative modes of entry include passive diffusion or utilization of another transporter and may also include interactions on the outer surface of the plasma membrane or other membrane receptor interactions. In contrast, the parent CHO cell line represents a cell type with high PAT activity.[4] Comparison of conjugate cytotoxicity in these two CHO lines provided an important screen to detect selective conjugate delivery via the PAT. For example, a conjugate with high utilization of the polyamine transporter would be very toxic to CHO cells but less so to CHO-MG cells.[4,7] In short, highly selective PAT ligands give high CHO-MG/CHO $IC_{50}$ ratios.

As shown in Table 1, all compounds were cytotoxic to L1210 cells. This result suggested that each compound, even those not containing the ideal polyamine message, could enter murine cells and exert their cytotoxic effect. Insight into the PAT selectivity of these compounds was gained by comparisons of their respective cytotoxicity in the CHO and CHO-MG cell lines. Derivatives **5** and **7**, as well as "water-soluble anthracene" controls **10**–**12**, had no preference for either CHO cell line and gave virtually identical $IC_{50}$ values in both CHO-MG and CHO cells. In addition, the norspermidine analogue **6** showed very limited PAT selectivity (CHOMG/CHO $IC_{50}$ ratio of 1.8). These observations were consistent with these materials entering the cell through non-PAT mediated pathways. In contrast, dramatic differences in cytotoxicity were observed with **4** ($IC_{50}$ ratio of 148), with **8** (ratio of 24), and to a lesser degree with **9** (ratio of 3.5). Since the ratios for **4** and **8** are significantly higher than

**Table 1.** Biological Evaluation of Polyamine Derivatives in L1210, CHO, and CHO-MG Cells[a]

| compd (tether)[b] | $IC_{50}$ in $\mu$M | | | |
|---|---|---|---|---|
| | L1210 | CHO-MG $IC_{50}$ in $\mu$M | CHO $IC_{50}$ in $\mu$M | (CHOMG/CHO) $IC_{50}$ratio |
| **4**: Ant-$CH_2$(4,4) | 0.30 ± 0.04 | 66.7 ± 4.1 | 0.45 ± 0.10 | 148 |
| **5**: Ant-$CH_2$NEt(4,4) | 22.2 ± 1.2 | 21.9 ± 0.9 | 22.2 ± 0.7 | 1 |
| **6**: Ant-$CH_2$(3,3) | 1.8 ± 0.4 | 3.4 ± 0.5 | 1.9 ± 0.4 | 1.8 |
| **7**: Ant-$CH_2$NEt(3,3) | 2.2 ± 0.1 | 4.0 ± 0.3 | 5.3 ± 0.4 | 0.8 |
| **8**: Ant-$CH_2$(4,3) | 0.4 ± 0.1 | 9.5 ± 1.1 | 0.4 ± 0.1 | 24 |
| **9**: Ant-$CH_2$(3,4) | 0.7 ± 0.3 | 8.8 ± 1.2 | 2.5 ± 0.7 | 3.5 |
| **10**: Ant-$CH_2$(4) | 6.3 ± 0.3 | 7.6 ± 0.4 | 7.7 ± 0.5 | 1 |
| **11**: Ant-$CH_2$(3) | 2.2 ± 0.1 | 2.4 ± 0.2 | 2.3 ± 0.3 | 1 |
| **12**: Ant-$CH_2$NEt(3) | 5.1 ± 0.1 | 6.6 ± 0.4 | 7.7 ± 0.7 | 0.9 |

[a] Measurements are ± 1 SD. Data for **4**–**10** have been previously reported,[4,7,48,53] while those listed for **11** and **12** are new. [b] Ant = anthracen-9-yl. Cells were incubated for 48 h with the respective conjugate.

the other members of the series, these two compounds were deemed to have good PAT selectivity (with **4** being superior). Indeed, the CHOMG/CHO $IC_{50}$ ratios in Table 1 suggested that PAT targeting is influenced strongly by both the polyamine sequence and the degree of substitution at the $N^1$-position. For example, **8** was more selective than its regioisomer **9** and N-ethylation of **4** (to form compound **5**) completely abolished the superior PAT selectivity of **4** (compound **5**, ratio of 1). Therefore, this panel of compounds represented a homologous series of polyamine motifs with different PAT selectivity as measured in CHO and CHO-MG cells. In this regard they made excellent probes to evaluate polyamine transport in novel assay systems in multicellular organisms such as the *Drosophila* imaginal disc epithelium described below.

***Drosophila* Epithelium Studies with Anthracene−Polyamine Conjugates.** Our primary objective was to determine if *Drosophila* imaginal discs have an active PAT with characteristics similar to those of a vertebrate PAT. Therefore, we tested the library of anthracene−polyamine conjugates on developing leg imaginal discs in in vitro culture. In preliminary experiments, the optimal concentration of the PAT-selective conjugate **4** was determined for these studies. Compound **4** was chosen as the benchmark compound because it displayed the greatest selectivity for PAT in CHO cells (Table 1) and provided a reference point for comparison of data from the other compounds. Data from preliminary experiments with **4** established that 18 $\mu$M in the culture medium was the lowest concentration sufficient to consistently inhibit development of 100% of the dissected imaginal discs (data not shown). Subsequently, all experiments with compounds **4**–**12** were conducted at 18 $\mu$M concentration.

When leg imaginal discs are cultured in vitro in the presence of ecdysone, the folded epithelium is rapidly reshaped into the tubelike structure of the presumptive leg by the morphogenetic process of eversion (Figure 3).[49,51] In all imaginal disc assays, discs were scored as everted (developed) or noneverted (undeveloped). In some cases partial eversion was observed, and these discs were scored as everted. For each compound tested, the percent inhibition of development was determined ([(number of undeveloped discs)/(total number of discs)] × 100). Compounds that showed significant inhibition of imaginal disc eversion were also tested in the presence of 100 $\mu$M spermidine (**2a**) as a competitive PAT antagonist to determine selectivity for PAT. Data for compounds **4**–**12** and **2a** are shown in Table 2.

On the basis of results in L1210 and CHO cells, compounds **4**–**12** could be classified into three groups with respect to biological activity (Table 1). These groups are (i) high PAT selectivity and high toxicity (**4**, **8**), (ii) low to moderate PAT selectivity and moderate toxicity (**6**, **7**, **9**–**12**), and (iii) low PAT selectivity and low toxicity (**5**). These groupings permit a direct comparison of the behavior of compounds **4**–**12** in vertebrate cell culture and *Drosophila* imaginal discs.

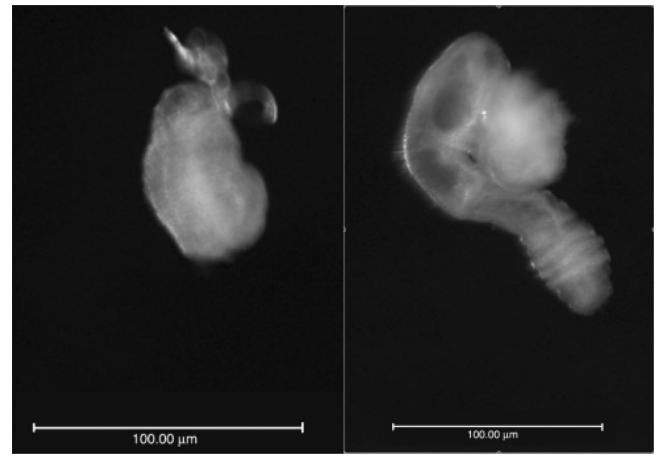


**Figure 3.** Left image: leg imaginal disc prior to incubation with ecdysone showing the "noneverted" phenotype. Right image: leg imaginal disc after 15 h of incubation with 1 $\mu$g/mL ecdysone showing the "everted" phenotype. The scale bar in each image represents 100 $\mu$m.

**Compounds with High PAT Selectivity and High Toxicity in L1210 and CHO Cells.** Compounds **4** and **8** exhibit high PAT selectivity and high toxicity in L1210 and CHO cells with **4** showing 6-fold greater specificity for PAT. In *Drosophila* leg imaginal disc culture compound **4** strongly inhibited disc eversion (97.6% inhibition). The inhibition by **4** was dramatically reduced (i.e., development of the discs was "rescued") when spermidine (100 $\mu$M) was added to the culture medium (8.7% inhibition). Eversion in the presence of **4** + spermidine (**2a**) was indistinguishable from experiments conducted in the absence of **4** ($P = 0.651$; one-way ANOVA with Tukey's post-hoc test). In contrast, eversion in the presence of **4** was significantly different from **4** + spermidine or a control assay ($P < 0.001$). We also used Trypan blue exclusion[55] to determine if noneverted imaginal disc cells were inviable or developmentally arrested. After treatment with **4**, imaginal discs stain positive for Trypan blue whereas everted control discs do not, indicating that **4** is toxic to imaginal discs. Thus, **4** shows high PAT selectivity and toxicity in the leg imaginal disc culture.

Inhibition of eversion by **8** was also significant (73.9%), and discs were rescued by the addition of 100 $\mu$M spermidine (23.8% inhibition; $P < 0.001$). Results with **8** + spermidine vs control are not significantly different ($P = 0.115$), although we observed considerable variance in the data with spermidine. It is noteworthy that competitive inhibition of **8** with spermidine is less effective than with **4**. This is consistent with results from

**Table 2.** Biological Evaluation of Polyamine Derivatives in *Drosophila* Imaginal Disc Cells[a]

| compd (tether) | % inhibition of disc eversion in presence of 18 $\mu$M compound | % inhibition of disc eversion in the presence of compound and 100 $\mu$M spermidine, **2a** | % inhibition of disc eversion in absence of compound (control) |
|---|---|---|---|
| **4**: Ant-$CH_2$(4,4) | 97.6 ± 1.7 | 8.7 ± 2.7 | 6.2 ± 1.0 |
| **5**: Ant-$CH_2$NEt(4,4) | 5.5 ± 1.2 | ND | 1.4 ± 0.95 |
| **6**: Ant-$CH_2$(3,3) | 85.4 ± 10.2 | 100[b] | 4.9 ± 2.75 |
| **7**: Ant-$CH_2$NEt(3,3) | 88.5 ± 11.6 | 100 ± 0[c] | 6.9 ± 1.4 |
| **8**: Ant-$CH_2$(4,3) | 73.9 ± 7.5 | 23.8 ± 8.9 | 4.3 ± 1.45 |
| **9**: Ant-$CH_2$(3,4) | 7.8 ± 2.6 | 9.9 ± 3.9 | 6.2 ± 2.8 |
| **10**: Ant-$CH_2$(4) | 78.4 ± 13.3 | 100 ± 0[c] | 6.7 ± 1.4 |
| **11**: Ant-$CH_2$(3) | 95.2 ± 4.0 | 100 ± 0[c] | 7.4 ± 0.9 |
| **12**: Ant-$CH_2$NEt(3) | 75.7 ± 2.9 | 90[b] | 1.8 ± 1.8 |
| **2a**: spermidine (100 $\mu$M) | 4.8 ± 0.6 | | |

[a] Measurements are indicated as the mean ± SEM. Unless otherwise indicated, data are from three to eight replicate experiments. [b] One replicate. [c] Two replicates.

CHO cells that indicate that **4** has greater PAT selectivity and that some of the toxicity associated with **8** may be due to uptake via a PAT-independent mechanism. In summary, compounds **4** and **8** exhibit high PAT selectivity and high toxicity in L1210 and CHO cells and *Drosophila* imaginal discs.

**Compounds with Low to Moderate PAT Selectivity and Moderate Toxicity in L1210 and CHO Cells.** Compounds **6**, **7**, and **9–12** all exhibit low to moderate PAT selectivity and moderate toxicity in L1210 and CHO cells. Compounds **6**, **7**, and **10–12** strongly inhibit imaginal disc eversion (Table 2), and addition of spermidine fails to rescue the imaginal discs from inhibition by these conjugates. Thus, all of these compounds show low selectivity for PAT in imaginal discs and are toxic to development. The one exception to these observations is compound **9**. In contrast to observations in L1210 and CHO cells, this compound exhibits negligible toxicity in imaginal discs. Interestingly, in all cases, inhibition of eversion in the presence of spermidine was greater than with the compound alone. However, where statistical analysis was possible, none of the differences between compound and compound + spermidine were significant (e.g., $P = 0.38$ for **10**).

**Compounds with Low PAT Selectivity and Low Toxicity in L1210 and CHO Cells.** Compound **5** exhibits low PAT selectivity and relatively low toxicity in L1210 and CHO cells. In agreement with these results, **5** has no inhibitory effect on the development of cultured imaginal discs.

In summary, the results obtained with the conjugate library **4–12** in the imaginal disc assay tracked closely with the previous CHO screens and provided strong evidence for a PAT with vertebrate characteristics in *Drosophila* leg imaginal discs.

**Broader Implications.** The polyamines are essential for normal growth and development. In recent years it has become apparent that the polyamines have important roles in multiple cellular processes including cell proliferation, specific binding of protein factors to DNA, apoptosis, and epigenetic control of gene expression.[37a,56] Given this diverse array of functions, it is hardly surprising that polyamines have been linked to a variety of human pathologies. For example, cancer cells import exogenous polyamines,[1,3] presumably to sustain rapid growth. Thus, the PAT is a promising target for anticancer therapies utilizing toxic compounds that selectively gain entry into the cell via the PAT. While studies of the efficacy of candidate PAT-selective toxic compounds in cell culture have been invaluable, it would be useful to assay these compounds in an intact epithelium that more closely approximates a natural in vivo environment. To this end we have developed a novel assay for PAT-selective compounds in an intact and developing *Drosophila* imaginal disc epithelium. A major strength of the *Drosophila* imaginal disc assay is that the epithelium can be cultured in vitro, where it mimics in vivo development.

Previous testing of a library of polyamine−anthracene conjugates in L1210 and CHO/CHO-MG cells revealed varying degrees of PAT selectivity and toxicity among the compounds.[4,7,53,54] Two compounds, **4** and **8**, showed high PAT selectivity in these assays, and these compounds were the only library members to show PAT selectivity in imaginal discs as judged by competitive inhibition with spermidine. Indeed, of all the remaining compounds that showed toxicity in the imaginal disc assay (**6**, **7**, **10–12**), none of them were competitively inhibited by spermidine. The parallel between polyamine transport in imaginal discs and CHO/CHO-MG is even more striking when the relative selectivity of **4** and **8** for PAT is considered. In CHO/CHO-MG cells, **4** exhibits a 6-fold greater affinity for PAT than **8**. In the imaginal disc assay spermidine blocks uptake of both **4** and **8** and yet is clearly more effective in preventing uptake of **4**, consistent with the greater affinity of **4** for PAT in CHO/CHO-MG cells. In general there is strong concordance in the behavior of all compounds tested in CHO/CHO-MG cells and imaginal discs. The only exception was compound **9** that exhibited relatively low selectivity for PAT in CHO/CHO-MG cells. Collectively, our data point to the existence of a vertebrate-like PAT in *Drosophila* imaginal discs and reinforce the usefulness of the imaginal disc assay in the future testing of PAT-selective toxic compounds.

The existence of a PAT with biochemical properties similar to those of the mammalian PAT was recently reported in *Drosophila* S2 cells.[57] The PAT in S2 cells is a $H^+$ dependent transporter that shows specific affinity for spermine and spermidine but not putrescine and has pharmacological properties suggesting similarity to the vertebrate Slc22 (solute carrier 22) family of solute carriers. Whether the same PAT is utilized in imaginal discs and S2 cells is unknown, and it will be interesting to determine if imaginal discs lacking a functional Slc22 transporter are resistant to compounds **4** and **8**.

The discovery of a PAT in *Drosophila* imaginal discs raises the possibility of testing polyamine−drug conjugates against developing cancers in vivo. A number of *Drosophila* tumor suppressor genes with human orthologues have been linked to hyperplastic or neoplastic overgrowth of imaginal discs.[48d] Many cancer models rely on knockouts of both copies of a gene in all cells of a tissue or the body. In reality, cancer has a clonal origin with malignant cells developing in the background of an otherwise normal epithelium. In *Drosophila*, site-specific mitotic recombination in imaginal discs can be used to generate mitotic clones homozygous for a mutation in a tumor suppressor gene in a background of heterozygous "wild-type" cells.[58] Such

clones are readily detected by the inclusion of appropriate cell markers in the genetic makeup of the animals tested.[59] The ability of specific polyamine−drug conjugates to inhibit the growth of mitotic clones homozygous for mutations in tumor suppressor genes could be quantitatively assayed in animals that are fed the polyamine conjugates. Two recent reports describe the development of a mitotic recombination system in mice.[60] In one of these studies the pattern of tumorigenesis associated with p53 mitotic clones was markedly different from that seen in p53 knockout animals, illustrating the need to study tumorigenesis in a more realistic cellular environment.

Finally, while the pathways utilized in the biosynthesis of polyamines from ornithine, and their catabolism, have been well characterized, the mechanism of uptake of exogenous polyamines is poorly understood. Numerous studies point to the existence of an active polyamine transport process across the cell membrane of higher eukaryotes, and yet identification of the PAT in a multicellular organism remains elusive. We note that the new imaginal disc assay described herein can be used to screen directly for future PAT selective compounds. Moreover, imaginal discs homozygous for mutations in candidate polyamine transporter encoding genes can be assayed for resistance to highly PAT-selective compounds such as **4**. In this regard, the assay provides two benefits to the medicinal chemistry community (PAT drug screening and PAT gene screening). We anticipate that this assay will be of value in achieving the ultimate goal of identification and characterization of a mammalian PAT.

## Conclusions

The imaginal disc epithelium of *Drosophila melanogaster* was used to develop a novel screening assay for PAT-selective molecules. Screening of a library of polyamine−anthracene conjugates (previously tested in CHO and L1210 cells) indicated that these compounds had similar PAT selectivity and toxicity profiles in vertebrate cell culture and in the *Drosophila* imaginal discs. Collectively our studies indicate the presence of a PAT with vertebrate-like characteristics in imaginal discs and confirm the presence of PAT in *Drosophila*. Major advantages of the imaginal disc system include the ability to assay novel PAT-selective compounds in an intact and developing epithelium and the potential to harness the power of *Drosophila* genetics in the search for PAT in a multicellular eukaryote.

## Experimental Section

**Polyamine−Anthracene Conjugates and Chemicals.** Preparation of the polyamine−anthracene conjugates has been previously reported, and each analogue was characterized by $^1$H NMR, $^{13}$C NMR, and mass spectral and elemental analyses. All reagents and chemicals used were obtained from commercial sources. BSA fraction V (Sigma no. A-9647) and 20-hydroxyecdysone (Sigma no. H-5142) were obtained from the Sigma-Aldrich Company.

***Drosophila* Stocks and Larval Collections.** The wild-type Oregon-R variant of *Drosophila melanogaster* was used for all experiments. Twenty females and five males were allowed to mate and lay eggs on standard cornmeal medium containing 0.05% bromophenol blue for 24 h at 25 °C. Larvae fed on medium containing bromophenol blue can be monitored with respect to developmental stage.[61] Following removal of adults, larval development was monitored until larvae ceased feeding and began to wander on the walls of the culture container. Wandering larvae with light-blue guts were selected for dissection. These larvae are within 7 h of pupariation but have not yet been exposed to the metamorphic pulse of 20-hydroxyecdysone that triggers imaginal disc morphogenesis. Imaginal discs dissected from larvae at this stage of development are competent to develop upon exposure to 20-hydroxyecdysone in in vitro culture.

**Imaginal Disc Culture and Scoring.** Leg imaginal discs were dissected in room temperature Ringer's solution (130 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2 \cdot 2H_2O$) containing 0.1% BSA (w/v). BSA was added to the Ringer's solution immediately prior to use. Dissected discs were left in Ringer's solution for no more than 1 h prior to culture, and typically 100 discs could be dissected in this time. To begin cultures, discs were transferred to 1 mL of minimal Robb's medium at 25°C (see below). Generally, three cultures each containing 30 leg imaginal discs were set up in parallel as follows: (a) treatment 1, 18 $\mu$M compound and 1 $\mu$g/mL 20-hydroxyecdysone; (b) treatment 2, 18 $\mu$M compound, 100 $\mu$M spermidine, and 1 $\mu$g/mL 20-hydroxyecdysone; (c) treatment 3, 1 $\mu$g/mL 20-hydroxyecdysone (eversion control).

Imaginal discs were incubated in 12-well plastic culture plates for 15 h at 25°C. After 15 h, the discs were scored as everted or noneverted. Fully everted discs (the leg is fully extended from the disc; see Figure 3 right panel) and partially everted discs (leg protruding from epithelium but not fully extended) were scored as everted. Noneverted discs showed no sign of development (Figure 3, left panel). For each compound tested, a percent inhibition of development was determined ([(number of noneverted discs)/(total number of discs)] × 100%) and is listed in Table 2.

**20-Hydroxyecdysone.** 20-Hydroxyecdysone was dissolved in absolute ethanol to a final concentration of 1 mg/mL and stored in aliquots at −20 °C. Before use, the stock solution was diluted to 0.1 mg/mL in absolute ethanol and the diluted solution (10 $\mu$L) was added to minimal Robb's medium (1 mL).

**Robb's Minimal Medium.** Minimal Robb's medium consisting of 40 mM KCl, 0.4 mM $KH_2PO_4$, 40 mM NaCl, 0.4 mM $NaH_2PO_4 \cdot 7H_2O$, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.2 mM $MgCl_2 \cdot 6H_2O$, 1 mM $CaCl_2 \cdot 2H_2O$, 10 mM glucose, 0.2 mM L-asparagine, 4.0 mM L-glutamine, 0.16 mM glycine, 0.64 mM L-leucine, 0.32 mM L-proline, 0.16 mM *R*-serine, and 0.64 mM L-valine, pH 7.2) was prepared and stored at −20° C. Immediately prior to use, 10 $\mu$L of 10% BSA (w/v) was added to 1 mL of medium.

**Acknowledgment.** We are grateful to David Jenkins of the Department of Biology for help with statistical analysis and to Robert Ruggiero for thoughtful comments on the manuscript. M.I. was partially supported by an American Cancer Society Florida Division Undergraduate Summer Fellowship. This work was supported by NIH Grant GM065884 to L.v.K., and partial support was provided by the Broad Medical Research Program to O.P.

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JM701198S