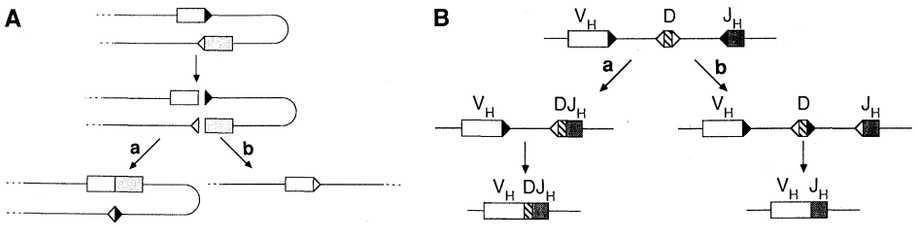

Fig. 4. (**A**) model for signal sequence replacement by alternative resolution of an intermediate in Ig gene rearrangement. Open and shaded boxes represent different Ig coding segments; filled and open triangles represent recombinational signal sequences bearing 23- and 12-bp spacer regions. For explanation, see text. Loss of nucleotides from the coding sequences and addition of novel nucleotides at the junctions are not included in the model, but these processes are presumed to occur after cleavage of DNA and before joining. (**B**) Altered targeting of Ig heavy chain gene segments as a consequence of signal sequence replacement. Boxes representing V_H, D, and J_H segments are indicated. Filled triangles represent recombinational signals carrying 23-bp spacers; open triangles, recombinational signals with 12-bp spacers.

coding sequences would likely have been joined to sites within the V_H coding sequence, rather than to a specific site at its 3′ flank.

In principle, signal sequence replacement can alter the targeting of Ig gene segments. The assembly of heavy chain genes, for example, normally involves the joining of a D segment to a J_H segment and subsequent joining of a V_H segment to the DJ_H unit (*2, 3, 20*) (Fig. 4B, a). If the signal sequences of a J_H segment were replaced by the signal sequences of a D segment, this would permit the direct joining of a V_H segment to J_H (Fig. 4B, b).

The existence of heavy chain genes that lack D segments would be consistent with the occurrence of signal sequence replacement in vivo. The nucleotide sequence of one functional μ gene, 22.11, contains a precise fusion of V_H and J_H coding segments (*21*). The Ig heavy chain genes that lack D sequences are not necessarily the result of direct V_H-to-J_H joining, in that V_H-to-DJ_H joining can be so imprecise as to completely remove the D sequence (*10*). Nevertheless, because of the precision of the V_H-J_H junction, it seems unlikely that the 22.11 μ gene was formed by imprecise V_H-to-DJ_H joining. Our observations suggest an alternative possibility—that the gene was assembled by direct V_H-to-J_H joining, via signal sequence replacement.

Note added in proof: Analysis of a fourth signal sequence replacement product revealed precise joining with respect to the V_H heptamer sequence and loss of one nucleotide from the DJ_H coding sequence, in agreement with the results presented above.

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Structural Rearrangement of the Retinoblastoma Gene in Human Breast Carcinoma

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Structural changes of the human retinoblastoma gene have been demonstrated previously in retinoblastoma and some clinically related tumors including osteosarcoma. Structural aberrations of the retinoblastoma locus (RB1) were observed in 25% of breast tumor cell lines studied and 7% of the primary tumors. These changes include homozygous internal deletions and total deletion of RB1; a duplication of an exon was observed in one of the cell lines. In all cases, structural changes either resulted in the absence or truncation of the RB1 transcript. No obvious defect in RB1 was detected by DNA blot analysis in primary tumors or cell lines from Wilms' tumor, cervical carcinoma, or hepatoma. These results further support the concept that the human RB1 gene has pleiotropic effects on specific types of cancer.

TUMOR DEVELOPMENT IN CERTAIN types of cancer is thought to be the result of the unmasking of one or more recessive genes by two mutations. The mutations can result in a reduction to homozygosity of the altered allele, as has been demonstrated in a number of human cancers (*1–11*). Retinoblastoma is the best studied example in which the mutation of a recessive gene is suspected to play a role in malignant transformation. Complementary DNA (cDNA) clones of the putative RB1 gene have recently been isolated (*13–15*). Structural aberrations at RB1, including examples of internal deletions of both alleles of the RB1 gene (*15*), were demonstrated in many primary retinoblastoma tumors and cell lines. The RB1 transcript is either absent or truncated in most of the tumor samples. The complete inactivation of both alleles of the RB1 gene in retinoblastoma appears to be required for tumorigenesis.

Clinically, surviving patients with the familial form of the disease are at increasing risk for other specific types of primary malignancies such as fibrosarcoma, melanoma, and osteosarcoma (*16*), which suggests that a single pleiotropic recessive locus is the link between retinoblastoma and osteosarcoma. While breast tumors have seldom been associated with retinoblastoma, mothers of children with osteosarcoma are at higher risk for breast tumors (*17*). Studies of restriction

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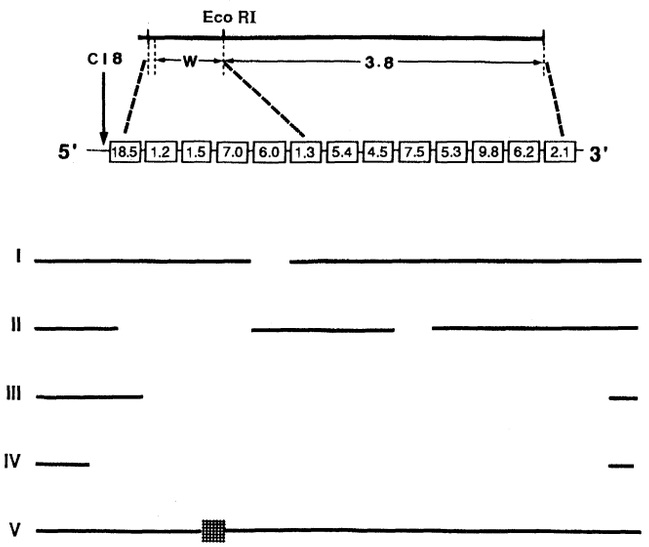

Fig. 1. Structural aberrations of the RB1 gene in human breast tumors and cell lines. Probes 3.8, W, and C18 are described in the text. The open boxes at the top represent Hind III fragments detectable with probes 3.8 and W. The numbers inside the boxes represent the size of the DNA fragments in kilobases. Deletions of RB1 in the tumors are shown as gaps below. Duplication is shown as a shaded box. The following tumors or cell lines are shown: I, 622; II, BT-549; III, MDA-MB-468; IV, 410, 411, 346, and DU4475; and V, MDA-MB-436.

fragment length polymorphisms (RFLPs) also have recently demonstrated a reduction to homozygosity of chromosome 13 in hereditary breast tumors (*9*). We report here the detection of specific structural changes of RB1 in primary tumors and established cell lines from human breast tumors.

Because of the complexity of the locus and the presence of a GC-rich region in the 5′ end of the cDNA clone, we chose to analyze RB1 with three probes (Fig. 1). Probe 3.8 represents the 3′ segment and probe W the 5′ segment of the RB1 gene. Together they represent the majority of the RB1 cDNA clone without the stretch of GC-rich region at the 5′ end. To circumvent the lack of a useful probe in the GC-rich region we made use of probe C18, which detects a 5.3-kb Hind III fragment and is situated 5′ to the first exon, to see if any deletion had extended beyond the 5′ end.

DNA from 18 human primary hepatocellular carcinomas, 1 hepatoma cell line (PLC/PRF/5); 7 human cervical carcinoma cell lines (CaSki, C-4I, C-4II, C-33A, HT-3, ME 180, and MS751); 10 primary Wilms' tumor samples, 2 Wilms' tumor cell lines (G401, Wiltu-1), 41 primary human breast tumors, 10 lymph node metastases, and 16 established human breast cell lines (BT-20, MCF-7, HSO578T, DU4475, BT-483, MDA-MB-468, T47DX, BT-549, MDA-MB-231, MDA-MB-436, MDA-MB-157, MDA-MB-175, MDA-MB-453, ZR-75-30, BT-474, MDA-MB-415) from pleural effusion or metastasis were analyzed. RNA, when available, was also analyzed. The clinical and molecular characterization of the 41 primary breast tumors and the 10 lymph node metastases have been described (*17*). Of the 41 samples, 11 were obtained from premenopausal and 30 from postmenopausal women. All samples were frozen in liquid nitrogen immediately after surgical excision and were never cultured in vitro.

Of the various types of tumor we examined, structural aberrations were found at a high frequency only in breast carcinoma. These included 3 of 41 primary breast tumors, 1 of 10 lymph node metastasis, and 4 of 16 human breast tumor cell lines (Fig. 2). Results from those tumors that have no obvious changes (hepatoma, Wilms' tumor, and cervical carcinoma) are not shown. The various structural changes are summarized schematically (Fig. 1). These changes included: (i) homozygous internal deletion of one region of the RB1 gene, (ii) homozygous internal deletions of two regions of the RB1 gene, (iii) homozygous 3′ deletion, (iv) homozygous total deletion, and (v) duplication of a portion of the RB1 gene.

Homozygous internal deletions are exemplified by an infiltrating ductal breast carcinoma 622 as well as the breast tumor cell line BT-549. Carcinoma 622 was an infiltrating ductal breast carcinoma from a postmenopausal woman. When compared with other tumor DNA in the same gel (Fig. 2B, lanes 1 to 3), the 6.2-kb exon-containing fragment resulting from digestion with Hind III was found to be partially deleted in 622; this resulted in the generation of a novel 4-kb fragment (Fig. 2B, lane 2). The presence of stromal tissues apparently contributed to background hybridization signals in the 6.2-kb region. Homozygous internal deletions were also found in the breast carcinoma cell line BT-549, which was established from pleural effusion. The ductal breast carcinoma line BT-549 had homozygous internal deletions in two regions of RB1 (Fig. 2A, lane 18; Fig. 2C, lane 2). These include a homozygous total deletion of the 7.0-, 1.5-, and 1.2-kb Hind III fragments and part of the 18.5-kb Hind III fragment to generate a novel 10-kb fragment. Analysis with Bgl II and Eco RI led to the same conclusion. For example, the normal 8.0-kb and 3.3-kb Eco RI fragments were deleted in this tumor (Fig. 2C, lane 2). The other homozygous deletion region in this tumor includes the exons in the Hind III 7.5-kb fragment (Fig. 2B, lane 18).

Examples of possible homozygous deletion of DNA sequences on chromosome 13 including all of RB1 are provided by two primary breast carcinoma samples and an established breast tumor cell line. The two primary breast carcinoma samples are infiltrating ductal carcinomas 410 and 346 (Fig. 2, A and B, lanes 5 and 9, respectively). Sample 411 is a lymph node metastasis from the same patient as 410. In all three samples, the hybridization signal with the RB1 probes was much reduced even when equal amounts of DNA were present in each lane, as demonstrated by ethidium bromide staining and hybridization to unrelated probes λMS31 (*18*) and pGCWP (*19*) (Fig. 2B). This reduction in hybridization signal intensity indicates that RB1 is missing from the majority of the cells in these samples. Again, the background hybridization is very likely due to the presence of stromal tissues in the tumor samples. Moreover, there may well be heterogeneity among the tumor cells themselves in a given tumor sample. Both the primary infiltrating ductal tumor 410 and its lymph node metastasis 411 showed reduced RB1 hybridization signals. Sample 410 was a poorly differentiated carcinoma obtained from a premenopausal woman and sample 346 was also a poorly differentiated carcinoma but from a postmenopausal woman. In addition, structural rearrangement of the c-*myc* gene has been documented in 346 (*20*).

The problem of stromal tissue contamination does not exist in established cell lines. Cell lines DU4475 [established from a metastatic cutaneous nodule (Fig. 2, A and B, lane 14)] and MDA-MB-468 [a breast adenocarcinoma line established from pleural effusion (Fig. 2, A and B, lane 16)] showed homozygous deletion of part or all of the RB1 gene. Neither of the RB1 probes hybridized to the tumor DNA of DU4475, although there was hybridization with probe pGCWP (*19*), which is unrelated to the RB1 gene (Fig. 2B). Results from the digestion with Eco RI led to identical conclusions (Fig. 2C, lane 3). Hybridization of a probe C18, 5′ to the RB1 gene, also did not detect a signal (Fig. 2D, lane 4). The structural aberration in this tumor is, therefore, a total deletion of the RB1 gene. In the tumor cell line MDA-MB-468 most of the RB1 gene is homozygously deleted; DNA sequences corresponding to the extreme 5′ end of the RB1 gene and beyond were

detected as a 18.5-kb Hind III fragment with probe W (Fig. 2A, lane 16) and a 5.3-kb Hind III fragment with probe C18 (Fig. 2D, lane 6). The structural change in this tumor was therefore classified as a homozygous 3′ deletion.

In breast tumor cell line MDA-MB-436 (Fig. 2A, lane 20) there appears to be a duplication of a part of the RB1 gene that resulted in a change in the length of restriction fragments analyzed with Hind III and Eco RI. An extra 5.0-kb Hind III fragment can be detected by hybridization to part of probe W. The precise unit of duplication remains to be defined by molecular cloning. However, restriction fragment patterns revealed by Eco RI (Fig. 2C, lane 5) can best be explained by the duplication of part of probe W such that a 5.0-kb Hind III fragment was generated between the Hind III 1.5-kb and 7.0-kb regions. As a result, the normal 8.0-kb Eco RI fragment (Fig. 2D) was increased to about 13 kb in size. The absence of the normal 8.0-kb fragment indicates that the change is homozygous. No hybridization was detected when the plasmid vector pGEM-2 was used as a probe, indicating that the change is not an artefact of plasmid contamination.

The remaining tumor samples had no obvious structural changes detectable by DNA blot analysis. The apparent change in the breast tumor line T-47DX (Fig. 2A, lane 17) is due to a rare RFLP of the normal 1.5-kb Hind III fragment. This RFLP has been observed in the DNA of other normal human subjects.

In breast tumors, as in retinoblastomas, structural changes in RB1 correlate well with the changes in its transcription (Fig. 3). The breast tumor cell lines HSO578T and MDA-MB-231, which show no obvious structural defects of RB1, contained a normal-sized transcript of the RB1 gene. On the other hand, the breast tumor cell lines MDA-MB-468 and DU4475 which had homozygous deletion of part or all of the RB1 gene, and the tumor cell line MDA-MB-436, which has a duplication of part of the RB1 gene, had little or no Rb transcripts. The breast tumor cell line BT-549, which had a homozygous internal deletion, had a truncated transcript of 1.7 kb. A normal 4.7-kb transcript was detected in the normal human breast myoepithelial cell line HBL 100.

In summary, structural changes of the RB1 gene were observed in about 25% of the breast tumor cell lines but only in 7% of the primary tumors. It is possible that the loss of the RB1 gene function enhances the establishment of cell lines. In fact, in all the tumor cell lines in which disruption of RB1 is observed, the cell population appears to be homogeneous with respect to the RB1 genotype. Our report here suggests that the RB1 gene may be the recessive locus that is implicated in breast tumors. None of the breast tumor patients here were known to be predisposed to the development of retinoblastoma. Similarly, breast tumors are not the frequent second primary tumors found in familial retinoblastoma patients. One possible explanation is that more than one genetic event needs to take place for tumorigenic transformation to occur. Two different types of tumor may therefore have common requirements for the mutation of a particular locus, but each tumor also has its own peculiar set of loci that needs to be altered. For retinoblastoma, other nonran-

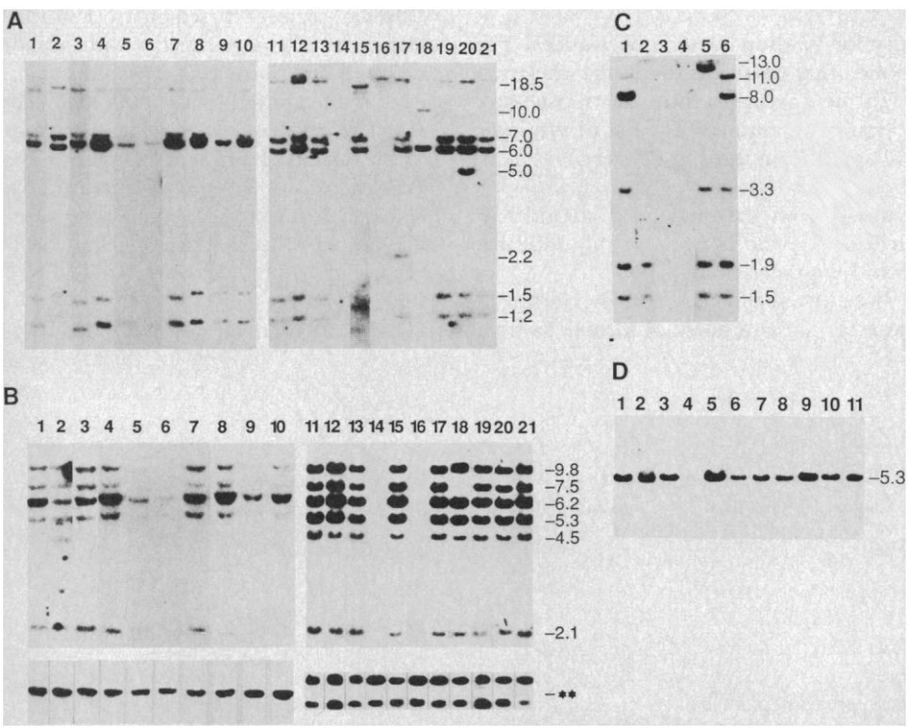

Fig. 2. DNA blot analysis of primary human breast tumor samples and cell lines. High molecular weight DNA was digested with Hind III (**A**, **B**, and **D**) or Eco RI (**C**) for DNA analysis with probes as described (*15*). (A) probe W; (B) probe 3.8; (C) probe W; (D) probe C18. At the bottom of (B), is shown a rehybridization of the filters to unrelated probes: * (lanes 1 to 10) to probe λMS31 (*18*) and ** (lanes 11 to 21) to probe pGCWP (*19*). This was done to check on the quantity of DNA used in each lane. For (A) and (B), lanes 1 to 10 are breast tumor samples and lanes 11 to 21 are breast cell lines obtained from American Type Culture Collection. Lanes 1, 621; 2, 622; 3, 623; 4, 446; 5, 410; 6, 411; 7, 423; 8, 323; 9, 346; 10, 328; 11, BT-20; 12, MCF-7; 13, HSO578T; 14, DU4475; 15, BT-483; 16, MDA-MB-468; 17, T-47DX; 18, BT-549; 19, MDA-MB-231; 20, MDA-MB-436; 21, normal human breast myoepithelial cell HBL 100. (C) Lane 1, MDA-MB-175; lane 2, BT-549; lane 3, DU4475; lane 4, MDA-MB-468; lane 5, MDA-MB-436; and lane 6, MDA-MB-231. (D) Lanes 1 to 11 correspond to lanes 11 to 21 of (A) and (B).

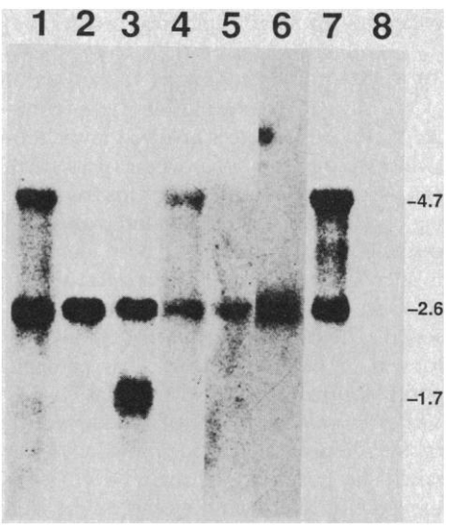

Fig. 3. RNA blot analysis of tumor cell lines. Polyadenylated RNAs were isolated and analyzed as described (*15*). Hybridizations were done with probe 3.8 and a cDNA of the human catalase gene. The RB1 gene probe detects a 4.7-kb transcript while the catalase probe detects a 2.6-kb transcript. Lanes 1 to 5, breast tumor cell lines HS0578T, MDA-MB-468, BT-549, MDA-MB-231, and MDA-MB-436, respectively; lane 6, DU4475; lane 7, normal human myoepithelial cell line HBL 100. Because the size of the RB1 gene transcript is only slightly larger than the 28S ribosomal RNA, it was necessary to include a control lane of ribosomal RNA (lane 8) to monitor the conditions of washing.

dom chromosomal changes involving chromosome 1 and especially chromosome 6 have been reported (*12, 21–23*), and for breast cancer, loss of heterozygosity on chromosome 11 (*10*) in addition to chromosome 13 also gives support to this concept. Inheritance of a mutation in one of these loci will probably enhance the transformation process but be insufficient for tumorigenesis.

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Recognition and Transport of Adenine Derivatives with Synthetic Receptors

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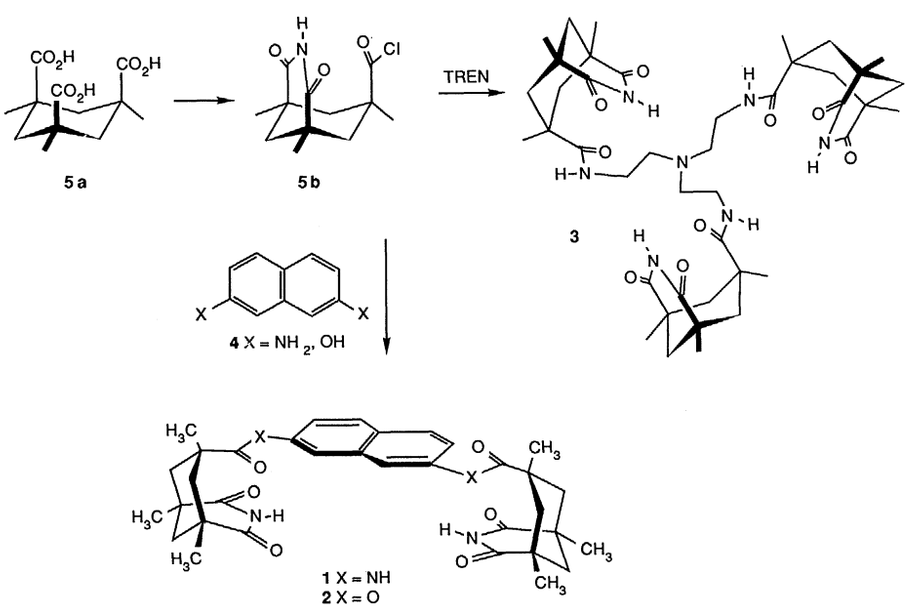
Several new synthetic agents show high affinity for binding adenine derivatives. The structures feature complementary hydrogen bonds that cause the molecular chelation of the purine nucleus. The high lipophilicity of the new agents permits the transport of adenosine and deoxyadenosine across organic liquid membranes. The use of synthetic receptors for small biological targets may have application in drug delivery.

MODEL RECEPTORS HAVE BEEN useful for exploring the intermolecular forces involved in the molecular recognition of adenine derivatives (*1*). Watson-Crick and Hoogsteen base pairing, bifurcated hydrogen bonds, and aryl stacking interactions have all been identified in such systems and their relative contributions to binding selectivity have been assessed. The binding studies have been performed in organic solvents that compete poorly for hydrogen bonds, such as $CDCl_3$. The extraordinary affinity of structures such as the diimide **1** for adenine derivatives raised the possibility of their use in more competitive hydrogen-bonding media. Accordingly, these substances (**1**, **2**, and **3**) were tested for their ability to extract adenine from its aqueous solutions (*2*). We report these results, including their application as vehicles for transport of adenine derivatives across liquid membranes.

The new molecular chelates were prepared by acylating the appropriate spacer groups **4** or TREN (2,2′,2″-triaminotriethylamine) with the imide acid chloride **5b** (scheme 1); the latter is readily available from Kemp's triacid **5a** (*3*).

Both the diamide **1** and the diester **2** extracted one equivalent of adenine **6a** from H_2O into $CDCl_3$. The nuclear magnetic resonance (NMR) spectra of the extracts were consistent with simultaneous Watson-Crick, Hoogsteen, and aryl stacking interactions in the complexes (**7a**) (*4*). The more flexible TREN derivative **3** extracted ~100% of capacity (based on a 1:1 stoichiometry). Parallel experiments with adenosine **6b** (0.1*M*) showed that even this compound was extracted by the molecular chelates. The relative efficiency of extractions was the diamide **1** (45% of capacity) > the TREN derivative **3** (15% of capacity) >> the diester **2** (undetected). With deoxyadenosine **6c**, the order was diamide **1a** (~90% of capacity) > diester **2** (30%) > TREN **3** (10%).

Transport of these adenine derivatives



Scheme 1.

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