High-performance liquid chromatography and photoaffinity crosslinking to explore the binding environment of nevirapine to reverse transcriptase of human immunodeficiency virus type-1

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Abstract

Nevirapine (BI-RG-587) is a potent inhibitor of the polymerase activity of reverse transcriptase of human immunodeficiency virus type-1. Nevirapine, as well as several other non-nucleoside compounds of various structural classes, bind strongly at a site which includes tyrosines 181 and 188 of the p66 subunit of reverse transcriptase. The chromatography which was utilized to explore this binding site is described. BI-RH-448 and BI-RJ-70, two tritiated photoaffinity azido analogues of nevirapine, are each crosslinked to reverse transcriptase. The use of several HPLC-based techniques employing different modes of detection makes it possible to demonstrate a dramatic difference between the two azido analogues in crosslinking behavior. In particular, by comparing HPLC tryptic peptide maps of the photoadducts formed between reverse transcriptase and each azido analogue, it can be shown that crosslinking with BI-RJ-70 but not with BI-RH-448 is more localized, stable, and hence exploitable for the identification of the specifically bonded amino acid residue(s). In addition, comparison of the tryptic maps also makes it feasible to assess which rings of the nevirapine structure are proximal or distal to amino acid side chains of reverse transcriptase. Finally, another feature of the HPLC peptide maps is the application of on-line detection by second order derivative UV absorbance spectroscopy to identify the crosslinked amino acid residue.

1. Introduction

Human immunodeficiency virus type-1 (HIV-1) is the causative agent of acquired immunodeficiency syndrome (AIDS). Reverse transcriptase, an enzyme of HIV-1, catalyzes the polymerization of proviral DNA and is essential to the replication of HIV-1. Hence drugs that inhibit reverse transcriptase represent potential therapies against AIDS (see ref. 1 and references cited therein).

In the USA the only three drugs approved for the treatment of AIDS are the nucleoside analogues 3'-azido-3'-deoxythymidine (AZT), 2',3'-dideoxyinosine (ddI), and 2',3'-dideoxycytosine (ddC) [2-4]. Nucleoside analogues inhibit re-

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verse transcriptase by a complex mechanism that includes chain termination of the nascent nucleic acid in the process of reverse transcription [5,6]. Unfortunately, nucleoside analogues are associated with clinically significant side effects (*e.g.*, bone marrow suppression and peripheral neuropathy [7–9]).

Several non-nucleoside drugs, such as TIBO [10], L-697-661 [11], HEPT [12], BHAP [13], and nevirapine [14,15], also inhibit reverse transcriptase and are under investigation [16,17]. The mechanism of the non-nucleoside drugs does not involve chain termination and is specific for the polymerase activity of HIV-1 reverse transcriptase. Thus it is hoped that the non-nucleoside drugs will not inhibit polymerization of human DNA and that as a consequence fewer and/or less severe clinical side effects will be manifested [15].

Although the various non-nucleoside drugs are different in molecular structure, they evidently bind to a similar region of the p66 subunit of reverse transcriptase [18–22]. We were the first to report the identification of tyrosine residues 181 and 188 as important components of this critical region adjacent to the putative active site of reverse transcriptase [19]. Subsequent studies involving mutagenesis [20,21] and X-ray crystallography [22] corroborated the identification of the tyrosines and increased further an already intense interest in understanding the structure of the binding region of reverse transcriptase for non-nucleoside drugs [23].

In our earlier research, the identification of the tyrosine residues 181 and 188 was achieved through photoaffinity crosslinking of an azido analogue (BI-RJ-70) of nevirapine to reverse transcriptase [19]. The crosslinking was extensive and highly specific, as tryptic peptide mapping by HPLC and N-terminal peptide sequencing showed that approximately 75% of the starting amount of BI-RJ-70 covalently attached to the tyrosine residues 181 and 188.

The chromatography used with BI-RJ-70 is now described in detail in this paper. Furthermore, BI-RH-448, a new tritiated photoaffinity probe and azido analogue of nevirapine, is introduced and characterized. By using several HPLC-based techniques employing different modes of detection to compare BI-RH-448 with BI-RJ-70, a dramatic difference between the two azido analogues in crosslinking behavior can be demonstrated. In particular, a comparison of HPLC tryptic peptide maps of the photoadducts formed between reverse transcriptase and each azido analogue shows that crosslinking with BI-RJ-70 is more localized, stable, and hence more exploitable than with BI-RH-448 for the identification of the covalently modified amino acid residues.

Moreover, the tryptic maps are consistent with the A-ring of the nevirapine structure oriented proximal to the tyrosines, and with the C-ring oriented distal from the tyrosines as well as from other amino acids. This assessment was not possible previously [19]. Finally, another feature of the HPLC peptide maps is the novel application of on-line detection by second order derivative UV absorbance spectroscopy to identify the crosslinked amino acid residue.

2. Experimental

2.1. Photoaffinity crosslinking

Preparation of HIV-1 reverse transcriptase [18,19,24], synthesis of nevirapine and analogues [25,26], and photoaffinity crosslinking of azido analogue to reverse transcriptase [18,19,21] were described in detail previously.

2.2. Apparatus

A Hewlett-Packard 1090 (Avondale, PA, USA) high-performance liquid chromatograph integrated with a Hewlett-Packard 1040M diode array detector was used for HPLC. Zero-order UV absorbance spectra were collected on-line and reviewed with a Hewlett-Packard 98561A-300 series computer and 9153B disk drive. Hewlett-Packard revision 5.22 software was used to acquire and plot these spectra, as well as to convert them to second order derivative spectra. A C_{18} Delta-Pak column (150×2.1 mm) from Millipore-Waters (Milford, MA, USA) was used

for HPLC of reverse transcriptase, azido analogues, and tryptic digestions.

HPLC fractions were collected with a HeliFrac from Pharmacia LKB (Piscataway, NJ, USA), or with a 201 fraction collector from Gilson (Middleton, WI, USA). Sequences of amino acids of peptides in HPLC fractions were determined with a 477A pulsed-liquid protein sequencer from Applied Biosystems (Foster City, CA, USA). Radioactivity in HPLC fractions and in cycles of sequencing were measured with an LS 5000 TA scintillation counter from Beckman (Fullerton, CA, USA). Peptide synthesis was carried out on an Applied Biosystems 430A synthesizer.

2.3. Reagents and solvents

Trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone was from Worthington (Freehold, NJ, USA). Water was HPLC-grade from a Milli-Q and Milli-RO system from Millipore (Milford, MA, USA). Acetonitrile was from Burdick and Jackson (Muskegon, MI, USA) and trifluoroacetic acid was sequenal grade from Pierce (Rockford, IL, USA). All other chemicals were reagent grade.

2.4. Procedures

Reverse transcriptase crosslinked to azido analogue was subjected to digestion by trypsin under denaturing conditions [19,27]. Briefly, to 35–250 μl of solution containing 40–1000 μg (0.34–8.5 nmol) of reverse transcriptase crosslinked to azido analogue, an approximately equal volume of 8 M urea in 400 mM ammonium bicarbonate (pH 7.8) was added. After vortexing, typically 2–4 volumes of water were then added. Next 10–25 μl of trypsin in water were added either all in one aliquot, or in two equal aliquots, the second aliquot following the first by 1.25 h. The final ratio of trypsin–reverse transcriptase was 1:25 or 1:12.5 (w/w).

Following a 2.5-h incubation at 37°C, the digest was frozen at −80°C, or treated with 1% trifluoroacetic acid to a final pH of 2, and/or injected onto the C_{18} reversed-phase HPLC

column. Conditions for HPLC are given in legends of figures. Fractions of HPLC effluent of peptide maps were collected in intervals of 0.5–2.0 min corresponding to volumes of 100–400 μl. An aliquot of 5–20 μl of each collected fraction was sampled for liquid scintillation counting off-line, and the measurement of radioactivity was then adjusted and reported to reflect the radioactivity in the entire fraction.

After the aliquots were taken for liquid scintillation counting, 100–800 μl of the collected fractions of effluent of HPLC tryptic peptide mapping were applied to the protein sequencer. At the completion of each Edman cycle, a known percentage of the product was transferred on-line to an Applied Biosystems 120A PTH analyzer for identification of the amino acid in that cycle. The remaining percentage of product in each cycle was collected and measured off-line for radioactivity by liquid scintillation counting. The measured radioactivity was then adjusted and reported to reflect all the radioactivity of the entire product of each cycle.

Peptide synthesis was performed with small-scale rapid cycles and *tert.*-butoxycarbonyl chemistry followed by cleavage with hydrofluoric acid. Synthesized peptide was purified by semi-preparative HPLC employing conditions similar to those used for tryptic peptide mapping as described in the legend of Fig. 2.

3. Results and discussion

3.1. Crosslinking azido analogue to reverse transcriptase

Regions of reverse transcriptase accessible to nevirapine and other non-nucleoside drugs were explored by photoaffinity crosslinking by BI-RH-448 or BI-RJ-70, two structural and functional azido analogues of nevirapine [18,25,26]. The structures of BI-RH-448 and BI-RJ-70 (Fig. 1) were designed to achieve a bi-directional probing of reverse transcriptase, as the azido group was located on the C-ring of BI-RH-448 and on the A-ring of BI-RJ-70. To enhance detection, radioactive tritium was incorporated on the
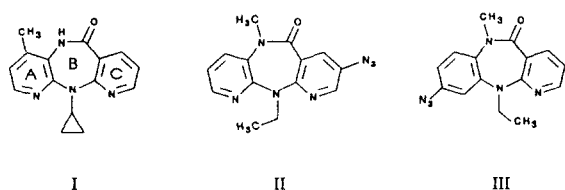
Fig. 1. Chemical structures of nevirapine (I), BI-RH-448 (II), and BI-RJ-70 (III). See text for position of tritium label.

methyl carbon at the 5-nitrogen of the B-ring of BI-RH-448 and on the 8-carbon of the C-ring of BI-RJ-70.

BI-RH-448 or BI-RJ-70 was separately cross-linked to reverse transcriptase. The crosslinking was effected by irradiating the azido analogue after binding to reverse transcriptase. At the start of irradiation the ratio of the molar concentration of azido analogue to that of reverse transcriptase was typically about 1:1 [18,19,21]. During irradiation the azide moiety was converted to a nitrene or other reactive intermediate that covalently bonded (crosslinked) the azido analogue to proximal backbone and/or side chains of the amino acid residues of reverse transcriptase [18,19,28,29]. The covalent attachment to reverse transcriptase abrogated its catalytic function [15,18].

3.2. Tryptic peptide mapping by HPLC of photoadduct

After irradiation the photoadduct formed between reverse transcriptase and BI-RH-448 or BI-RJ-70 was digested with trypsin, as was also a control of reverse transcriptase which had not been exposed to an azido analogue. The peptide fragments generated by digestion were resolved by reversed-phase HPLC (Fig. 2). At 210 nm approximately 70 major and minor peaks and shoulders were observed and exhibited generally comparable retentions and sizes in all three peptide maps (Fig. 2A, B, C). Seventy features in the maps were consistent with a prediction of 65 peptides if proteolysis of reverse transcriptase by trypsin was complete and specific at arginines and lysines. Another indication that the diges-
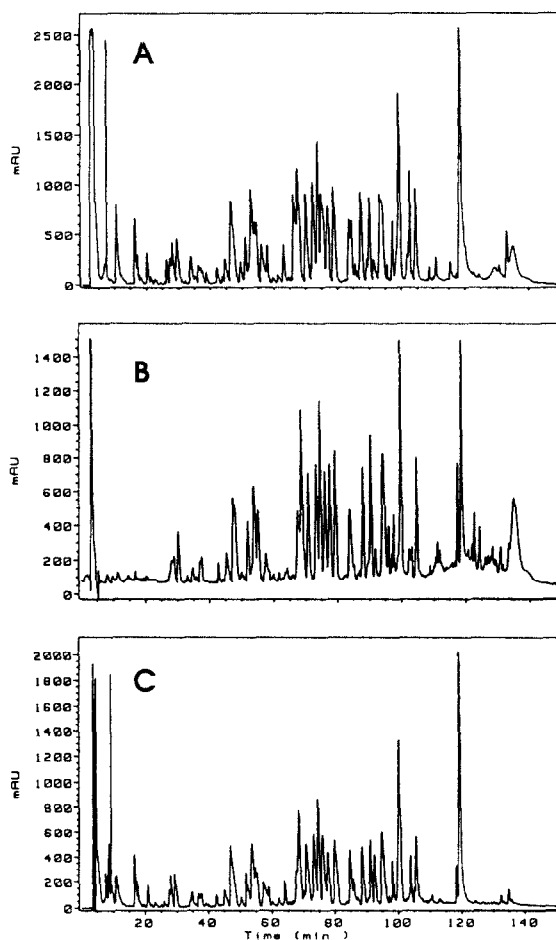
Fig. 2. Reversed-phase HPLC of tryptic peptides of 200–250 μg of reverse transcriptase. Solvent A was 0.06% (v/v) trifluoroacetic acid in water (pH 2.5) and solvent B was 0.052% (v/v) trifluoroacetic acid in water–acetonitrile (30:70, v/v). A gradient of 0–40% B in 90 min, 40–70% B in 40 min, 70–100% B in 20 min, 100% B for 5 min, 100–0% B in 15 min, and 0% B for 10 min was programmed with a flow-rate of 0.20 ml/min. Detection was by absorbance at 210 nm. (A) Reverse transcriptase exposed to no azido analogue. (B) Reverse transcriptase crosslinked to BI-RH-448 by irradiation. (C) Reverse transcriptase crosslinked to BI-RJ-70 by irradiation.

tions were thorough was that little signal was observed in the retention window (125–135 min) of undigested reverse transcriptase.

From one map to another in Fig. 2, a few differences in size and location of peaks were evident. The differences, however, were not

necessarily due to the presence or absence of azido analogue. Rather, the differences were often attributable to different conditions of the chromatography, such as amount and/or volume of sample injected onto the column [30], or the composition of solution containing the reverse transcriptase. Therefore, as in previous studies in which HPLC was utilized to locate the binding sites of proteins that were crosslinked to probes [31–47], a mode of detection more selective than absorbance at 210 nm in Fig. 2 was required to identify peaks that corresponded to peptide crosslinked to azido analogue.

3.3. Selective detection at 335 nm

A more selective spectroscopic method of detection was achieved from a comparison of the spectral properties of control reverse transcriptase and the azido analogues (Fig. 3). Reverse transcriptase which had not been irradiated eluted at 38 min under the HPLC conditions given in Fig. 3A. The spectrum of the protein peak was measured on-line via diode-array detection and exhibited a maximum in UV absorbance at approximately 278 nm (Fig. 3A), due to the presence of aromatic amino acid residues. Of note there was negligible absorbance at wavelengths greater than 320 nm.

When chromatographed, BI-RJ-70 which had not been irradiated eluted at 36 min (Fig. 3B). Unlike reverse transcriptase, however, BI-RJ-70 exhibited finite absorbance at wavelengths greater than 320 nm (Fig. 3B). BI-RH-448 also exhibited finite absorbance at wavelengths greater than 320 nm and eluted earlier than reverse transcriptase (data not shown).

Similarly, when BI-RJ-70 was irradiated in the presence of reverse transcriptase, the resulting photoadduct of BI-RJ-70 crosslinked to reverse transcriptase eluted at 38 min and exhibited finite absorbance at wavelengths greater than 320 nm (Fig. 3C). This absorbance was clearly due to the BI-RJ-70 component of the photoadduct. Not surprisingly, the crosslinking of BI-RJ-70 to reverse transcriptase had little effect on the retention of the reverse transcriptase (cf. Fig. 3A and C), as the molecular masses of reverse
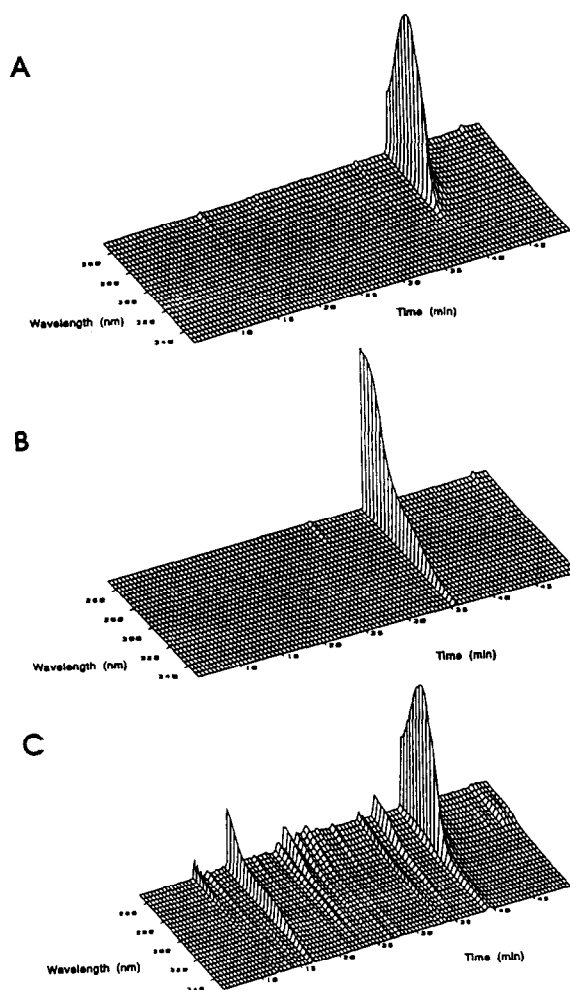

Fig. 3. Reversed-phase HPLC and on-line spectral scanning of reverse transcriptase, BI-RJ-70, their photoadduct, and photolytic derivatives of BI-RJ-70. Conditions were the same as those given in the legend of Fig. 2, except that the gradient was 0–70% B in 35 min, 70–100% B in 5 min, 100% B for 5 min, 100–0% B in 10 min, and 0% B for 10 min. (A) Reverse transcriptase exposed to no azido analogue and no irradiation. (B) BI-RJ-70 exposed to no reverse transcriptase and no irradiation. (C) Reverse transcriptase crosslinked to BI-RJ-70 by irradiation.

transcriptase and BI-RJ-70 were approximately 117 000 [24] and 294 [25], respectively.

In Fig. 3C numerous peaks that eluted earlier than the 36-min peak of unirradiated and un-crosslinked BI-RJ-70 (cf. Fig. 3B) were due to a molar excess (4:1) of BI-RJ-70 to reverse transcriptase at the start of irradiation. These peaks

were likely photolytic, derivative by-products of BI-RJ-70, because they were also produced when BI-RJ-70 was irradiated in the absence of reverse transcriptase (data not shown). The identities of the by-products were not determined, but of greater practical significance, all of them, like their precursor BI-RJ-70, exhibited finite absorbance at wavelengths greater than 320 nm (Fig. 3C).

From the data in Fig. 3 it followed that detection at a wavelength greater than 320 nm, e.g., 335 nm, provided a means to locate selectively tryptic peptides of reverse transcriptase which were crosslinked to azido analogues and to render the remainder transparent. Thus the wavelength of 335 nm was exploited to pinpoint the locations of peptides crosslinked to BI-RH-448 or BI-RJ-70 in peptide maps.

3.4. Peptides crosslinked to azido analogue

The same analyses (i.e., injections) illustrated with detection at 210 nm in Fig. 2 were replotted with detection at 335 nm in Fig. 4. In contrast to the three tryptic peptide maps in Fig. 2, the differences among the three chromatograms in Fig. 4 were striking. As there was no azido analogue present, no peaks corresponding to peptides crosslinked to analogue were observed for control reverse transcriptase in Fig. 4A. In Fig. 4B most of the peptides crosslinked to BI-RH-448 eluted within the 22-min retention window of 84–106 min, which was earlier than the 22-min retention window of 98–120 min that contained most of the peptides crosslinked to BI-RJ-70 in Fig. 4C.

Analogously, unirradiated BI-RH-448 eluted at 68 min, which was earlier than the elution time of 87 min for unirradiated BI-RJ-70 (data not shown). Irradiation of either azido analogue in the absence of reverse transcriptase yielded products that also absorbed at 335 nm and eluted earlier than the respective unirradiated azido analogue (data not shown, but cf. Fig. 3C). These findings corroborated that the peaks that eluted within 84–106 min in Fig. 4B and within 98–120 min in Fig. 4C corresponded to peptides
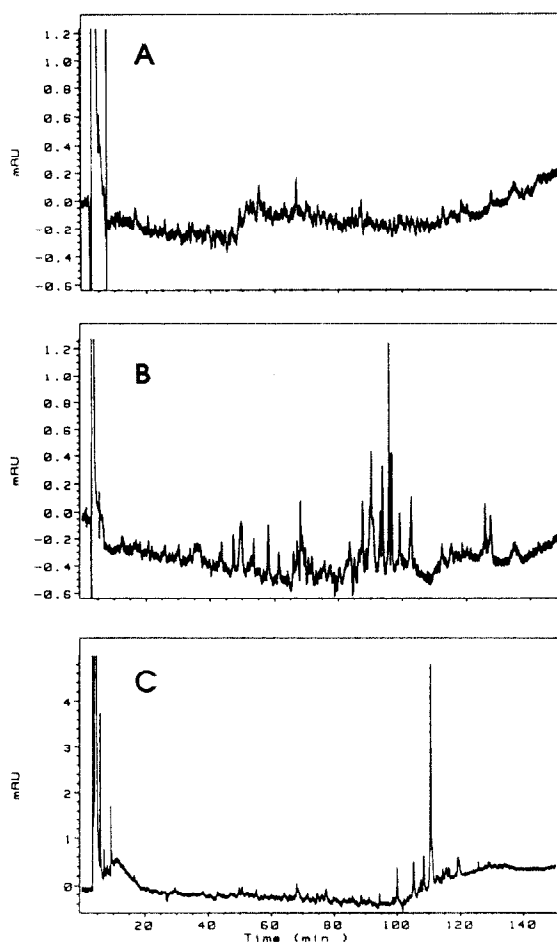

Fig. 4. Reversed-phase HPLC of tryptic peptides of 200–250 μg of reverse transcriptase. Conditions are identical to those given in the legend of Fig. 2, except detection was by absorbance at 335 nm. (A) Reverse transcriptase exposed to no azido analogue. (B) Reverse transcriptase crosslinked to BI-RH-448 by irradiation. (C) Reverse transcriptase crosslinked to BI-RJ-70 by irradiation.

crosslinked to analogue and not merely to non-peptide photolytic derivatives of azido analogue.

3.5. Crosslinking differences between BI-RH-448 and BI-RJ-70

Other differences between Fig. 4B and 4C were noteworthy. For instance in Fig. 4C one peak at 111 min predominated and was at least 5 times larger than any of the others. In com-

parison the largest peak (96 min) in Fig. 4B was no more than twice as large as several of the next largest peaks. Another difference was that there were many more additional peptides crosslinked to BI-RH-448 in Fig. 4B than there were peptides crosslinked to BI-RJ-70 in Fig. 4C.

Thus the HPLC profiles of Fig. 4B and 4C illustrated the dramatic difference between BI-RH-448 and BI-RJ-70 in their probing of the binding environment of reverse transcriptase. In addition, the profiles were also consistent with and diagnostic of a more specific, localized, and/ or stable crosslinking of BI-RJ-70 than of BI-RH-448 to reverse transcriptase. Unlike other studies of crosslinking [28,29,31–47], our study takes advantage of tryptic peptide mapping by HPLC with selective detection for the purpose of comparing qualitatively the specificity, localization, and/or stability of the crosslinking of two photoaffinity azido analogues of a drug to its target protein.

3.6. Detection by radioactivity

The utility of detection at 335 nm in Fig. 4B and 4C was further corroborated by monitoring radioactivity in fractions of HPLC effluent collected from the two analyses. The radioactivities in the fractions of peptides crosslinked to BI-RH-448 in Fig. 5A and to BI-RJ-70 in Fig. 5B generally coeluted with their respective absorbances at 335 nm in Fig. 4B and 4C.

In Fig. 5B 75% of the radioactivity of BI-RJ-70 collected from the column was concentrated in the 22-min retention window 98–120 min containing most of the peptides crosslinked to BI-RJ-70 (cf. Fig. 4C). In contrast to Fig. 5B, in Fig. 5A only half that much or 38% of the radioactivity of BI-RH-448 collected from the column was concentrated in the 22-min retention window (84–106 min) containing most of the peptides crosslinked to BI-RH-448 (cf. Fig. 4B). Thus as was the case with absorbance at 335 nm in Fig. 4B and 4C, the profiles of radioactivities in the peptide maps showed a more specific, complete, and/or stable crosslinking of BI-RJ-70 (Fig. 5B) than of BI-RH-448 (Fig. 5A) to reverse transcriptase.
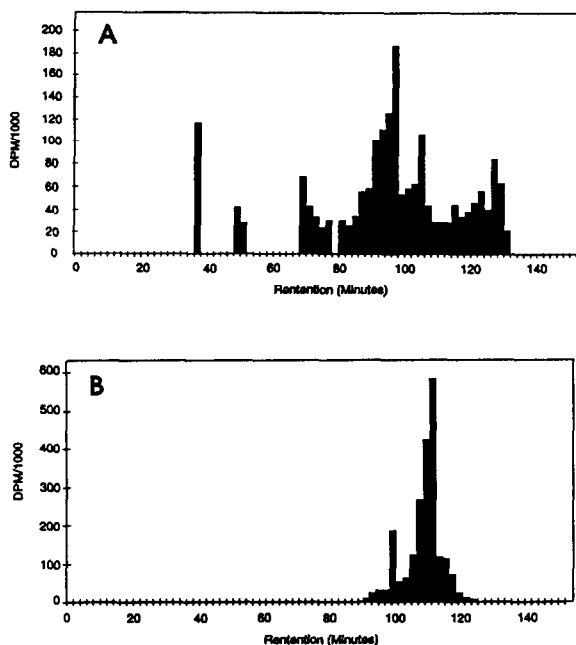

Fig. 5. Radiochromatograms of reversed-phase HPLC of tryptic peptides of 200–250 μg of reverse transcriptase. Conditions are identical to those given in the legend of Fig. 2, except detection was by measurement of radioactivity in fractions of HPLC effluent collected every 2 min. (A) Reverse transcriptase crosslinked to BI-RH-448 by irradiation. Approximately 4 million DPM were loaded onto and collected from the column. (B) Reverse transcriptase crosslinked to BI-RJ-70 by irradiation. Approximately 2 million DPM were loaded onto and collected from the column.

3.7. N-terminal sequencing of HPLC tryptic peptide fractions

The positions and identities of the amino acid residues that were crosslinked to azido analogue were determined by N-terminal protein sequencing. For amino acids crosslinked to BI-RH-448, 11 fractions of HPLC effluent within 84–106 min in Figs. 4B and 5A were analyzed. For amino acids crosslinked to BI-RJ-70, 11 fractions of HPLC effluent within 98–120 min in Figs. 4C and 5B were analyzed. At the conclusion of the chemistry of every cycle of Edman degradation and derivatization in sequence analysis, at least 60% of the volume of product was shunted off-line for measurement of radioactivity (Tables 1 and 2), while the remaining volume was directed

Table 1
Radioactivity (in DPM) in cycles of Edman sequencing of fractions of tryptic peptide map of reverse transcriptase crosslinked to BI-RH-448.

	Fraction[a]										
	84–86 2%	86–88 2%	88–90 4%	90–92 5%	92–94 5%	94–96 8%	96–98 2%	98–100 2%	100–102 2%	102–104 4%	104–106 2%
Cycle 1	568	864	573	369	258	492	319	639	337	371	521
2	−[b]	334	417	274	2328	6905	824	418	344	–	310
3	527	293	415	–	937	5034	260	305	272	–	–
4	332	420	–	–	1609	1941	–	–	258	–	–
5	–	321	2271	1376	1175	1469	263	392	314	–	–
6	449	404	1998	1268	1791	1346	–	369	331	–	260
7	–	889	1569	1122	2330	583	–	428	–	–	298
8	–	483	820	762	1389	465	–	294	–	–	254
9	–	–	505	540	936	689	–	1547	1036	1368	389
10	–	–	319	435	624	332	–	967	913	956	–
11	–	–	378	542	567	445	–	553	585	444	–
12	–	–	519	1277	443	291	–	431	417	442	–
13	–	–	752	1230	390	389	–	–	383	259	–
14	–	–	545	710	352	314	–	–	–	–	–
15	–	–	328	426	284	–	–	330	–	–	–
16	–	–	–	366	–	–	–	–	–	–	–
17	–	–	–	288	–	–	–	–	–	–	–
18	–	–	–	–	–	–	–	–	–	–	–
19	–	–	–	–	–	–	–	–	–	–	–
20	–	–	–	–	–	–	–	396	–	–	–

[a] First line (e.g. 84–86) indicates collection time (e.g. from 84 to 86 min) in the tryptic peptide map; second line (e.g. 2%) indicates percentage fraction accounted for (e.g. 2%) of total radioactivity collected in the tryptic peptide map.
[b] Radioactivity was less than background, which was defined as 250 DPM.

on-line for identification by HPLC of the phenylthiohydantoin (PTH) amino acid.

In such a manner radioactivity associated with specific amino acid positions was successfully captured. Successful capture of radioactivity has also been reported in a few other studies [32,37,46]. On the other hand, there have been numerous studies in which radioactivity was not captured [33–35,38,40,41,43–45], possibly because nucleotide analogues that were likely less hydrophobic than the azido analogues of nevirapine were employed as photoaffinity probes. Crosslinking nucleotide analogues to amino acids or their derivatives may have decreased solubility in the non-polar solvents utilized to extract the derivatized amino acids in the sequencer. Apparently and fortuitously, BI-RH-448 and BI-RJ-70 were hydrophobic enough not to prevent this solubilization of crosslinked amino acids and/or their derivatives.

The pattern of radioactivity associated with amino acid positions of peptides crosslinked to BI-RH-448 (Table 1) differed dramatically from that of BI-RJ-70 (Table 2). In the peptides crosslinked to BI-RH-448 (Table 1), each of 10 different cycles, including 2, 3, 4, 5, 6, 7, 9, 11, 12, and 13, were associated with radioactivity greater than that in the preceding cycle and thus not attributable to sequencer lag. Each of the 10 cycles likely corresponded to a different amino acid position, as the data in and the discussion of Fig. 2 suggested that the tryptic cleavages of reverse transcriptase were extensive and specific at arginines and lysines. Thus, the data of Table I implied that no fewer than 10 amino acids were crosslinked to BI-RH-448. In contrast to Table

Table 2
Radioactivity (DPM) in cycles of Edman sequencing of fractions of tryptic peptide map of reverse transcriptase crosslinked to BI-RJ-70.

	Fraction[a]										
	98–100 7%	100–102 1%	102–104 2%	104–106 4%	106–108 9%	108–110 15%	110–112 24%	112–114 4%	114–116 4%	116–118 3%	118–120 2%
Cycle 1	–[b]	–	–	–	–	–	–	818	–	–	–
2	–	–	–	–	–	–	–	–	–	–	–
3	–	–	–	–	–	–	–	–	–	–	–
4	–	–	–	–	–	–	–	–	–	–	–
5	–	–	–	–	–	–	–	–	–	–	–
6	–	–	–	–	–	–	–	–	–	–	–
7	–	–	–	–	–	–	–	–	–	–	–
8	3368	–	816	1324	9104	8080	25660	3238	8514	1842	1916
9	2488	–	548	634	7100	3620	7900	914	2316	744	624
10	1108	–	–	–	1394	960	2748	470	870	390	278
11	590	–	–	–	–	492	724	614	732	–	–
12	442	–	–	–	–	–	444	308	264	–	–
13	404	–	–	–	–	–	–	268	–	–	–
14	336	–	–	–	–	322	–	–	–	–	–
15	384	–	–	256	380	374	956	424	670	316	658
16	–	–	–	–	330	294	300	322	362	–	–
17	–	–	–	–	–	–	–	–	–	–	–
18	–	–	–	–	–	–	–	–	–	–	–
19	–	–	–	–	–	–	–	–	–	–	–
20	–	–	–	–	–	–	–	–	–	–	–

[a] First line (e.g. 98–100) indicates collection time (e.g. from 98 to 100 min) in the tryptic peptide map; second line (e.g. 7%) indicates percentage fraction accounted for (e.g. 7%) of total radioactivity collected in the tryptic peptide map.
[b] Radioactivity was less than background, which was defined as 250 DPM.

1, in the peptides crosslinked to BI-RJ-70 (Table 2), radioactivity greater than that in the preceding cycle was measured primarily in only two amino acid cycles, specifically 8 and 15, suggesting that amino acids at these two positions were crosslinked to BI-RJ-70.

Hence the data in Tables 1 and 2 again pointed to a more specific and localized crosslinking of BI-RJ-70 than of BI-RH-448 to reverse transcriptase. Furthermore, none of the 10 positions identified as containing amino acids crosslinked to BI-RH-448 was the same as the two positions, i.e., 8 and 15, identified as containing amino acids crosslinked to BI-RJ-70. Therefore, none of the amino acids crosslinked to either azido analogue was crosslinked to the other azido analogue. Evidently, the two azido analogues probed different amino acids within

the same region of reverse transcriptase, or probed two different regions. Because of the related structures of the azido analogues, it was more likely that the same region was probed. However, it could not be ruled out that different regions were probed as well, as discussed in the next sections.

3.8. Amino acids crosslinked to azido analogue

To identify the specific amino acids crosslinked to BI-RH-448 or to BI-RJ-70, peptides crosslinked to each azido analogue were isolated and then analyzed by protein sequencing. Isolation of the peptides crosslinked to azido analogue was achieved in high purity by modification of the HPLC conditions described for the tryptic peptide maps of Figs. 2 and 4.

For example in Fig. 4B the largest peak at 335 nm corresponding to peptide crosslinked to BI-RH-448 was at 95.5 min in the fraction collected at 94–96 min. Coeluting in the same fraction were also peptides which were not crosslinked to BI-RH-448 and which were likely at concentrations greater than that of the peptide at 95.5 min crosslinked to BI-RH-448 (cf. Figs. 2B and 4B). To isolate the peak at 95.5 min, four injections like that in Fig. 4B were made, and in each injection a 200-μl fraction was collected around 95.5 min. The four 200-μl fractions were pooled and subjected to a second HPLC step which employed pH 6.5 instead of the pH 2.5 employed in the first step (Fig. 4B). In the second HPLC step (Fig. 6), excellent resolution
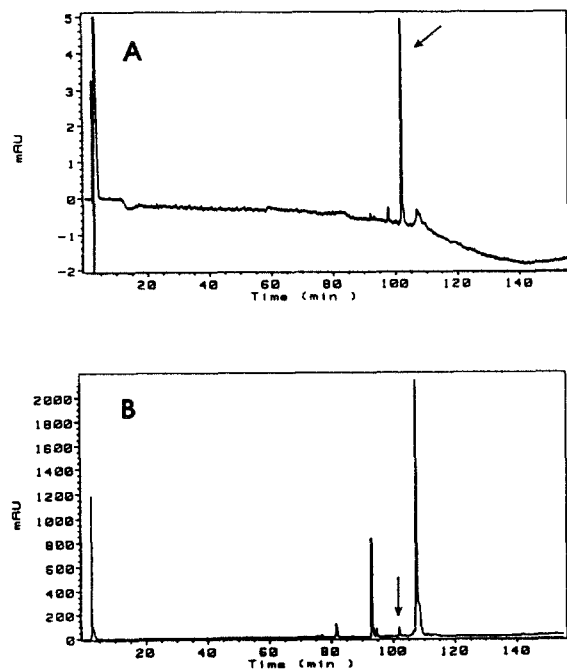

Fig. 6. Reversed-phase HPLC of a pool of four 200-μl fractions collected around 95.5 min in HPLC tryptic peptide maps comparable to that depicted in Figs. 2B and 4B. The pool of fractions was diluted to 1000 μl with water and then injected in 10 consecutive 100-μl loadings while the gradient was held at 100% A (0.05 M sodium phosphate, pH 6.5) and 0.20 ml/min. After injecting the 1000 μl a gradient identical to that described in the legend of Fig. 2 was applied, except solvent A was 0.05 M sodium phosphate and solvent B was 0.05 M sodium phosphate in water–acetonitrile (30:70, v/v). Detection was by UV absorbance. (A) 335 nm, (B) 210 nm.

of peptides crosslinked and not crosslinked to BI-RH-448 was obtained.

In Fig. 6A the HPLC fraction containing the largest peak (102 min) detected at 335 nm coeluted with the largest concentration of radioactivity (not shown) and did not appear to be contaminated with other peaks at 335 nm or 210 nm (Fig. 6B). Protein sequencing of this peak identified a peptide whose N-terminus started with leucine 425 of reverse transcriptase (Fig. 7). As illustrated in Fig. 7, tryptophan 426 corresponded to the amino acid residue released in cycle 2 of sequencing. The greatest level of radioactivity measured was also associated with cycle 2 of sequencing (Fig. 7), in agreement with the analysis of a comparable parent fraction (cf. fraction 94–96 min in Table 1). Thus it was concluded that BI-RH-448 was crosslinked to tryptophan 426.

In Fig. 6A fractions of other but smaller peaks at 335 nm and of smaller levels of radioactivity were analyzed by protein sequencing as well (data not shown). Again, peptides whose N-terminus started with leucine 425 were identified repeatedly whenever there was enough peptide to measure. Radioactivity was usually the greatest in amino acid position 2 corresponding to tryptophan 426, although for a few fractions of Fig. 6A levels of radioactivity too high to be attributable to sequencer lag were measured also in position 3, corresponding to tyrosine 427. In
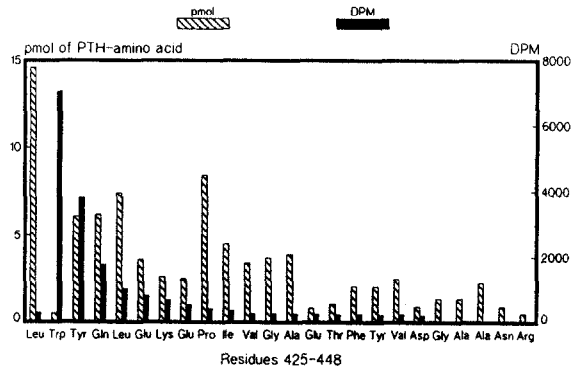

Fig. 7. Sequencing of the peptide corresponding to the peak indicated by the arrow at 102 min in Fig. 6. Conditions are given in the Experimental section.

Fig. 6A the different retentions of peptides all crosslinked to BI-RH-448 and all starting with leucine 425 at the N-terminus were due presumably to varying C-termini and/or to different atoms of tryptophan 426 and/or of tyrosine 427 to which BI-RH-448 was crosslinked.

3.9. Derivative UV absorbance spectroscopy

To corroborate that BI-RH-448 was crosslinked to tryptophan 426, on-line spectral scanning and second order derivative UV absorbance spectroscopy were carried out on the largest peak (102 min) at 335 nm in Fig. 6A. In the spectrum obtained (Fig. 8A), a minimum in absorbance was evident at 281–285 nm, which was indicative of the presence of tyrosine [48,49]. In the spectrum of Fig. 8B, which was of synthetic peptide not crosslinked to BI-RH-448
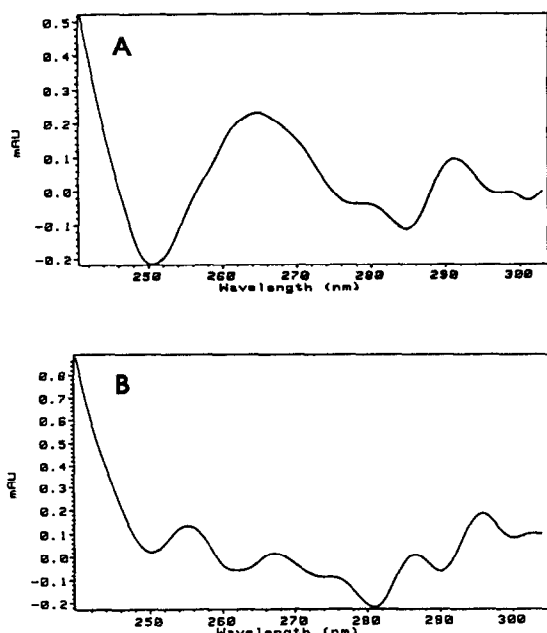

Fig. 8. Second order derivative UV absorbance spectra. Photodiode array detection was used to take spectra on-line at the apexes of eluting peptide peaks. (A) Peptide eluted at 102 min (Fig. 6A) was identified as amino acids 425–448, with tryptophan 426 crosslinked to BI-RH-448 (see text). (B) Synthetic peptide (amino acids 425–448) not crosslinked to BI-RH-448 eluted at 92 min (data not shown) under conditions of HPLC identical to that of Fig. 8A.

but otherwise of sequence of amino acids identical to the peptide in Fig. 8A, minima in absorbance were seen at 281–285 nm and at 288–292 nm, which were indicative of the presence of tyrosine and tryptophan, respectively [48,49]. In contrast, in Fig. 8A a minimum in absorbance at 288–292 nm was totally lacking, corroborating that BI-RH-448 was crosslinked to and thereby altered the spectrum of tryptophan 426.

In fact in Fig. 8A the largest minimum in absorbance was observed at approximately 250 nm, which was characteristic of products of irradiated BI-RH-448. Such a dominant minimum was not seen in Fig. 8B, as the synthetic peptide was never exposed to BI-RH-448. Other studies like ours have used on-line spectral scanning to locate in HPLC peptide maps peaks that corresponded to peptides crosslinked to azido reagents [33–35,38,40]. Our study, however, is the first to exploit on-line derivative spectroscopy to identify the precise amino acid to which the azido reagent was crosslinked.

By similar approaches attempts were made to elucidate the other amino acids that were crosslinked to BI-RH-448 and that corresponded to amino acid positions 4, 5, 6, 7, 9, 11, 12, and 13, in which radioactivity was measured in protein sequencing (Table 1). However, the peptides associated with the radioactivities in these positions were distributed so widely in Fig. 4B and were of concentrations so low that unambiguous identification has not been achieved to date. This was unfortunate, because these identifications potentially offer additional information about the binding environment of reverse transcriptase for nevirapine and its analogues.

3.10. Correlations to techniques other than HPLC

The amino acid residues which crosslinked to BI-RJ-70 and which corresponded to amino acid positions 8 and 15 (Table 2) were successfully identified as tyrosines 181 and 188, as reported previously [19]. The identifications of the tyrosines were supported by subsequent studies involving mutagenesis of reverse transcriptase. For instance the potency of nevirapine decreased

300–800 fold when tyrosine at position 181 or 188 or both was substituted with other amino acids [20,21]. Moreover a recent report in which the structure of reverse transcriptase co-crystallized with nevirapine was studied by X-ray crystallography showed that nevirapine was proximal to tyrosines 181 and 188 [22].

In comparison, mutagenesis studies performed in conjunction with the BI-RH-448 crosslinking experiments showed a far less dramatic decrease in the potency of nevirapine or its analogues. When tryptophan 426 or tyrosine 427 or glutamine 428 was substituted with the respective residue (alanine, phenylalanine, asparagine) from HIV-2 reverse transcriptase, the losses in inhibitory activity were only 2–6 fold [50]. Also, crystallographic studies of reverse transcriptase showed that tryptophan 426 was not proximal to nevirapine [22]. Indeed, based on other studies involving mutagenesis of reverse transcriptase, it was speculated that tryptophan 426 was in a region near the surface of reverse transcriptase [51]. This hypothesis was consistent with several of our findings: (i) tryptophan 426 was accessible to BI-RH-448, (ii) the effect of tryptophan 426 on the binding of nevirapine and its analogues to reverse transcriptase was modest at best, and (iii) the levels of tryptophan 426 crosslinked to BI-RH-448 were measurably smaller than that of tyrosines 181 and 188 crosslinked to BI-RJ-70.

Although the role, if any, which tryptophan 426 and tyrosine 427 played in binding nevirapine and its analogues was not clear, the use of BI-RH-448 contributed to our understanding of how non-nucleoside drugs bind to reverse transcriptase. For instance, the dramatic difference between BI-RH-448 and BI-RJ-70 in crosslinking behavior was consistent with the two analogues orienting with a similar directionality into the binding pocket. Furthermore, as nevirapine, BI-RH-448, and BI-RJ-70 exhibited comparable inhibition (IC50 90 nM, 150 nM, and 160 nM, respectively) against the polymerase activity of reverse transcriptase [14,18], the relatively diffuse crosslinking of BI-RH-448 to reverse transcriptase strongly suggested that immediately proximal to the C-ring of nevirapine or its analogues there were no amino acid side chains

with high reactivity towards nitrenes or other intermediates. The testing of this observation has not been possible by X-ray crystallography because of insufficient resolution to date [22,52].

4. Conclusions

HPLC-based techniques, most notably tryptic peptide mapping, on-line derivative UV absorbance spectroscopy, and off-line protein sequencing, were employed to demonstrate that two azido analogues of nevirapine differed markedly in their crosslinking behavior to reverse transcriptase of HIV-1. The HPLC techniques made it possible to carry out the unique comparison of BI-RH-448 and BI-RJ-70, and as a result to define better the structure of the binding pocket of reverse transcriptase for non-nucleoside inhibitors. For instance, the HPLC techniques made it feasible to show that tyrosines 181 and 188 were crucial components of the binding pocket, that the A-ring of nevirapine was oriented proximal to the tyrosines, and that the C-ring was likely oriented distal from the tyrosines as well as from other amino acids.

The identification of the tyrosines was subsequently used to assist in the design of mutant reverse transcriptase in mutagenesis studies [21], and in the assignment of amino acid positions in X-ray crystallography studies [22]. Both of these non-HPLC techniques were then able to corroborate the tyrosines 181 and 188 initially identified with the HPLC approach [19]. However, the finding that the C-ring of the azido analogues of nevirapine was oriented not proximal to the tyrosines, or for that matter, not proximal to any other amino acids in the binding pocket of reverse transcriptase for nevirapine analogues, remains achievable so far by HPLC methodology only. Testing of this finding by other techniques (*e.g.*, mutagenesis or X-ray crystallography) is eagerly awaited. Finally, as a practical note, the results reported demonstrate that all azido analogues of a given inhibitor may not be of equivalent utility in identifying amino acid residues located at the binding site. There-

fore, an accurate and/or complete definition of a binding site may demand the use of more than one azido analogue.

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